MELANOCORTIN RECEPTORS: FROM CLONING TO SELECTIVE LIGANDS

Ruta Muceniece

Latvian Institute of Organic Synthesis
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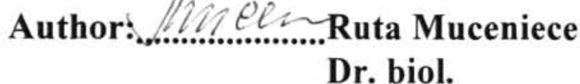

Author: .... **Ruta Muceniece**
Dr. biol.

Latvian Institute of Organic Synthesis
University of Latvia

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Professor, Dr. habil. biol.

Janis Baltkajs (Medical Academy, Riga)
Professor, Dr. habil. med.

Alexander Zharkovsky (Tartu University, Tartu)
Professor, Ph.D., MD.

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Anotācija

MSH-receptori kā fizioloģiska struktūrvienība ir pazīstami kopš 1957. gada. Alfa-melanocītstimulējošā hormona/adrenokortikotropā hormona (MSH/AKTH) peptīdu iespējamās saistīšanās vietas pie receptoriem ir atrastas gan smadzeņu gan perifērijas audos. Vēlākos gados receptoru pētījumos tika izmantotas melanomas šūnu līnijas. Šīs testsistēmas deva informatīvus rezultātus, bet tikai tagad ir zināms, ka šie agrāk iegūtie dati attiecas uz MC1 receptoru. 1992. gada aizsāktā molekulārā receptoru gēnu klonēšana parādīja piecu MC-receptoru apakštipu eksistēšanu. Šie pieci MC-receptori tika nosaukti pēc numuriem - MC1, MC2, MC3, MC4 un MC5. Tie pieder pie ar G-proteīnu saistītiem receptoriem, kuriem ir septiņi hidrofobi transmembrānas fragmenti un tie visi stimulē ciklisķā adenozīnmonofosfāta (cAMF) veidošanos. Iedarbojoties uz smadzenēs, imūnās sistēmas šūnās un periferiālos audos esošajiem receptoriem melanokortīni uzrāda neierobežoti plašas to pielietošanas iespējas. Viens veids kā selektīvi regulēt MC-receptoru apakštipus būtu farmakoloģiski ietekmēt tos procesus, kas notiek šūnās, kuras tieši producē MC-receptorus, vai arī izmainīt šo šūnu vai audu attīstības apstākļus.

Šajā darbā ir aprakstīti jaunu sintētisku vielu, kuras selektīvi aktivē visus līdz šim atklātos MC-receptorus vai selektīvi antagonizē dabīgo hormonu vai citu agonistu ietekmi uz šiem receptoriem, pētījumi. Dabīgo hormonu, to sintētisko analogu un ne-melanokortīnu vielu darbības salīdzinājums ļauj izdarīt secinājumus, ka alfa-MSH ir selektīvs MC1 receptora agonists, MC2 ir tikai AKTH receptors, MC3 receptoram ir relatīvi augsta gamma-MSH piesaistīšanas spēja, kas savukārt ir ļoti zema MC4 receptoram, bet MC5 receptors atgādina MC1 receptoru ar piebildi, ka visi dabīgie hormoni saistās pie MC5 ar daudz zemāku afinitāti kā pie MC1. Ar fāgu displeja skrīningu atrastie peptīdi uzrāda labāku selektivitāti pret MC1 receptoru. Bez jau zināmās MC1 lomas melanoģenēzes procesos tieši pēdējā laikā zinātniskā literatūrā parādās dati par MC1 selektīvu vielu nozīmi imūnās sistēmas funkcionēšanā. Ar šādām vielām varētu ārstēt imūnās sistēmas slimības, ietverot iekaisumus vai jebkuru radniecīgu gadījumu, kurā ir jāregulē makrofāgu, neutrofilu, monocītu, keratinocītu, melanocītu vai endotēlijšūnu darbība.

Pārbaudot ar radioligandu saistīšanās metodi simtiem jaunu sintētisku peptīdu tika atrasti savienojumi ar augstu selektivitāti un spēju stimulēt cAMF MC-receptoru

proteīnus producējošās šūnās. Tika atklāts, ka 26 locekļu gredzena cikliskie peptīdi, kur ciklu veido Cys4 un Cys 11, ar D-Nal7 aizvietotu Phe7 alfa-MSH molekulā, ir selektīvi MC4 ligandi, bet savukārt 29 locekļu gredzeni ir selektīvi MC3 ligandi. Saskaņā ar datiem, kas tika iegūti pēc MC4-receptora proteīnu neproducējošām pelēm (knock-out mice), MC4 selektīvas vielas var tikt pilnveidotas līdz aptaukošanās, anoreksijas vai bulēmijas ārstēšanas līdzekļiem.

Šis darbs ietver arī receptoru struktūras pētījumus, kādi ir iespējami ar mutētu dabīgo receptoru klonu veidošanu. Iegūtie mutanti tika ekspresēti šūnās un raksturoti pēc spējas piesaistīt MSH/AKTH peptīdus. Šāda tipa rezultāti ir nozīmīgi trīs dimensiju struktūras kompjūtermodelēšanas programmām, jo līdz šim vēl nav izdevies attīrīt un kristalizēt nevienu membrānās esoša receptora proteīnu, kas ļautu ar spektriem noteikt šo receptoru uzbūvi. Tika radīti daudzi mutanti un šo datu kopsavilkums rāda, ka ligandi saistās receptora transmembrānu (TM) segmentu 1, 2, 3, 6 un daļēji 7 iekšpusē. TM4 un TM5 praktiski nepiedalās tā sauktās "saistīšanās kabatas" (binding pocket) veidošanā.

Zīdītāju šūnās ekspresēto MC-receptoru koncentrācija ir nepietiekama receptoru proteīna attīrīšanai, kas arī ir iemesls meklēt citus veidus kā iegūt lielāku daudzumu receptoru. Ir zināms, ka bakulovīrusa ekspresijas sistēma ir viena no visefektīvākajām biometodēm liela daudzuma proteīnu ieguvei. Tieši mēs pirmo reizi parādījām, ka inficējot insektu Sf9 šūnas ar bakulovīrusu nesošu attiecīgā MC-receptora gēnu, tika iegūta augsta MC1 proteīna koncentrācija. Mūsdienās šādas šūnas tiek lietotas zāļu kompānijās liela apjoma vielu skrīningam un tuvākajā nākotnē tiks izmantotas receptoru attīrīšanai.

АННОТАЦИЯ

Рецепторы МСГ (меланоцит-стимулирующего гормона) известны с 1957 года как физиологические участки воздействия этого гормона. Так называемые сайты (участки) связывания для МСГ/АКТГ пептидов были найдены в мозгу и в периферии. Изучение этих рецепторов было также произведено на клетках меланомы. Эти тест-системы дали сравнимые результаты, но как сейчас известно, полученные данные относились к рецептору МС1. Начиная с 1992 года, методом молекулярного клонирования были идентифицированы пять различных подтипов МС-рецепторов, которые были названы, в порядке их открытия, МС1, МС2, ... МС5-рецепторами. Было установлено, что они принадлежат к классу рецепторов, связанных с Г-протеином и имеют семь гидрофобных трансмембранных фрагментов, которые стимулируют образование цАМФ.

Пептидные гормоны (меланокортины), действуя через МС-рецепторы, локализованные в центральной нервной системе и периферии, оказывают настолько широкий спектр действия, что их способности стать предшественниками лекарств просто безграничны. Одним из перспективных направлений является селективное регулирование МС-рецепторов путем фармакологического воздействия на клетки и ткани, производящие МС-рецепторные белки.

Настоящая работа обобщает прогресс в разработке новой группы химических соединений, селективно активирующих МС-рецепторы, а также являющихся антагонистами других гормонов и агонистов этих рецепторов. Можно сделать следующие основные заключения по результатам работы:

1. Сравнительное изучение природных меланокортинов, их синтетических аналогов, а также соединений «не-меланокортинной» природы позволило определить особенности различных подтипов МС-рецепторов. Было установлено, что α-МСГ является селективным лигандом МС1-рецептора, в то время как АКТГ связывается только с МС2; γ-МСГ имеет относительно высокое сродство к МС3, но не к МС4; МС5 в целом напоминает МС1 рецептор, с той разницей, что все меланокортины связываются с ним намного хуже.

2. Пептиды, найденные методом скрининга фагового дисплея показывают более высокую селективность к МС1. Помимо давно известного меланоцит-стимулирующего эффекта в настоящее время накапливается все больше данных об огромном значении селективных МС1 лигандов в модулировании иммунной системы. Такие соединения могут быть весьма полезны в лечении заболеваний иммунной системы, связанных с

действием макрофагов, нейтрофилов, моноцитов, кератиноцитов, меланоцитов, клеток эндотелия и др.

3. Тестирование сотен синтетических пептидов выявило новые соединения с селективным воздействием на отдельные подтипы МС-рецепторов в сочетании с эффективной стимуляцией или ингибированием образования цАМФ в клетках, производящих МС-рецепторы.

4. Нами было найдено, что 26-членные циклические пептиды Cys4, D-Nal7, аналоги Cys11-α-МСГ являются высокоселективными лигандами для МС4-рецептора, в то время как 29-членные пептиды являются селективными лигандами для МС3. Данные, полученные в результате экспериментов на мышах без гена МС4, показывают, что селективные лиганды МС4-рецепторов могут стать перспективными препаратами для лечения нарушений обмена веществ: лишнего веса, анорексии, булемии и др.

Данная работа включает также структурную характеристику рецепторов, полученную путем изучения мутантных клонов МС1, МС3, МС4 и МС5-рецепторов, которые были охарактеризованы методом радиолигандного связывания, а также по способности стимулировать синтез цАМФ в клетках, производящих мутантные гены. Полученные результаты являются весьма информативными для компьютерного моделирования трехмерной структуры рецепторного белка, так как до сих пор не существует данных о кристаллической структуре мембраносвязанных белков. Изучение мутантных генов показало, что сайты связывания лиганда находятся внутри трансмембранных фрагментов ТМ1, ТМ2, ТМ3, ТМ6 и ТМ7, в то время, как ТМ4 и ТМ5 прямо не участвуют в связывании.

Концентрация рецепторного белка в клетках позвоночных, производящих МС-рецепторы, является недостаточной для его выделения и очистки. Известно, что метод инфицирования клеток бакуловирусом является одним из наиболее эффективных методов производства белка, соответствующего генам вируса. В этой связи этот метод был избран нами для увеличения производства МС-рецепторов в клетках. Нами впервые было показано, что заражение инсектных клеткок Sf9 бакуловирусом, несущим ген соответствующего МС-рецептора, приводит к получению высокой концентрации МС-рецепторного белка. В настоящее время такие клетки широко используются фармацевтическими компаниями для масштабного скрининга соединений, и в будущем, вероятно, будут использоваться также для очистки рецепторного белка.

ANNOTATION

Since 1957 MSH-receptors have been known as physiological entities. Binding sites for MSH/ACTH peptides have been identified in number of brain and peripheral tissues. Receptor studies were later also performed by binding on melanoma cell lines. These test systems gave comparable results and it is now known , that data obtained than with these systems refer to MC1 receptor. Starting at 1992 by the use of molecular cloning genes encoding five different subtypes of MC-receptors have been identified. These five MC-receptors are termed MC1, MC2, MC3, MC4, MC5 and they belong to the class of G-protein coupled receptors which have seven hydrophobic transmembrane domains and are coupled in a stimulatory fashion to cAMP. Acting through these five (localised in central nervous system, immune system and periphery) receptors melanocortin peptides exert so broad array of peripheral and central effects, that their investigation has no borders. One way is to provide means and methods to selectively regulation of MC-receptors by pharmacological affecting processes and conditions related to tissues and cells expressing the MC-receptors.

This study describes the design of a new group of chemical compounds which activate MC-receptors selectively and with high potency as well as which antagonise the action of other hormones and agonists on these receptors . Comprehensive testing of natural peptides and their synthetic analogues as well as non-melanocortins allowed to draw general conclusions. Such as, that αMSH is selective for the MC1 receptor, the MC2 binds ACTH, but not MSH peptides, the MC3 has relatively high potency for γMSH, but MC4 low, that MC5 has the same potency order as MC1, but MSH peptides bind to MC5 with much lower affinities. Peptides found by phage display screening show higher selectivity for MC1. Besides already known effect of MC1 in melanogenesis there is growing number of data showing importance of MC1 receptor selective substances in immune system modulation. Such substances can be used in the treatment of immunological diseases, including inflammation or any related condition involving the action of macrophages, neutrophils, monocytes, keratinocytes, melanocytes and endothelial cells.

Screening hundreds of synthetic peptides revealed novel compounds with high selectivity and affinity for MC-receptor subtypes in combination with effective stimulation of cAMP formation in MC-receptor expressing cells . We discovered that 26 member ring cyclic peptides Cys4, D-Nal7, Cys11-αMSH analogues posses for

MC4 receptor, 29 member rings for the MC3. According to data from knock-out mice MC4 selective substances have potential in treatment of overweight, anorexia and bulimia.

The present study includes also structural characteristic of these receptors by generation of mutated clones of human MC1, MC3, MC4, MC5 receptors. These mutants were functionally expressed in eukaryotic cells and characterised in radioligand binding assay. Results of this nature is important for 3D modelling of receptors protein as there is no data available of membrane bound receptor protein crystalline structure. Mutagenesis study showed that ligand binding pocket is buried within the TM1, TM2, TM3, TM6 and TM7, but TM4 and TM5 did not affect binding.

Concentration of expressed MC-receptors in mammalian cells is inefficient for receptor protein purification what is reason to search for other possibilities. It is known, that baculovirus expression system is one of the most efficient method for high level protein production. It was shown by us first time that infection of insect Sf9 cells with baculovirus carrying corresponding MC-receptor gene gives very high MC-receptor protein level. Nowadays such cells are used in drug companies for large scale screening and in the future will be used for receptor purification.

The thesis is based on the following papers, which are referred to in the text by Roman numerals:

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Abbreviations

ACTH	adrenocorticotropic hormone
bp	base pair
C	carboxyl
cAMP	adenosine 3:5-cyclic monophosphate
CNS	central nervous system
cpm	counts per minute
DNA	deoxyribonucleic acid
EL	extracellular loop
IBMX	isobutylmethylxantine
MC	melanocortin
MSH	melanocyte stimulating hormone
mRNA	mesenger ribonucleic acid
N	amino
PCR	polymer chain reaction
POMC	proopiomelanocortin
TM	transmembrane

INTRODUCTION

Approximately three decades ago the first time in scientific literature ACTH/MSH peptides (melanocortins) were mentioned (1, 2, 3). Since than up to nowadays a large number of central and peripheral effects of natural melanocortins and their synthetic analogues has been described. It was proved that:

1. ACTH and αMSH are not only present in the pituitary, but also in the other brain structures and periphery.
2. Melanocortins are formed from large precursor - proopiomelanocortin (POMC), which can be processed to ACTH, αMSH, γMSH and βLPH, which later can be processed further to βMSH and β-endorphin (4, 5). In the brain POMC-containing cell bodies are mainly localised in the arcuate nucleus of the hypothalamus (6) and nucleus tractus solitarius (7). Small amounts of POMC have been detected also in the various peripheral tissues and αMSH immunoreactivity has been found in the skin (8).
3. Peripheral targets for αMSH are melanocytes and melanoma cells, for ACTH - adrenal gland, but lacrimal, Harderian glands, as well as peripheral nerves respond to all ACTH/MSH peptides (for a review, Eberle 1988) (9). Although it was confirmed long time ago that ACTH/MSH-like peptides may modulate brain functions (conditioned avoidance behaviour, excessive grooming, stretching, yawing, release of pituitary hormones etc. (9), the exact their targets in the brain were not established. The melanocortin peptides are reported to have anti-inflammatory (10), antipyretic activity (11), protective actions against renal injury after ischemia in mice and rats (12), possibility to stimulate nerve regeneration (13), to influence blood flow, blood pressure, heart rate (14, 15) and to exert behavioural effects mainly related to attention, learning, memory, social, sexual behaviour (9).

The existence of specific receptors for the melanocortins has been shown in melanoma cells (16. 17, 18), peripheral tissues (19, 20) and in the brain (21,22), but the first cloned receptor was nominated by Chhajlani and Wikberg at 1992 (23) by applying PCR (polymerase chain reaction) on the human genomic DNA and primers designed by homology to the TM3 and TM6 of known G-protein coupled receptors (GPCR). The same human melanocortin receptor named MC1 and mouse MC1 receptors were independently cloned by Mountjoy et al. at the same year (24). Than human MC2 (24), MC5 (25), MC3 (26), MC4 (27) were cloned. Nowadays are cloned also MC receptors of other species: bovine MC1 (28), chicken MC1 (29), mouse MC2 (30), bovine (31), rat MC3 (32), mouse MC3 (33), rat MC5 (34), mouse MC5 (35), ovine MC5 (36) etc.

The MC receptors belong to the class of G-protein coupled receptors which are all built from a single peptide forming 7 transmembrane domains and they all couple in stimulatory fashion to cAMP.

The MC1 is present on melanocytes and melanoma cells (9). Recent data also indicates that MC1 is expressed in limited areas (periaqueductal gray) of rat and human brain (37) as well as in the testis (28). Also very recently MC1 is shown to be present on macrophages (38), neutrophils (39), glioma cells and astrocytes (40), monocytes and endothelial cells (41), testis and ovary (42). This suggests a specific role of MC1 in cutaneous and reproductive physiology as well as pathophysiology. The MC2 is ACTH receptor. It is present in the cortex of adrenal gland (9). MC3 is found in distinct areas of the brain, placental, gut tissues (26). MC4 is found to be present only in the brain (27).
MC5 receptor is expressed in the brain as well in several peripheral tissues (34, 35).
Taking together all expression data obtained in different species one can describe localisation of MC receptor subtypes as following:
MC1 - melanocytes, melanoma, macrophages, monocytes, brain (limited areas, such as periaqueductal gray), adipose tissues, testis.,ovary.
MC3 - brain, placenta, duodenum, pancreas, stomach, heart.
MC4 - brain (widely distributed in almost every brain region, including the cortex, thalamus, hypothalamus, brain stem and spinal cord).
MC5 - brain, skin, adrenal gland, spleen, thymus, bone marrow, uterus, stomach, thyroid, pineal gland, mammary gland.

The most studied and clarified physiological roles of the melanocortins were the effects of αMSH on pigmentation and of ACTH on steroid production in the adrenal gland. Recent knowledge about MC receptor localisation may help to establish and explain a large number of other effects attributed to the MSH peptides. A diversity of effects is induced by natural melanocortic peptides. These effects should be mediated by different subtypes of the MC-receptors. There are growing number of publications showing that MC1 receptors play important role in the modulation of inflammation an effect claimed to be caused by the inhibition of the production of NO synthase (10). MSH peptide is also known in increase the formation of interleukin 10 (IL-10) in monocytes. Role of MSH peptides in cutaneous biology is described as ability to stimulate pigment formation of the skin, to modify keratinocytes proliferation and differentiation.

The most important achievement in the revealing of specific physiological role of each MC receptor subtype is generation of knock-out MC4 and MC5 mice (inactivation of the corresponding receptor by targeted gene disruption). MC4 knock-out mice developed a obesity syndrome similar to aguoti obesity syndrome (43), associated with hyperinsulinemia, hyperglycemia and elevated expression of neuropeptide Y in the hypothalamus (44). MC5 knock-out mice gave strong evidence that this receptor plays important role in the regulation of exocrine glands functions (45). Taking into account already known data about MC1 and MC2 significance and above described data about MC4 and MC5 properties, only MC3 physiological role remains unclierified. The obtained MC3 localisation in the heart and relatively high affinity of this receptor for γMSH (which is known to influence the cardiovascular system) lead to speculation that MC3 receptor may be involved in hypotensive, bradycardic responses (46). However, Van Bergen 1997 (15) published data that effects of γMSH on the cardiovascular system are not mediated by any of the cloned MC receptors.
Brain or peripheral tissues as source of MC-receptors in radioligand binding studies or melanophores from lower vertebrates like frogs, lizards for testing agonist/antagonist properties were performed since 1957 (9). Nowadays mainly cloned receptors are used in screening experiments to find selective binders to each of receptor subtype.
Thus, knowledge of chemistry, mechanisms of action, physiological effects of the melanocortin peptides have been reviewed and enlarged by use of molecular techniques and especially by creating knock-out mice models.

AIMS

The overall aim of this work was to study all MC receptors.
The specific aims of present studies were:

1. To clone hMC5 receptor.
2. To prove that MC1 receptor is expressed in melanoma cells.
3. To show characteristic binding profile for all MC receptors.
4. With help of mutations into wild type receptor structure to find important/unimportant structure elements for receptor-ligand interactions.
5. To find selective substances for each MC receptor subtype between MSH/ACTH like peptides as well as between non-MSH peptides (like TRH, dynorphines etc).
6. To develop large scale expression system in insect cells using recombinant baculovirus expression system.
7. To screen MC1 active substances found by phage display library.

METHODS

brief description of methods used in corresponding papers.

1.Cloning of hMC5 (Than called MC2 receptor).

The oligonucleotide primers for polymerase chain reaction (PCR) were designed on the nucleotide sequence of the cloned MC1 receptor. The primers were synthesised with EcoRl and BamHl linkers. Human genomic DNA was subjected to PCR using the above described primers. One product was observed which was cloned into the pGEM7zf+ vector (Promega Corp., USA) and the resulting plasmid, termed G8, was sequenced using the chain termination method. 10 million clones of the human placental DNA genomic library (Stratagene, USA) were screened with ^{32}P-labelled G8 probe. Positive plaques were picked and 2400 bp Sac 1 fragment from the genomic clone MC5, was subcloned into the pGEM5zf+ vector and sequenced. The entire coding region of the MC5 genomic DNA clone was inserted into pRC/CMV vector and used for transformation cells and later in binding studies.

2.The localisation of MC1 in WM266-4 human melanoma cells and melanoma tissues was investigated by applying antipeptide antiserum prepared by immunising rabbits with a 11-mer synthetic peptide from hMC1 receptors N-terminus. The immunoreactivity was detected with enzyme-linked immunosorbent assay (ELISA). The specific immunoreactivity was demonstrated on the surface of the cells by using either biotin-avidin immunoalkaline phosphotase ·(Vectastain-ABC kit I, Vector Red, Vector Lab). or TRITC-staining method.

3. **Mutagenesis studies** were carried out in 4 different ways: **1**) truncations at the N-terminus. These constructs were made by PCR, checked on agarose gels by electrophoresis, purified by Qiagen or Jetpure beads prior cloning and sequencing.

2) multiple mutations in certain area of the clone which were introduced simultaneously. The constructs were made according to the so called Megaprimer approach (47). These modifications were made on hMC3 extracellular loop (EL) 2, TM4, TM5 and mMC5 EL1. EL3.

3) chimeras for melanocortin 1/3 receptors were created by a modification of the megaprimer approach.

4) mutations were done by introducing single amino acid mutations separately in the two putative start codons of the hMC3 at the N-terminal part and two mutations into hMC1.

4. Cells and expression.

Eucaryotic cells were grown in Dulbecco`s modified Eagles medium (Gibco, BRL) with 10% foetal calf serum (Gibco BRL) and IX antibiotic/antimycotic solution (Gibco BRL). Cells which naturally express MC receptors: WM-266-4, B16-F1, Y1 were used in experiments when they were about 90-100 % confluent. COS-1, COS-7, HEK-293, CHO, G4F cells were transfected on petri dishes with the corresponding to aim of studies DNA (approximately 1 μg DNA for every $1x10^6$ cells) mixed with liposomes in serum free medium Optimem. MC1 and MC5 receptor clones have been cloned into the pRc/CMV vector. The MC3 and MC4 receptors, cloned into CNV/Neo vector, were gift from Dr. Ira Gantz, USA. The different mutants were cloned into pRc/CMV vector, but the chimeras into pcDNA 3.1.

The liposomes used were either the commercially available Lipofectin (BRL, USA) or produced according to Campell (48). After 6 h Optimem was replaced with the previous medium and cells were cultivated for 48 h. Cells were than detached from the petri dishes by incubation in Hank`s balanced salt solution (Gibco, BRL) with 0.5-1 mM EDTA for about 3 min, then scraped off, centrifuged and used for radioligand binding, or cAMP determination, or localisation studies.

Baculovirus expression system is established as one of the most efficient method for high level production ·(49) and were used for insect Sf9 cells infection . Sf9 cells were grown at density 2 million cells/ml and infected with baculovirus carrying appropriate MC receptor gene and after 48 h were used in assays.

5.Radioligand binding.

The radioligands used were labelled with ^{125}I in our lab or purchased from Amersham, Malme. [^{125}I]-Nle^4-D-Phe^7-MSH; [^{125}I-MSH], [$^{3\text{-}125}$ I-iodotyrosyl^{23}]-ACTH(1-39) were labelled by the Chloramine T method or iodobeads techniques and purified by HPLC.

The binding assays were performed on whole intact cells into binding buffer - MEM with Earles salts, 25 mM HEPES, pH=7.0, 0.2 % bovine serum albumin, 1mM 1,10-phenantroline, 0.5 mg per litre leupeptin and 200 mg per litre bacitracin. Cells were distributed in microtiter 96-wells plates, centrifuged and after removing buffer incubated for 2 h at 37° with binding buffer containing for competition analysis constant labelled ligand concentration and appropriate concentrations of unlabelled competing ligand. For saturation analysis 12 concentrations of labelled ligand in the range of 0.02 up to 20 nM were used. Non-specific binding was determined in the presence of 3 μM appropriate unlabelled ligand. Untransfected cells were used as

control. After incubation the cells were washed with ice-cold buffer and detached from the plates with 0.1 M NaOH solution. Radioactivity was counted in gamma counter and the data analysed with a software package for radioligand binding studies (Wan System, Umea, Sweden). The data was analysed by 1) fitting in to the four parameter logistic function using non-linear least squares regression and then the Ki values were obtained from IC_{50} values according to the equation of Cheng and Prusoff (49) or by 2) fitting it to formulas derived from the law of mass action by the method generally refereed to as computer modelling.

6. cAMP determination.

Cells were harvested and incubated for 30 min at 37° with Dulbeccos modified Eagles medium containing 0.5 mM IBMX and appropriate concentrations of tested substances. After incubation cAMP was extracted with perchloric acid, centrifuged and the protein free supernatant was neutralised with 5 M KOH/TRIS solution. 0.05 ml of this neutral supernatant was added to a 96 well microtiter plate. The content of cAMP was than estimated according to binding protein method (50). Samples were harvested by filtration on Whatman GF/B filters using semiautomatic Brandel cell harvester. Each filter was rinsed with 3 ml 50 mM TRIS/HCl pH=7.4. The filters were punched out and put into scintillation vials with scintillation fluid EcoScint and counted on beta-counter. The readings were converted into cAMP concentration by constracting curves from standards.

RESULTS AND DISCUSSION

1.hMC5 cloning (paper I).

The cloned DNA were coded for protein of 325 amino acids which possessed seven hydrophobic segments, a characteristic of G-protein coupled receptors. This receptor showed identical order of affinity for the melanocortin peptides as MC1, but the affinities and the fold differences in the affinities were different from earlier described MC receptors. These results suggested that we had cloned a new MC receptor subtype.

2.hMC1 localisation (papers II and III).

It was shown earlier that αMSH can bind to melanoma cells, but it was not known what subtype of MC receptors are localised on tumour cells. By applying an antipeptide antiserum specific for MC1 receptor we demonstrated that MC1 is located on melanoma cells as well as in specimens of melanoma tumours.

MC1 receptor had a patchy distribution with the receptor being arranged in clusters to limited areas on the surface of the melanoma cells.

3.3D receptor structure studies

G-protein coupled receptors belong to the largest family of receptors with approximately 1000 members in mammals and over 100 in humans for peptide hormones. MC-receptor family has the shortest amino acid sequence among the G-protein coupled receptors as they are correspondingly MC1 (317), MC2 (297), MC3 (361), MC4 (333) and MC5 (325) amino acids long. They have short N-(25-39) and C-terminal regions (17-21) amino acids long as well as a very small second extracellular loop ca 9 amino acids. The all MC receptor subtypes share considerable amino acid identity, highest between the MC4 and MC5 (60% of identity, generally highest homology for all MC-receptors are in transmembrane (TM) segments TM1, TM3 and TM7.

1) Site directed mutagenesis (paper IV-VI).

Our site directed mutagenesis studies of hMC1 receptor showed that mutations of Asp-117 (D117) in the TM3, or His-260 (H260) in TM6 to Ala resulted in loss of affinity for αMSH but not for NDP-MSH. These data were used to molecular modelling and resulted in conclusion of putative interaction of D117 and H260 in the MC1 receptor with His6 and Glu5 in the MSH peptides. Due to this hypothesis we tested MSH peptides with exchanged His6 in the MSH peptide with Tyr6 and Glu5 to Asp5 (SHU9119), Melanotan II and did not find support to this idea. These data

combined may indicate that D117 and H260 mutations cause conformational changes in the receptor which can not be linked to any specific amino acid in the MSH peptides.

2)Truncated clones - (papers VII-VIII).

The genomic DNA sequence for the hMC3 shows a 361 amino acid long open reading frame. MC3 receptor Met38 aligns with Met1 in the hMC1. The N-terminal of mouse and rat MC3 are lacking the first ATG corresponding to the proposed transaction initiation codon of hMC1.Therefore MC3 receptor has for 37 aminoacids shorter N-terminal region as MC1. In order to investigate the importance of longer N-terminal chain we made 3 mutant clones MC3 ATG1, MC3 ATG2 and MC3 with deleted sequence between first and second ATG (Mc3-Mto M). Binding results showed that all MC3 clones are similar to the original wild type. Further we created 11 truncated clones of hMC1, hMC3, hMC4 and hMC5 receptors in order to investigate the putative participation of N-terminal region in ligand binding. The results show that 27, 25, 28 and 20 amino acids could be deleted from N-terminus of MC1, MC3, MC4 and MC5, respectively, including all potential N-terminal glycosylation sites in the MC1 and MC4 receptors, without affecting ligand binding.

3) Transmembrane regions (paper IX-X).

MC receptors belong to seven TM G-protein coupled receptors. TM4 and TM5 show low amino acid homology within the MC receptor family. In order to investigate the participation of different TM domains of MC receptors TM4 and TM5 of the MC3 were separately changed so that their amino acid sequences corresponded identically with MC1. These data provided evidence that these domains do not participate in ligand binding. Creating a series of chimerical MC1/MC3 receptors showed that residues within TM1 must participate in they selective binding. Moreover, the data on the chimeras MC1/MC3 indicate that TM1, TM2, TM3, TM6 and end domains of TM7 are making up the binding pocket for the MSH peptides.

4. Characterisation of all MC-receptor subtypes (paper XI-XIV).

All new cloned MC receptors have been characterised by radioligand binding and cAMP determination approaches.

Table. Ligand potency order

MC1	NDP-MSH αMSH βMSH ACTH γMSH
MC2	ACTH
MC3	NDP-MSH γMSH βMSH αMSH ACTH
MC4	NDP-MSH βMSH αMSH ACTH γMSH
MC5	NDP-MSH αMSH βMSH ACTH γMSH

The MC1 and MC5 have the same potency order but for MC5 ligand affinities are more than 100-fold lower than for MC1. MC3 receptor binds γMSH with 2-3 fold higher affinity as αMSH. However γMSH binds better to MC1 and thus is not selective binder to MC3. The main difference between the MC4 and the MC1 and MC3 is that γMSH binds with particularly low affinity to MC4.

ACTH1-39 has similar binding potency as αMSH for the all mentioned MC receptors but only because of first 13 amino acids which are identical to αMSH. The MC1-MC5 receptors do not have a binding epitope for ACTH beyond the sequence of αMSH.

MC2 receptor binds only ACTH, but not others melanocortin peptides. It was proved using mouse adrenocortical cell line Y1, that expresses MC2. These cells were found to bind only ACTH. When other MC subtype DNAs were transfected into these cells, characteristic binding of the iodinated NDP-MSH appeared for each of the receptor subtype.

5. Search for selective ligands to each of MC receptor subtype (papers XV-XX).

a) Screening of non-peptides and non-melanocortin peptides.

We have done relatively small screening of non-peptide substances and did not find binders with calculable Ki values for their binding to MC receptors.

Screening of non-melanocortin peptides such as TRH, its analogs, SP analog spentin, LHRH, Tyr6-LHRH, GHRH, somatostatin, enkephalins, endorphins, dynorphins and their analogs showed that few of them bind with micromolar Ki values to MC receptors, mostly MC1. TRH turned out to be also full agonist for receptor MC1 in the transfected COS cells and natural mouse melanoma cells B16F1. Dynorphin1-13 was partial agonist. Enkephalins, endorphins, LHRH, somatostatin, tymopentin, spentin did not bind at all with 1 mM highest concentration tested.

b) Binding of cyclic and linear MSH core peptides.

We showed that cyclic 5-, 6-, and 7-amino acid long core peptides had much lower affinities as their corresponding linear homologues. Moreover the relative loss of binding due to the cyclisation did not change as the ring size decreased. Therefore, decreasing the ring size does not seem to force the peptide into a more unfavourable conformation.

We synthesised 9 NDP-MSH analogues where the N- or C-terminals were deleted or exchanged by those of β- or γMSH and where the core residues His6, Phe7, Arg8, Trp9 were individually substituted by Glu6, D-Nal7, Lys8, His9, respectively. These results showed that N-terminal segment Ser1-Tyr2-Ser3 of NDP-MSH is not important for the binding to MC1. C-terminal Gly10-Lys11-Pro12-Val13 of NDP-MSH is clearly important for binding to all four MC receptors. These data indicated

that low affinity of γMSH for MC4 is due to its C-terminal Asp10-Arg11-Phe12. Substitution of D-Phe7 by D-Nal7 increased the affinity for MC4 but not for the other MC receptor subtypes.These results are valuable for molecular modelling and design of selective drugs for MC receptors.

c) MC4 receptor selective antagonists.

Synthesis of a novel series of cyclic MSH analogues lead to discovery that compounds with 26 membered rings of Cys4, D-Nal7, Cys11 αMSH4-11 displayed specific MC4 receptor selectivity. They also showed strong antagonistic properties in cAMP stimulation assay.

We found that a compounds with 29 members ring of Cys3, Nle10, D-Nal7, Cys11 αMSH3-11 had highest affinity for the MC3 receptor.

We were first who reported truly MC4 and MC3 selective ligands. These substances are used to clarify the physiological roles of the MC3, MC4 and MC5 receptors. It is already reported that HS014 increases food intake in freely moving rats.

d) ACTH4-10 analogues.

Phe-I7, Ala6 and D-Ala4, Gln5, Tyr6 substituted ACTH4/10 analogues were reported to be putative MC receptor antagonists. Analysis of their binding properties showed that His6 has a specially important role in binding to the MC receptors. Replacement of Phe7 by para-iodo-Phe resulted in loss of affinity for the MC1 receptor, but BIM22015 and ORG2766 did not bind to MC receptors at all.

e) Cyclic D-Nal7 and D-Phe 7 αMSH analogues.

The binding of the 2 cyclic lactam MSH4-10 analogues MTII and SHU9119 and 5 cyclic Cys4, Cys10-αMSH analogues were tested on COS cells transiently expressing the human MC receptors. Substitution of D-Phe7 by D-Nal7 resulted in a shift in favour of selectivity for the MC4 receptor. HS9510 showed the highest selectivity for the MC4 receptor among all the substances tested. However cyclic lactams displayed an over all highest affinity for the MC receptors than cyclic Cys-Cys αMSH4-10 analogues.

f) New αMSH analogues MSH-B, HP-228, GHRP-6 and 153N-6.

Recently new MSH analogues were isolated from pituitary gland of the see lamprey and MSH-B was found to be 10 times more potent than αMSH in a frog skin darkening test. Another synthetic analog HP-228 was shown to inhibit induction of nitric oxide synthase in vivo. MSH antagonist 153N-6 was identified by screening of a library of MSH5-13. A peptide GHRP-6 was identified in a search for a competitive MSH antagonist using frog skin bioassay. This compound is enkephalin analog also known as SK&F. All tested substances had the highest affinities for the MC1 receptor. MSH-B has similar potency as a αMSH and these two peptides induced also similar cAMP stimulation level in murine B16F1 melanoma cells.

6.Expression of MC1 receptor in insect cells (paper XXI).

Recombinant baculovirus-based insect cell expression system generally yields high expression levels of functional proteins what is not easily to reach in mammalian cells .In these studies insect Sf9 cells were engineered for expression of two different epitope-tagged versions of hMC1 Flag-MC1-His and hMC1-Myc. The virus infected Sf9 cells expressed functional MC1 in high numbers and displayed ligand binding pharmacology identical to that observed in mammalian cells.The expression level was pmoles of binding sites per mg of protein and could be suitable for purification of receptor by using immobilised metal chelation affinity chromatography or immunoaffinity chromatography.

7. Phage display (paper XXII).

A phage display system for the selection of peptides binding to heterologously expressed hMC1 on the surface of insect cells has been established. Insect cells were used to select binders from phage library, in which critical determinant Phe7-Arg8-Trp9 were kept, whereas the flanking regions were allowed to vary freely. This was first phage display which was used successfully with G-protein coupled receptors lacking an extracellular binding domains. From all screened peptides in this library MS04 was found to be the most selective binder to hMC1 described so far.

CONCLUSIONS

1. We and others have reported the cloning of five melanocortin receptors. They belong to G-protein coupled 7 transmembrane segments receptor family and are the shortest proteins in this family.

2. MC receptor localised on surface of malignant tumour cells and melanocytes is proved to be MC1.

3. All MSH/ACTH peptides bind to MC1, MC3, MC4 and MC5 receptors. but only ACTH can bind to MC2 receptor. Besides MC2 receptor shows considerably less sequence homology to the other MC receptors. For example, the homology of MC2 receptor to MC1 is only 25% and MC2 to the MC3 receptor is only 29%.
α-MSH is selective for MC1 receptor, the MC3 has relatively high binding affinity for γ-MSH. MC5 receptors shares the same potency order of natural MSH peptides as the MC1 but has much lower affinities. MC4 is special in a way that has very low affinity to γ-MSH peptides.

4. Taking together all mutagenesis studies conclusion is that TM1, TM2, TM3, TM6 and end domains of TM7 are making up the binding pocket for the MSH peptides. N-terminal regions, extracellular loops and TM4, TM5 do not play an important role for the ligand binding.

5. Radioligand binding studies of novel (synthesised in Uppsala, Department of Pharmaceutical Pharmacology) MSH/ACTH analogues confirm that we have found general roles for MC3 and MC4 receptors selective substances. Thus, we have discovered that 26 members cyclic [Cys^{4}, D-Nal^{7}, Cys^{11}] αMSH(4-11) derivatives displayed specific MC4 receptor selectivity, but 29 members ring analogues MC3 selectivity.
It is also shown that these substances are antagonists in MC3 and MC4 transfected cells. HS014 shows antagonistic properties also *in vivo* experiments and increased food intake in rats.

6. The MC1 receptor is functionally expressed in insect cells with concentration of 10 pmoles per mg of total protein, what is the highest reported expression level and may be used for receptor large scale expression, purification, crystallisation etc.

7. Screening of phage display library led to so far the most selective peptide MS04 for MC1 receptor. This peptide is used as lead substance for creating analogues, which bind at pM affinities to MC1.

8. The analysis of earlier published MC active substances shows that SHU9119 (MC4 receptor antagonist) is practically non-selective in binding studies.
153N-6, MSH-B and HP-228 is selective for MC1. ORG2766 and BIM22015 do not bind to the cloned MC receptors.

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MOLECULAR CLONING OF A NOVEL HUMAN MELANOCORTIN RECEPTOR

Vijay Chhajlani, Ruta Muceniece and Jarl E. S. Wikberg

Department of Pharmaceutical Pharmacology, Box 591, Biomedical Centre, Uppsala 75124, Sweden

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A human genomic clone designated MC-2 is isolated. The cloned DNA codes for a protein of 325 amino acids which possesses seven hydrophobic segments, a characteristic of G-protein coupled receptors. The MC-2 receptor is expressed in brain tissue but not in the melanoma cells. When the MC-2 DNA is expressed in COS-7 cells, it binds [^{125}I]-labelled [Nle^4, D-Phe^7]- α melanocyte stimulating hormone (NDP-MSH) which then could be displaced by melanotropic peptides α-MSH, β-MSH, γ-MSH and adrenocorticotropic hormone, but not by non-melanotropic peptide β-endorphine. The highest affinity of 5.18 nM was for the NDP-MSH peptide. The novel MC-2 receptor and the MC-1 receptor, described earlier by us (8) showed identical order of affinity for the melanocortin peptides, but the affinities and the fold differences in the affinities to the melanocortin peptides were different when compared to the earlier described MC-1 receptor. The results suggest that the MC-2 DNA codes for a novel melanocortin receptor. © 1993 Academic Press, Inc.

Melanocortin peptide family consists of α-MSH (melanocyte stimulating hormone), β-MSH, γ-MSH as well as ACTH (adrenocorticotropic hormone). All these peptides are generated from a common precursor, the pro-opiomelanocortin (POMC) by post-translational processing (1). Apart from pituitary, the melanocortin peptides are known to be expressed in other human tissues like skin, spleen and testis (2). Although the receptors for melanocortins are present in melanocytes, adrenocortical cells, immune cells and central nervous system (3), they display different pharmacological properties depending on the tissue they are expressed in (4, 5, 6, 7). Recently, we (8) and others (9, 10) have reported the cloning of melanocortin receptors. It is evident from these reports that a particular MSH receptor showing the highest affinity for α-MSH in radioligand binding (8) as well as cyclic AMP (9)

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assays is expressed only in the melanoma cells (originated from melanocytes) and not in the other tissues including CNS and immune system. Gantz et al (10) have recently described the cloning of a melanocortin receptor which is expressed in the brain, placental and the gut tissues but not expressed in the melanoma cells. In the present report we describe the molecular cloning of yet another novel melanocortin receptor which is expressed in brain but not in melanoma cells. We have called the novel receptor MC-2, for simplifying the nomenclature of different melanocortin receptors.

MATERIALS AND METHODS

Isolation of a genomic clone. The oligonucleotide primers for polymerase chain reaction (PCR) were designed based on the nucleotide sequence of the cloned MSH-receptor (8). The primers were synthesized with EcoRI and BamHI linkers at the 5´ ends to facilitate the cloning. The primer sequences were:

(i) upstream primer 5´GGGAATTCTACGCACTGCGCTACCACAGCATCGTG and

(ii) downstream primer 5´-GGGGATCCAATGCCCAGCAGGATGGTGAGGGTGA.

Human genomic DNA was subjected to PCR using the above described primers. The PCR was done in a final volume of 50 µl and contained 1 µg human genomic DNA, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 µM of each deoxynucleotide, 1 µM of each primer and 1 unit of enzyme Taq DNA polymerase (Perkin Elmer Cetus, USA). The PCR thermal profile used was 93°C for 60 seconds, 55°C for 40 seconds and 72°C for 60 seconds for a total of 25 cycles. Ten percent of the reaction mixture was analysed by agarose gel electrophoresis. One product was observed which was cloned into the pGEM7zf(+) vector (Promega Corp., USA) and the resulting plasmid, termed G8, was sequenced using the chain termination method (11).
Approximately 10^6 clones of a human placental DNA genomic library (Stratagene, USA) were screened with [^{32}P]-labelled G8 probe. Hybridization was done in a buffer (6 x SSC, 5 x Denhardt's solution, 10mM sodium phosphate pH 7.0, 1mM EDTA, 0.5% SDS and 0.1 mg/ml denatured salmon testis DNA) at 60°C for 12 hours. Positive plaques were picked and after repeating the screening two more times a positive plaque, designated MC-2, was isolated. A 2400 bp Sac I fragment from the genomic clone MC-2, was subcloned into the pGEM5zf(+) vector and both strands of the entire coding region were sequenced using the chain termination method (11).
Functional expression of the MC-2 DNA. The entire coding region of the MC-2 genomic DNA clone was inserted into the pRC/CMV vector (Invitrogen Corp. USA). COS-7 cells were grown in Dulbecco's modified Eagle's medium with 8 percent foetal calf serum and non-essential

amino acids. Eighty percent confluent cultures were transfected with 1 µg DNA and 40 µg lipofectin (BRL, USA) in serum free medium. Five hours after transfection the serum-containing medium was replaced and cells were cultivated for 20 hours. Cells were then scraped off, centrifuged, resuspended in serum-containing medium, plated on 48 well plates, and allowed to grow for 24 hours. The cells were then washed with 0.3 ml of binding buffer (minimum essential medium with Earle's salts, 25 mM HEPES pH 7.0, 0.2% bovine serum albumin, 1 mM 1,10-phenanthroline, 0.5 mg per litre leupeptine and 200 mg per litre bacitracin) and then incubated at 37°C for 2 hours with 0.2 ml of binding buffer containing 24,000 CPM of [^{125}I]-NDP-MSH and the appropriate concentration of unlabelled peptides. NDP-MSH was labelled with [^{125}I] and purified as described before (3). The plates were then put on ice, the cells washed with 0.3 ml of ice cold binding buffer, and detached from plates with 0.3 ml of 0.1N NaOH. Radioactivity was counted and data analysed by an iterative, non-linear curve fitting programme suitable for radioligand binding analysis.

RNA detection by PCR analysis. RNA from human brain tissue (purchased from Clontech, USA) and WM266-4 melanoma cells (made by Fast Track kit from Invitrogen Corp. USA) were reverse transcribed with SuperScript RNase H$^-$ reverse transcriptase (BRL, USA). PCR was performed on samples before and after reverse transcription to rule out the posibility of genomic DNA contamination in RNA preparations. Five µg of RNA was used for reverse transcription and then all of it was used as template in the first PCR. The first PCR was performed with primers (described below as number 1 & 2) specific for the 5'- and 3'-untranslated regions of the MC-2 DNA. Ten percent of the first PCR reaction was then subjected to a second PCR with primers (described below as number 3 & 4) specific for the coding region of the clone MC-2.

Primer 1: 5'-GGAAGCTTTCTTTGGTAGGCTG
Primer 2: 5'-GGTCTAGAGCCACAGAGAGGAG
Primer 3: 5'-CTGCATTTCTTGGATCT
Primer 4: 5'-AAGCTGCACATGGATGC

Both the PCRs were performed with Gene amplification kit (Perkin Elmer Cetus, USA). The PCR thermal profile used was 93°C for 60 seconds, 55°C for 40 seconds and 72°C for 60 seconds for a total of 40 cycles. Fifty percent of the reaction was analysed by agarose gel electrophoresis. The product at the 380 bp position was analysed by restriction enzyme analysis and was found to contain the expected pattern (data not shown).

RESULTS AND DISCUSSION

PCR primers were designed based on the consensus sequence of the human MSH-receptor (8). Primers were used to perform PCR on human genomic DNA, as many G-protein coupled receptors are known to be intronless. One

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-375  GTG TCC AGG GGC ACT CTT CCA GGT CTC AGG AAC GCA GGT CAG AAT GTG CAA GCC AGC TGC  -316
-315  CGG GCA CGT GGC TCA CCC CTG TAG TAC CAG CAC TTT GGG AGG CTG AGA GAG AAG ATC GCT  -256
-255  TGT GGC CAG GAG TTT GAG ACC AGA CTG GGG CTT CAT AGG GAG ACC CTG TCT CTT AAA AAA  -196
-195  AAA AAA AAA AAA GGA CTG AGT GAG CCG AGC CCA GTC CTC TCA TGC ACT GTG TCA TTC ATC  -136
-135  CCC TTT CTT AGG CTG TGT TGG TTC TAG GCT AGC TGC TGT CTT TCT TTG GTA GGC TGC TAA   -76
 -75  CCT CTT TGG ATT GTG AAT TTA AAA CAT GTT TTA CAG TAA ATT TGC TGC CAA GAC AAG AGG   -16
 -15  TGT ATT TCT CCA GCA ATG AAT TCC TCA TTT CAC CTG CAT TTC TTG GAT CTC AAC CTG AAT    44
   1                      Met Asn Ser Ser Phe His Leu His Phe Leu Asp Leu Asn Leu Asn    15
                               *

  45  GCC ACA GAG GGC AAC CTT TCA GGA CCC AAT GTC AAA AAC AAG TCT TCA CCA TGT GAA GAC   104
  16  Ala Thr Glu Gly Asn Leu Ser Gly Pro Asn Val Lys Asn Lys Ser Ser Pro Cys Glu Asp    35
                  *                           *

 105  ATG GGC ATT GCT GTG GAG GTG TTT CTC ACT CTG GGT GTC ATC AGC CTC TTG GAG AAC ATC   164
  36  Met Gly Ile Ala Val Glu Val Phe Leu Thr Leu Gly Val Ile Ser Leu Leu Glu Asn Ile    55
                                                  I

 165  TTG GTC ATA GGG GCC ATA GTG AAG AAC AAA AAC CTG CAC TCC CCC ATG TAC TTC TTC GTG   224
  56  Leu Val Ile Gly Ala Ile Val Lys Asn Lys Asn Leu His Ser Pro Met Tyr Phe Phe Val    75

 225  TGC AGC CTG GCA GTG GCG GAC ATG CTG GTG AGC ATG TCC AGT GCC TGG GAG ACC ATC ACC   284
  76  Cys Ser Leu Ala Val Ala Asp Met Leu Val Ser Met Ser Ser Ala Trp Glu Thr Ile Thr    95
                                              II

 285  ATC TAC CTA CTC AAC AAC AAG CAC CTA GTG ATA GCA GAC GCC TTT GTG CGC CAC ATT GAC   344
  96  Ile Tyr Leu Leu Asn Asn Lys His Leu Val Ile Ala Asp Ala Phe Val Arg His Ile Asp   115

 345  AAT GTG TTT GAC TCC ATG ATC TGC ATT TCC GTG GTG GCA TCC ATG TGC AGC TTA CTG GCC   404
 116  Asn Val Phe Asp Ser Met Ile Cys Ile Ser Val Val Ala Ser Met Cys Ser Leu Leu Ala   135
                                              III

 405  ATT GCA GTG GAT AGG TAC GTC ACC ATC TTC TAC GCC CTG CGC TAC CAC CAC ATC ATG ACG   464
 136  Ile Ala Val Asp Arg Tyr Val Thr Ile Phe Tyr Ala Leu Arg Tyr His His Ile Met Thr   155

 465  GCG AGG CGC TCA GGG GCC ATC ATC GCC GGC ATC TGG GCT TTC TGC ACG GGC TGC GGC ATT   524
 156  Ala Arg Arg Ser Gly Ala Ile Ile Ala Gly Ile Trp Ala Phe Cys Thr Gly Cys Gly Ile   175
                                              IV

 525  GTC TTC ATC CTG TAC TCA GAA TCC ACC TAC GTC ATC CTG TGC CTC ATC TCC ATG TTC TTC   584
 176  Val Phe Ile Leu Tyr Ser Glu Ser Thr Tyr Val Ile Leu Cys Leu Ile Ser Met Phe Phe   195

 585  GCT ATG CTG TTC CTC CTG GTG TCT CTG TAC ATA CAC ATG TTC CTC CTG GCG CGG ACT CAC   644
 196  Ala Met Leu Phe Leu Leu Val Ser Leu Tyr Ile His Met Phe Leu Leu Ala Arg Thr His   215
                  V

 645  GTC AAG CGG ATC GCG CTC TGC CCG GGG CCA GCT CTG CGC GGC AGA GGA CCA GCA TGG CAG   704
 216  Val Lys Arg Ile Ala Leu Cys Pro Gly Pro Ala Leu Arg Gly Arg Gly Pro Ala Trp Gln   235

 705  GGC GCG GTC ACC GTC ACC ATG CTG CTG GGC GTG TTT ACC GTG TGC TGG GCC CCG TTC TTC   764
 236  Gly Ala Val Thr Val Thr Met Leu Leu Gly Val Phe Thr Val Cys Trp Ala Pro Phe Phe   255
                                                              VI

 765  CTT CAT CTC ACT TTA ATG CTT TCT TGC CCT CAG AAC CTC TAC TGC TCT CGC TTC ATG TCT   824
 256  Leu His Leu Thr Leu Met Leu Ser Cys Pro Gln Asn Leu Tyr Cys Ser Arg Phe Met Ser   275

 825  CAC TTC AAT ATG TAC CTC ATA CTC ATC ATG TGT AAT TCC GTG ATG GAC CCT CTC ATA TAT   884
 276  His Phe Asn Met Tyr Leu Ile Leu Ile Met Cys Asn Ser Val Met Asp Pro Leu Ile Tyr   295
                                          VII

 885  GCC TTC CGC AGC CAA GAG ATG CGG AAG ACC TTT AAG GAG ATT ATT TGC TGC CGT GGT TTC   944
 296  Ala Phe Arg Ser Gln Glu Met Arg Lys Thr Phe Lys Glu Ile Ile Cys Cys Arg Gly Phe   315

 945  AGG ATC GCC TGC AGC TTT CCC AGA AGG GAT TAA CGA CAA AGT GCT CCT CTC TGT GGC TCT  1004
 316  Arg Ile Ala Cys Ser Phe Pro Arg Arg Asp                                           325
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Fig.1. Nucleotide and the predicted amino acid sequence of novel human melanocortin receptor. Transmembrane segments (underlined) were determined by hydropathy analysis according to Kyte and Dolittle (16). The asparagine linked glycosylation sites are marked (★) under the respective amino acids.

product, designated as G8 was obtained which contained a 306 nucleotide long sequence, showing G-protein coupled receptor characteristics and about 15% homology to the already cloned MSH-receptor (8). This DNA was then used to screen a human genomic library. A clone designated MC-2 was isolated. From this clone a 2400 bp Sac I restriction fragment was subcloned and sequenced. The cloned DNA contained an open reading frame of 975 bp beginning with GCAATGA which is in agreement with the Kozak

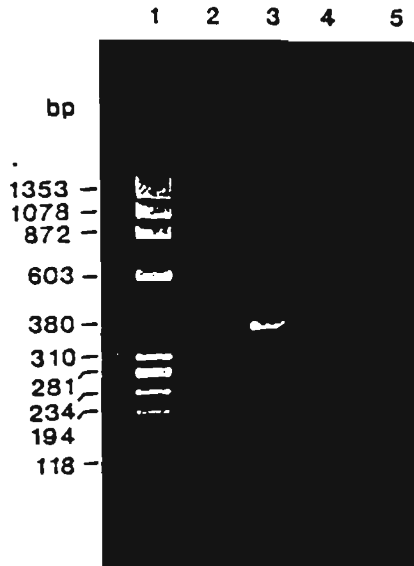

Fig.2. Agarose gel electrophoresis analysis of the PCR products generated from the human brain and melanoma cells mRNA. Lane 1-molecular weight markers, Lane 2-human brain mRNA without reverse transcription, Lane 3-human brain mRNA after reverse transcription, Lane 4-human melanoma cells mRNA without reverse transcription, Lane 5-human melanoma cells mRNA after reverse transcription. A specific product at the expected position of 380 bp is seen only in the human brain sample after reverse transcription.

consensus sequence for translation initiation sites (12). The entire protei consists of 325 amino acids with seven hydrophobic segments of amino acids, a characteristic of G-protein coupled receptors (Fig. 1). The N-terminal region contains three potential N-glycosylation sites at amino acid positions 2, 20 and 28.

The mRNA from human brain and melanoma cells was converted to cDNA and then subjected to two successive PCR amplifications using two different sets of primers. In the first PCR whole coding region was amplified and the product was subjected to second amplification with primers within the coding region. A specific product of 380 bp was seen in the mRNA sample from brain but not in the melanoma sample (Fig. 2). No product was observed when the PCR was performed on mRNA without converting it to the cDNA, thereby ruling out the possibility of genomic DNA contamination in the mRNA samples. The melanocortin receptor cloned earlier by us (8) and others (9) was found to be expressed only in the melanoma tissue and no where else. Gantz et al (10) recently described cloning of another melanocortin receptor which is not at all expressed in melanoma cells but expressed in brain, placenta and gut tissues. The novel receptor described in this report is also expressed in

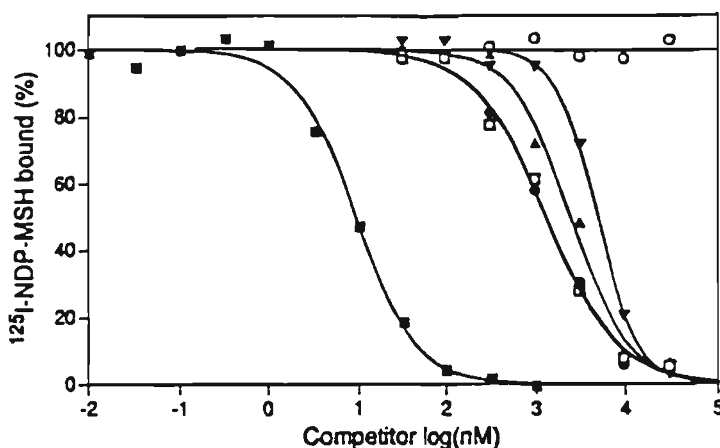

Fig.3. Relative potencies of melanotropins for inhibiting [^{125}I]-NDP-MSH binding to COS-7 cells transfected with MC-2 receptor DNA. Shown are competition curves for non-labelled NDP-MSH (■), α-MSH (●), ACTH (1-39) (□), β-MSH (▲), γ-MSH (▼) and β-endorphine (O).

brain but not in melanoma cells (Fig. 2). These results taken together raise a very interesting possibility, that the receptor expressed in melanoma tissue is only expressed there and the other melanocortin receptors (described in this report and in ref. 9 and 10) are expressed at least in brain and some selected peripheral tissues. Thus the receptor expressed in melanoma cells is the only one likely to be mediating the melanin synthesis, and by contrast the MC-2 (described in this report) and MC-3 (10) receptors are likely to be mediating the other diverse actions of melanocyte stimulating hormone. These actions include effect on attention, memory and learning (5,13), temperature control in the central nervous system (14), stimulation of the endocrine system (15) and modulation of the immune inflammatory responses (6).

When the MC-2 receptor DNA was expressed in COS-7 cells and analysed by radioligand binding analysis the [^{125}I]-NDP-MSH was displaced from the expressed receptor by melanotropic peptides (Fig. 3). The potency order was NDP-MSH (K_i = 5.18 ± 0.54 nM), α-MSH (K_i = 928 ± 314 nM) > ACTH (1-39) (K_i = 929 ± 389 nM) > β-MSH (K_i = 1.75 ± 0.67 μM) > γ-MSH (K_i = 3.45 ± 0.88 μM). The non-melanotropic peptide β-endorphine showed no affinity for the expressed MC-2 receptor. Although the potency order is identical to the one reported earlier by us for the MC-1 receptor (8), the differences in the affinities among different peptides is quite high. For example the difference in the affinities of NDP-MSH and α-MSH for the MC-1 receptor (8) was only four fold as compared to 185 fold for the MC-2 receptor described in this report. Also the novel MC-2 receptor shows several fold lower affinities as compared to the MC-1 receptor for the available melanotropic

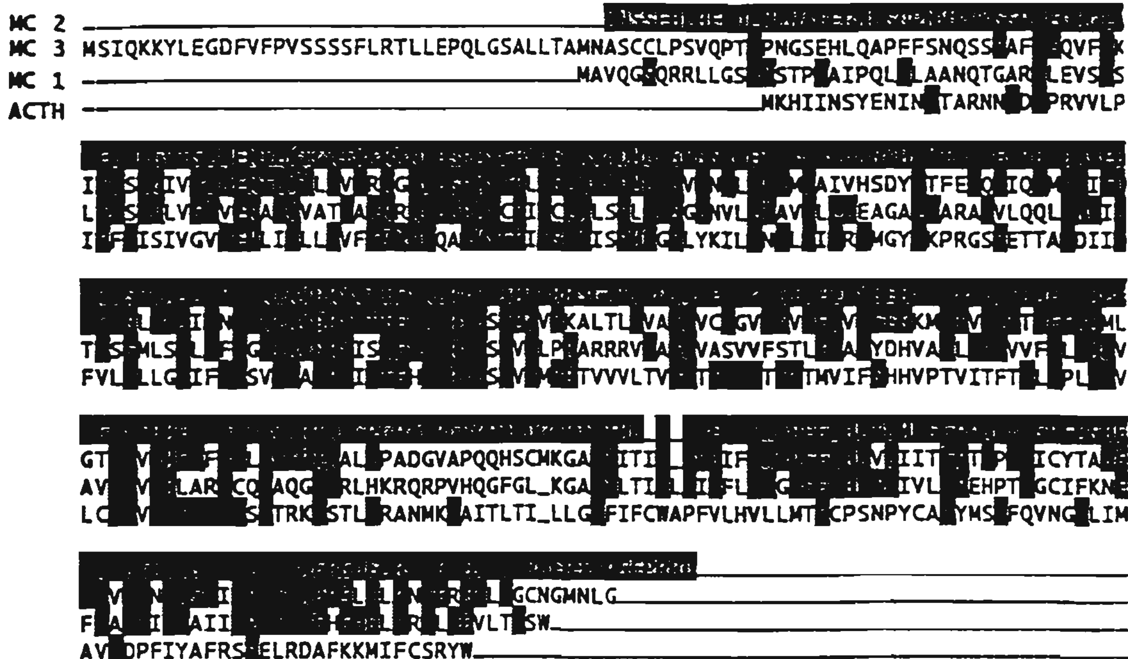

Fig.4. The amino acid sequence comparison of the melanocortin receptors. The novel MC-2 sequence is compared to the other published sequences. The amino acids common in all four receptors are marked by dark shading. MC-3 is reported by Gantz et al (10). The MC-1 is reported earlier by us (8). The ACTH receptor is reported by Mountjoy et al (9).

peptides. These results suggest that the novel MC-2 receptor is a subtype of the melanocortin receptors. Perhaps, the low affinity of the known melanocortin peptides could suggest that, there may exist a natural, high affinity, endogenous ligand for the novel MC-2 receptor, which is yet to be discovered.

In the following we suggest a nomenclature for different melanocortin receptors based on sequence homologies among these receptors. We suggest that the receptor expressed in the melanoma cells should be called MC-1 (melanocortin 1) as that was the one cloned first (8,9). The receptor described by us in this report should be called MC-2, as the receptor described by Gantz et al. (10) is already called MC-3. The ACTH receptor (9) should just be called ACTH receptor as it shows considerably less sequence homology to the other melanocortin receptors. When the predicted amino acid sequence of the cloned MC-2 DNA is compared to the other melanocortin receptors (Fig. 4), it shows highest homology of 53% to the MC-3 receptor described by Gantz et al (10). The cloned MC-2 receptor and the MC-3 receptor show 42% homology to the MC-1 receptor expressed in the melanoma cells (8, 9). The homology of ACTH receptor to MC-1 receptor

is only 25% and to the MC-2 and the MC-3 receptors is only 29%. This produces a very clear picture that the receptor expressed in the melanoma cells (8,9) and the ones described by Gantz et al (10) and by us in this report should be grouped together and the ACTH receptor (9) may perhaps have a group of its own.

In this study we have described a melanocortin receptor MC-2, which is expressed in brain but not in melanoma cells. This novel receptor has a distinct primary structure. The expressed MC-2 receptor showed affinity to the natural melanotropic peptide ligands, but not to the non-melanotropic peptide β-endorphine.

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CANCER LETTERS

Immunological localisation of melanocortin 1 receptor on the cell surface of WM266-4 human melanoma cells

Yun Xia[a,*], Ruta Muceniece[b], Jarl E.S. Wikberg[a]

[a]*Department of Pharmaceutical Biosciences, Division of Pharmacology, Box 591, Biomedical Centre, Uppsala University, S-751 24, Uppsala, Sweden*

[b]*Laboratory of Pharmacology, Institute of Organic Synthesis, Riga, Latvia*

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Abstract

The localisation of melanocortin 1 receptor (MC1R) in WM266-4 human melanoma cells was investigated by applying an antipeptide antiserum specific for the cloned human MC1R (MSH receptor). In enzyme-linked immunosorbent assay (ELISA), the immunoreactivity was detected in the membrane fraction of WM266-4 cells. The ELISA reactivity could be inhibited by an antiserum pre-absorbed with its specific synthetic peptide. In immunocytochemistry, the specific immunoreactivity was demonstrated on the surface of the cells by using either biotin-avidin immunoalkaline phosphatase- or TRITC-staining method. These results indicate that the MC1R is prominently present on the plasma membrane of WM266-4 human melanoma cells.

Keywords: Melanocortin 1 receptor; Antibody; Melanoma cell

1. Introduction

The current knowledge of melanocortin receptors (MCRs) present in melanoma cells is mostly drawn from studies using ligand binding in which melanocortin peptides like Nle^4, $D\text{-}Phe^7$ α-MSH or β-MSH were labelled as probes for MCRS. Data obtained with the use of such techniques suggest that the specific receptor for melanocortin peptides is present on the cell membrane of the melanoma cells [1–3], but also that some of these receptors may be localised intracellularly [4,5]. The stimulation of the melanoma cell MC receptor leads to accumulation of cAMP, increase in cytosolic calcium [6] and activation of protein kinase C [7]. These biological responses seem to be involved in the regulation of cell proliferation, differentiation, pigmentation, and metastatic potential of melanoma [8–11].

Five different genes encoding structurally distinct MCRs were recently cloned [12–16]. These MCRs show distinct pharmacological responses to melanocortins and exhibit specific tissue distribution. The melanocortin 1 receptor (MC1R), that was originally cloned from the melanoma cells, displays high affin-

* Corresponding author. Tel.: +46 18 174406; fax: +46 18 559718.

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ity for melanocortin peptides like α-melanocyte-stimulating hormone (α-MSH) and is mainly expressed in cells of melanocytic origin [12,13]. In a previous study we developed antipeptide antisera against human MC1R, which were useful to detect the MC1R both in a genetically engineered COS cells expressing these receptors and in specimens of melanoma tumours [17]. However, the subcellular localisation of MC1R in melanoma cells requires further determination as the delineation of intracellular staining from cell membranous staining was difficult with the use of frozen tissue sections of melanoma tumours. The apparent quite unique distribution of MC1Rs to the tumour cells of malignant melanoma prompts the possibility to use antibodies directed against the receptor for the diagnosis and treatment of the disease. In the present study we were therefore interested in localising MC1R in WM266-4 human melanoma cells by using immunocytochemical approaches. Our results demonstrate that immunologically detectable MC1R are prominently present on the cell surface of human melanoma cells.

2. Materials and methods

2.1. Cell culture

WM266-4 human melanoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 1% MEM non-essential amino acid and 1% MEM vitamin solution, 100 IU penicillin/ml and 100 μg streptomycin/ml at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells grown in monolayers were detached from the culture flasks and collected by low speed centrifugation (700 *g*). The following preparations of the cells were according to different experimental procedures.

2.2. Receptor binding studies

A ^{125}I [Nle^4, D-Phe^7]-α-MSH (^{125}I NDP-MSH) binding assay was carried out essentially as described previously [12]. Incubation with 0.2 nM of label and appropriate concentrations of the unlabelled NDP-MSH was for 2 h at 37°C in MEM medium with Eagle's salts, 25 mM Hepes (pH 7.4), 0.2% bovine serum albumin, 1 mM 1,10-phenanthroline, 0.5 μg leupeptin/ml and 200 μg bacitracin/ml.

2.3. Antipeptide antiserum

An anti-peptide antiserum was prepared by immunising rabbits with a 11-mer synthetic peptide (M2-Y) derived from the amino terminus of cloned human MC1R [12], as described [17]. The specificity of the M2-Y antiserum binding to the human MC1R has been previously documented [17].

2.4. Enzyme-linked immunosorbent assay (ELISA)

The pellet from the 700 × *g* spin of harvested cells was resuspended in cold hypotonic buffer (10 mM Tris–HCl, 1 μg of leupeptin/ml, 1 mM EDTA, pH 7.6), and kept on ice for 30 min. The cells were disrupted by using a stepped micro tip with Sonifier B15 (4 × 10 s at 60 W of power output). The homogenate was centrifuged at 15 000 × *g* for 30 min and the pellet, assumed to represent the crude membrane fraction, was then re-suspended in the coating buffer (10 mM phosphate, 137 mM sodium chloride, 0.5 μg of leupeptin/ml, pH 7.6). The solution of crude membrane fraction (100 μl) was added into each well of the 96-well EIA plate and incubated overnight at 22°C. The following ELISA procedure was performed as described previously [17,18]. In brief, the coated plate was washed with distilled water and loaded with 100 μl of 1.5% bovine serum albumin in PBS for 1 h at 37°C to block unoccupied sites. The antiserum which had been diluted to various concentrations with 1.5% BSA in PBS was added into each well, and the plate was incubated at 37°C for 1 h. The peroxidase-conjugated, affinity isolated goat antirabbit immunoglobulin (DAKO, Glostrup, Denmark) diluted 1:2000 with 1.5% BSA in PBS was added to the wells. After incubating the plate at 37°C for 1 h, 1,2-phenylenediamine (DAKO) was added as chromogen substrate according to the instructions of the manufacturer and the colour developed was quantitated at 490 nM using a kinetic microplate reader (NOVA Biolabs). When competition experiments were performed, the antiserum (1:100 dilution) was pre-incubated with various concentrations of M2-Y peptide at 37°C for 1 h before being added to the ELISA plates.

2.5. Immunocytochemistry

The cells seeded onto the poly L-lysine coated glass slide were covered by the culture medium for 1–2 h in an incubator to allow the cells to attach well. Attached cells were washed twice with 0.01 M PBS (pH 7.4), and fixed in 4% paraformaldehyde for 8 min at 4°C, and then exposed to the antipeptide antisera directly or after permeabilizing with 0.3% Triton X-100 in PBS for 10 min. The antiserum was used at dilution of 1:50–500, and incubation was carried out overnight at 4°C. Slides were then processed using the biotin-avidin immunoalkaline phosphates kit (Vectastain ABC kit I, Vector Red; Vector Laboratories; 'ABC-staining'). The Vector Red product has red colour and is also highly fluorescent. Thus, the staining could be visualised by both light and fluorescence microscopy. The biotinylated secondary antibody (1:200) and chromogen substrate were used according to the instructions of the manufacturer. Levamisole solution (1.25 mM) was included in the substrate solution to inhibit endogenous alkaline phosphates. As an alternative to the ABC-staining, TRITC-conjugated swine anti-rabbit immunoglobulin (DOKA) was applied as secondary antibody (1:40) after the application of the M2-Y antiserum.

3. Results

Initial receptor binding studies showed that non-labelled NDP-MSH competed with ^{125}I NDP-MSH binding in the WM266-4 cells with a K_i of 80 pM. This K_i-value was essentially identical with that for NDP-MSH binding to MC1R expressing COS cells [19]. In ELISA assay, an immunoreactivity was detected by using the M2-Y antiserum on the plates coated with crude membrane fraction of WM266-4 cells (Fig. 1a). In contrast, only a minor reactivity was seen when the antiserum was replaced with pre-immune serum (Fig. 1a). Moreover, the positive ELISA response was strongly and dose-dependently inhibited by pre-incubating the M2-Y antiserum with 0.06–1 mg/ml of M2-Y peptide (Fig. 1b).

When the M2-Y antiserum was applied in conjunction with the TRITC- or ABC-staining methods intense immunoreactivity was seen around the cell surface of non-permeabilised WM266-4 cells (Fig. 2a,c and d). However, the scope and intensity of labelling varied for different cells; some cells stained intensely, some faintly and some did not even stain at all. The proportion of cells staining positively ranked approximately 8–31% for the individual experiments. Pre-immune serum did not give any specific staining for either TRITC- (Fig. 2b) or ABC-staining (not shown). Moreover, the staining was blocked by pre-incubating the M2-Y antiserum with 0.5 mg/ml of the M2-Y peptide (not shown). When cells were perme-

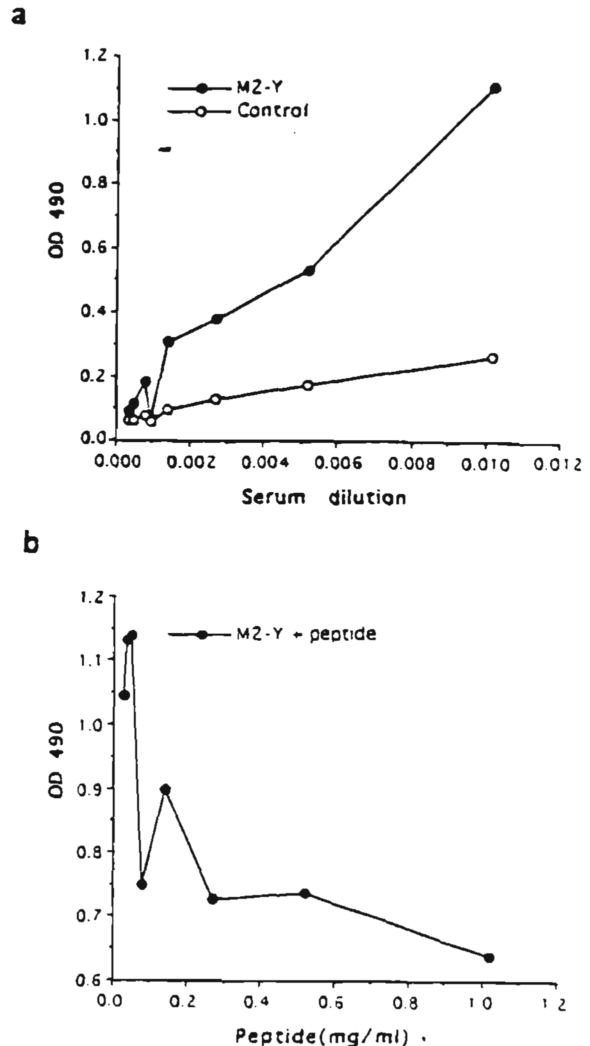

Fig. 1. The immunoreactivity for MC1Rs detected in the membrane fraction of WM266-4 melanoma cells by use of M2-Y antiserum. (a) ELISA responses of M2-Y antiserum (●) and pre-immune serum (O). (b) Inhibition of M2-Y antiserum ELISA reactivity by M2-Y peptide.

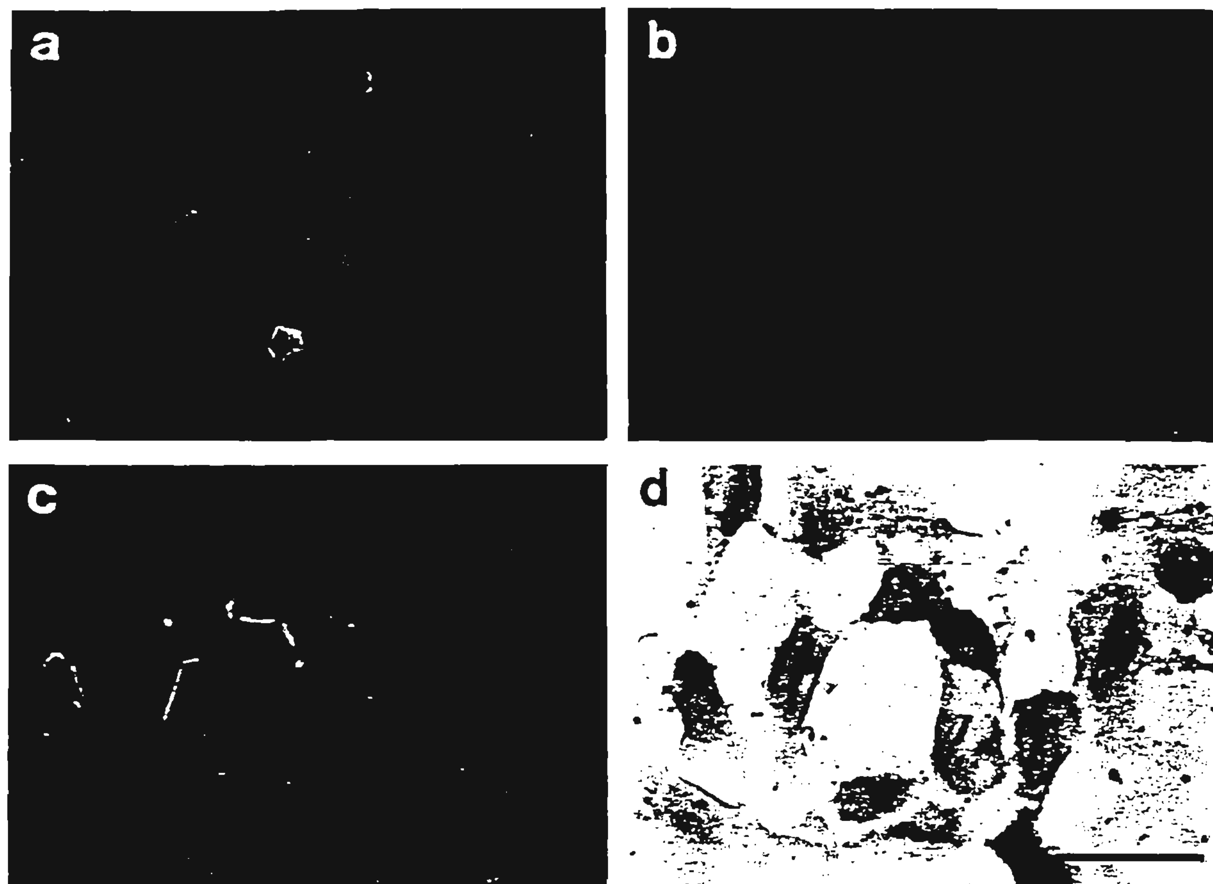

Fig. 2. Demonstration of cell surface localisation of MC1Rs on WM266-4 melanoma cells using TRITC-staining (a,b) and ABC-staining (c,d) with M2-Y antiserum. The fluorescent photographs (a) and (c) show the staining of the plasma membranes of the WM266-4 cells. In (b) is shown the fluorescent photograph of the control staining of cells with pre-immune serum. The corresponding light microscopic photograph of (c) is shown in (d). (a,b) Bar = 100 μm (c,d) Bar = 10 μm.

abilised with 0.3% Triton, intracellular staining could also be observed in some cells along with the intense staining of the cell membranes (Fig. 3).

4. Discussion

By use of immunocytochemical techniques we have demonstrated that prominent expression of MC1R is present on the cell surface of WM266-4 cells. However, we have also observed that not all cells are immunostainable and there was also a quite high variability in the proportion of cells staining between different experiments. This variability might reflect the differential expression of MC1R in the WM266-4 cell population. Earlier reports have suggested that the expression of MSH-receptors undergo a strong variation with the cell-cycle in melanoma cells and the majority of receptors is found to be present during the G2-phase [20–22]. There is some evidence that the cell density affects the responsiveness of melanoma cells to α-MSH [24,25] and it is possible that the density of the cells in culture may also influence the expression of MCRs. For other receptors, such as, e.g. for the α_{2A}-adrenoceptor in HT29 human adenocarcinoma cells, it has been reported that increasing cell density markedly increase their expression [23].

The present immunocytochemical localisation of MC1R on the surface of WM266-4 human melanoma cells is in agreement with previous studies where

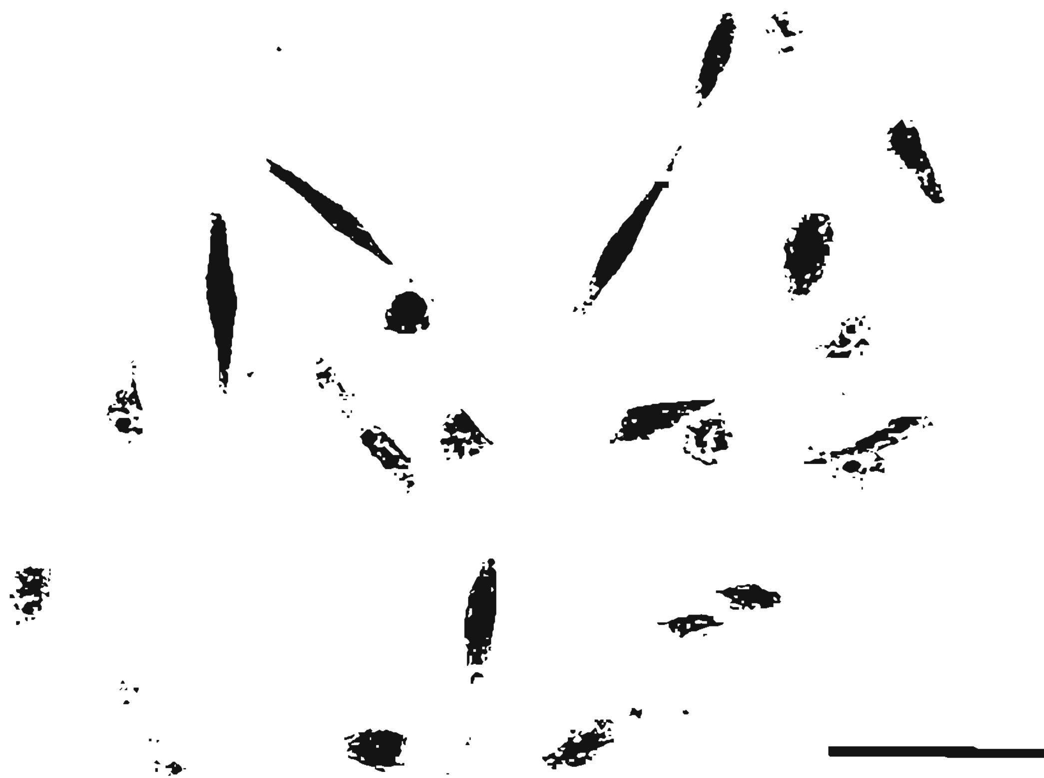

Fig. 3. Immuno-staining of MC1Rs in WM266-4 melanoma cells permeabilized with Triton using ABC-method. Shown is prominent immunostaining of the plasma membrane of the cells, as well as weaker staining in intracellular compartments. Bar = 50 μm.

autoradiography using [^{125}I]MSH as well as cytochemistry using a fluorescent FITC-MSH conjugate indicated that MSH receptors were present on the cell surface of melanoma cells [1,2,26]. However, these early studies also indicated that the MSH receptors had a patchy distribution with the receptors being arranged in clusters to limited areas on the surface of the melanoma cells. These observations are somewhat different to the present finding where the MC1R immunoreactivity was distributed quite uniformly along the plasma membrane of the WM266-4 cells. The reason for this difference is at present not clear, but could be due to differences in sensitivity and resolution of the methodologies used, or in differences in the cell lines employed.

Besides staining the cell membrane we also observed that M2-Y antiserum was capable of staining intracellular sites when the WM266-4 cells had been permeabilised with Triton. This result is compatible with previous studies where internal binding sites for MSH were detected in the melanoma cells [4,5].

In conclusion, by using immunocytochemical techniques we have demonstrated that the MC1R is localised to the cell surface of WM266-4 human melanoma cells. These cell surface receptors might in the future find use as a target for specific antibodies for the diagnosis and treatment of the malignant melanoma disease.

Acknowledgements

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Polyclonal antibodies against human melanocortin MC_1 receptor: preliminary immunohistochemical localisation of melanocortin MC_1 receptor to malignant melanoma cells

Yun Xia [a,*], Valdemar Skoog [b], Ruta Muceniece [a], Vijay Chhajlani [a], Jarl E.S. Wikberg [a]

[a] Department of Pharmaceutical Pharmacology, Box 591, Biomedical Centre, S-751 24 Uppsala, Sweden
[b] Department of Plastic Surgery, University Hospital, S-751 85 Uppsala, Sweden

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Abstract

Peptides of 11 and 15 residue lengths were synthesised according to the sequence of the N-terminal region of the human MC_1 melanocyte stimulating hormone receptor. The peptides were conjugated to thyroglobulin and used for preparation of antisera in the rabbit. Each of the conjugates raised antisera which showed high titre and specificity for its respective peptide antigen when evaluated in an ELISA test. Both types of antisera immunostained MC_1 receptor expressing COS-7 cells. By contrast, the sera did not stain control COS-7 cells not expressing the MC_1 receptor. Moreover, preimmune sera or antiserum preadsorbed with its respective peptide did not stain the MC_1 receptor expressing cells. The antisera were used to immunostain sections of normal human skin, as well as samples of cutaneous malignant melanoma tumours obtained from a patient. The cells of the melanoma tumours were very strongly immunostained with the MC_1 receptor antisera. By contrast, melanocytes which were present in the normal skin could not be visualised with our antisera.

Keywords: Melanocortin receptor; Antibody; Malignant melanoma

Introduction

Recently we and others have cloned five structurally related receptor cDNAs which encode proteins that bind melanocortin peptides such as melanocyte stimulating hormone (MSH) and adrenocorticotropic hormone (ACTH). These proteins, representing members of the G-protein coupled receptor family, are according to current nomenclature termed MC_1, MC_2, MC_3, MC_4 and MC_5 receptors(Chhajlani and Wikberg, 1992; Mountjoy et al., 1992; Gantz et al., 1993a,b; Chhajlani et al., 1993) [1]. Previous studies have indicated that the MC_1 receptor is expressed in melanoma cells (De Wied and Jolles, 1982; Chhajlani and Wikberg, 1992: Mountjoy et al., 1992), whereas the other melanocortin receptors are expressed in various other tissues such as brain, placenta and intestine (Chhajlani et al., 1993; Gantz et al., 1993a,b; Roselli-Rehfuss et al., 1993). Melanocortin receptors are known to mediate a variety of physiological responses of the melanocortin peptides, such as skin tanning, modulation of the immune system and modulation of various endocrinological and central nervous system functions (Smith et al., 1992; Catania and Lipton, 1993). The different types of responses are most likely mediated by specific types of the cloned melanocortin receptors. To study the physiology of the different subtypes of melanocortin receptors specific tools are required, e.g specific probes for

* Corresponding author.

[1] This nomenclature is the one proposed to the HUGO Nomenclature Committee and will likely be the final nomenclature agreed upon. Of these receptors the MC_1 receptor is the receptor originally cloned by us (Chhajlani and Wikberg, 1992) and Mountjoy et al. (1992). The MC_2 receptor is the presumed ACTH receptor cloned by Mountjoy et al. (1992). The MC_3 and MC_4 are the melanocortin receptors subsequently cloned by Gantz et al. (1992a,b), whereas MC_5 is yet another melanocortin receptor recently cloned by us (Chhajlani et al., 1993). Please note that the MC_5 receptor was originally termed MC_2 by us (Chhajlani et al. 1993). However, since the HUGO Committee had decided to reserve the term MC_2 for the receptor presumed to represent the ACTH receptor we have now decided to use the term MC_5 for our previously called 'MC_2' receptor, as this receptor is the fifth melanocortin receptor cloned.

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their cellular localization and pharmacological agonists and antagonists specific for the different types of melanocortin receptors. In the present study we have developed antisera directed towards the human MC_1 receptor. These antisera showed high specificity and could be used to immunostain MC_1 expressing cells, as well as cells of malignant melanoma tumours that had been obtained from a patient who had undergone surgical excision of the tumours.

2. Materials and methods

2.1. Preparation of immune sera

The amino acid sequence of the human MC_1 receptor was analysed with the antigenic index, according to Jameson and Wolf (1988) by using the University of Wisconsin GCG package (Madison, USA) in order to find suitable peptides showing high antigenicity. Moreover, the peptides were selected to avoid any similarities to the other cloned melanocortin receptors. Two peptides from the N-terminal region of the receptor, which corresponded to the amino acid residues 4–18 (peptide M1-Y) and 25–35 (peptide M2-Y), were selected from this analysis. The peptides were synthesised commercially (Multiple Peptide System; San Diego, CA, USA) and conjugated to bovine thyroglobulin, by using m-maleimidobenzoyl-N-hydroxysuccinimide as coupling reagent, to afford coupling of the terminal cystein of the peptide to thyroglobulin (Harlow and Lane, 1988). (For the M2-Y peptide the cystein of the native MC_1 receptor was utilised, whereas for the M1-Y peptide an extra cystein had been added at its C-terminus). thyroglobulin peptide conjugates (0.5 mg dissolved in 250 μl water) were emulsified at a ratio of 1:1 (v/v) in Freund's complete adjuvant and injected intramuscularly into Swedish loop rabbits. After three weeks, the rabbits were given booster injections with 0.5 mg of the conjugates in incomplete Freund's adjuvant and sera were collected 12–14 days after the boosters. From all the rabbits preimmune

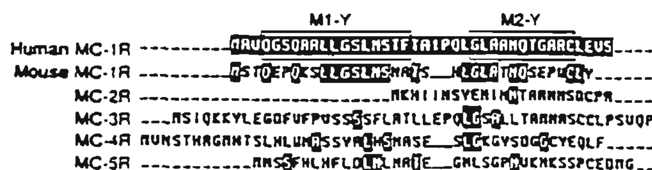
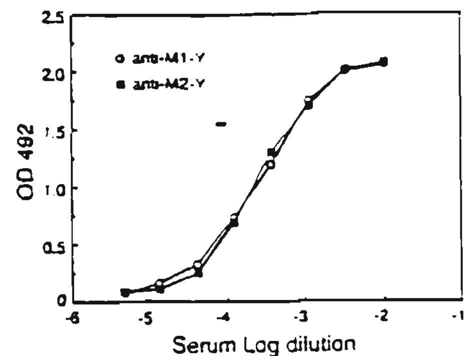


Fig. 1. Comparison of N-terminal amino acid sequences for the melanocortin receptors cloned to date. The sequeces were aligned by using the Mac Molly Tetra Package (Softgene, Berlin, Germany). The boxed sequences are the peptides (M1-Y and M2-Y) which were synthesised and used for immunisation of rabbits. As can be seen these peptides show no homology with other human melanocortin receptors.



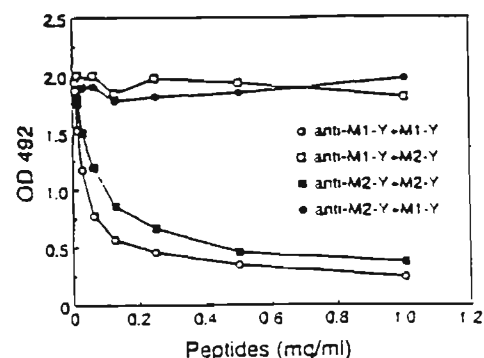


Fig. 2. Characterisation of antipeptide antisera with ELISA. The top panel shows ELISA responses of antisera against the respective synthetic peptides derived from the MC_1 receptor. Shown are curves for antibodies against peptide M1-Y (O), and peptide M2-Y (■). The bottom panel shows the interaction of antisera with synthetic peptides. Shown are dose-response curves of each antiserum with either the relevant or the irrelevant synthetic peptide. Each antiserum (1:100) was preincubated with peptide M1-Y or M2-Y at 37°C for 1 h before adding to the ELISA plates, as indicated in the panel.

sera had also been obtained before the immunisations. Sera were aliquoted and stored at −80°C until used.

2.2. Enzyme-linked immunosorbent assay (ELISA)

An ELISA method, similar to that of Appel et al. (1990), was applied to determine the titre and specificity of the antisera. In these assays, each well of a microtitre-plate (Costar 1/2 plates, Costar, Cambridge, USA) was coated with 70 pmol synthetic peptide in 0.1 M sodium carbonate buffer, pH 9.6 and incubated overnight at room temperature. The plates were then washed 10 times with deionised water to remove unbound peptide. The sites that had not become occupied by the peptide were thereafter blocked by incubating each well with 100 μl of 1.5% bovine serum albumin in phosphate buffered saline (33 mM phosphate, 145 mM NaCl), pH 7.2 for 1 h at 37°C. After washing 5 times with deionised water, 50 μl of the antiserum, which had been diluted to various concentrations with 1.5% bovine serum albumin in phosphate buffered saline, were added to each well followed by incubation at 37°C for 1 h. After washing 10 times with deionised

water, 50 µl peroxidase-conjugated, affinity isolated goat antirabbit immunoglobulins (DAKO, Glostrup, Denmark) diluted 1:2000 with [illegible]% bovine serum albumin in phosphate buffered saline were added to the wells, and the plate was incubated at 37°C for 1 h. After washing 10 times with deionised water, 1,2-phenylenediamine (DAKO) was then added as chromogen substrate according to the instructions of the manufacturer and the colour formed was quantified at 492 nM using a Titertech Multiscan microplate reader. When competition experiments were performed, the antiserum (1:100 dilution) was preincubated with various concentrations of the peptide at 37°C for 1 h before being added to the ELISA plates.

2.3. Cell culture and tissue specimens

COS-7 cells showing stable expression of the human MC_1 receptor were constructed by transfecting COS-7 cells with the human MC_1 receptor gene (a full account on the construction and properties of these cells will be published elsewhere). The cells were cultured in Dulbecco's modified Eagle medium with [illegible] mg/ml geneticin. Confluent cells were harvested and sedimented onto poly-L-lysine coated glass slides. The slides were covered with medium to allow the cells to grow for an additional [illegible] h. The slides were then rinsed two times in phosphate buffered saline and cells were fixed in acetone at [illegible] for [illegible] min. For control purposes non-transfected COS-7 cells were also cultured and treated in an identical fashion as the MC_1 expressing COS-7 cells. Malignant melanoma specimens were obtained from a [illegible]-year-old male patient who had tumours localised in the neck skin and subcutaneously in the arm. The diagnosis was confirmed by routine microscopic examination. The tumours were excised and divided and a minor piece taken for immunohistochemical staining. Normal skin specimens were obtained from patients undergoing plastic surgery for non-malignant conditions. The tissue specimens taken during operations were immediately frozen on dry ice. Cryostat sections (4 µm) were then cut and dried overnight and fixed as described above for the cultured cells.

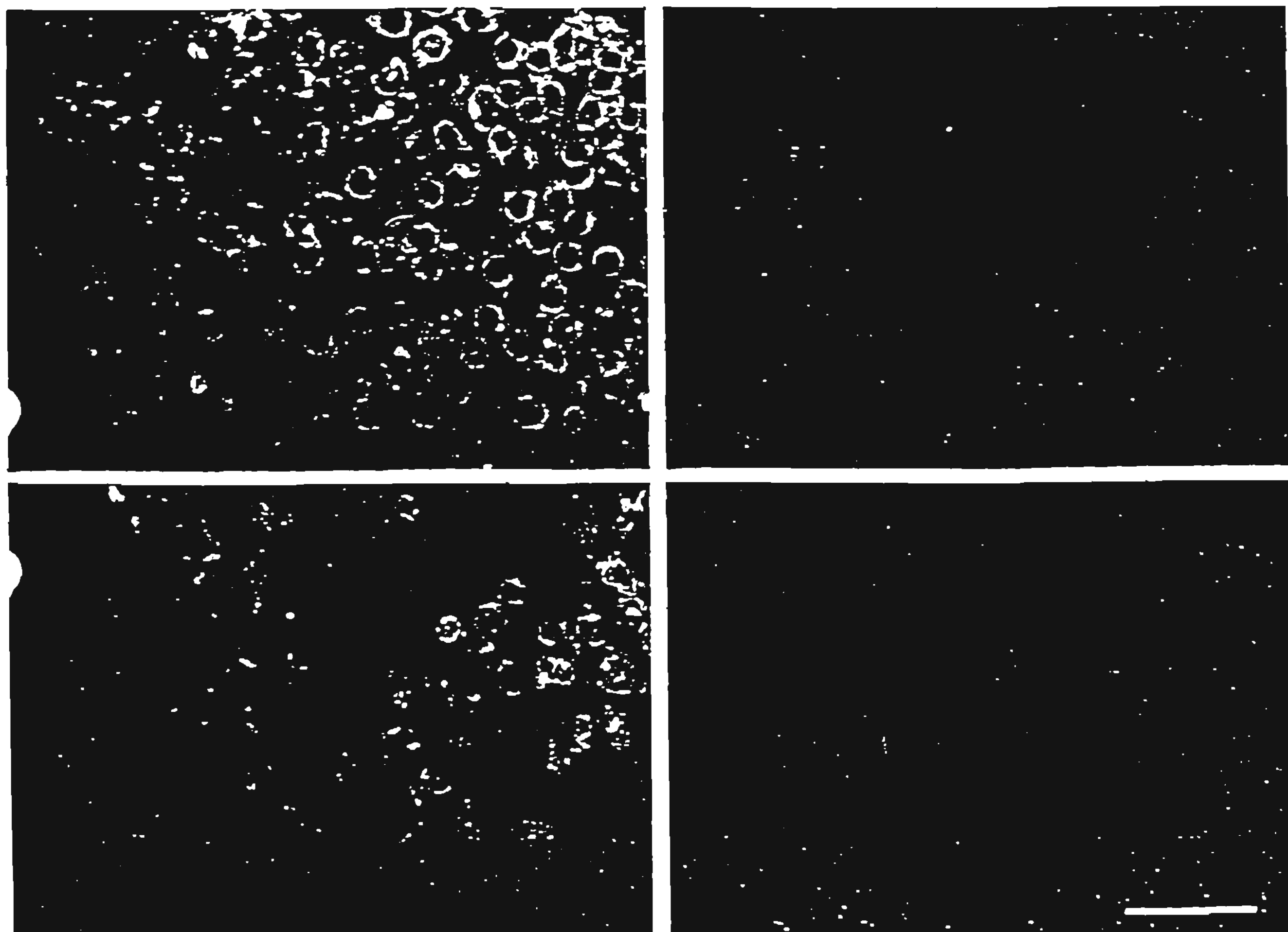


Fig. 3. Immunofluorescence staining of COS7 cells, with and without stable expression of MC_1 receptor, with the anti M1-Y and M2-Y sera. The left bottom and top panels show COS-7 cells of a cell line that had been genetically engineered to express the human MC_1 receptor. Shown in the right top and bottom panels are control COS-7 cells which do not express MC_1 receptors. In the left and right top panels are shown results for anti M1-Y serum. In the left and right bottom panels are shown results for anti M2-Y serum. Bar = 100 µM

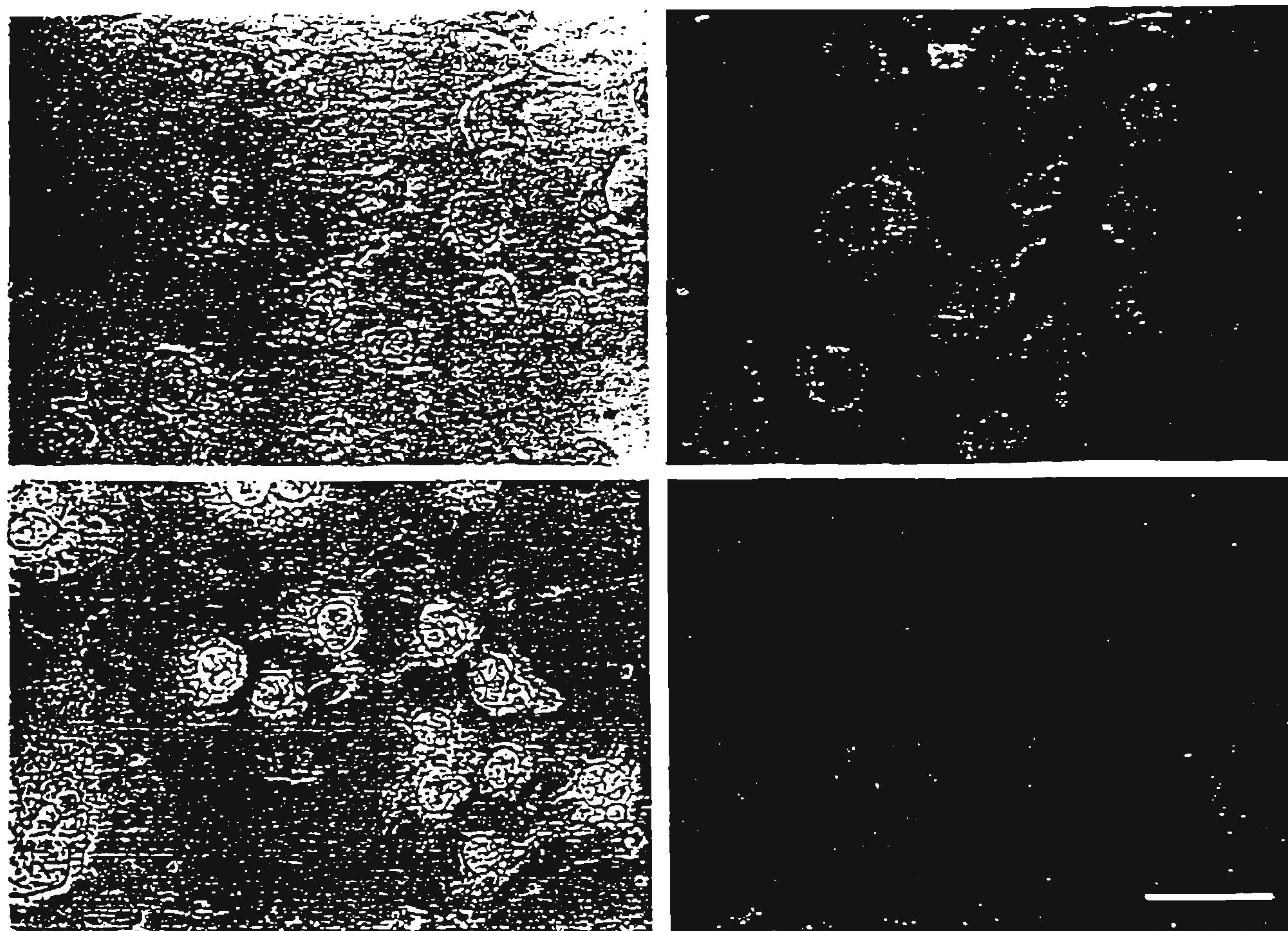


Fig. 4. Specificity of the anti-M1-Y serum for MC_1 receptor determined by fluorescent staining. The top right and left panels show the staining of the MC_1 receptor expressing cells with the M1-Y antiserum and its corresponding phase contrast photograph, respectively. The bottom right and left panels show the staining of the MC_1 receptor expressing cells with the M1-Y antiserum which was preabsorbed with the M1-Y peptide and its corresponding phase contrast photograph, respectively. Bar = 50 μM.

2.4. Immunostaining

The slides were processed either for indirect immunofluorescent or immunoperoxidase staining. The slides for peroxidase staining were pre-incubated with 0.6% hydrogen peroxide in methanol for 20 min and then for 1 h with 10% normal goat serum in PBS, in order to block non-specific staining. The slides used for immunofluorescent staining were not pre-incubated prior to exposure to antisera. Slides were incubated overnight at 4°C with the primary antisera, diluted 1:100 for cell staining and 1:50 for tissue staining in 1.5% bovine serum albumin in phosphate buffered saline, pH 7.2. The slides were then incubated with secondary antibodies for 1 h at room temperature. For immunofluorescent staining TRITC-conjugated affinity purified swine anti rabbit immunoglobulin was used at a dilution of 1:40. After staining, the slides were observed using a fluorescent microscope. For immunoperoxidase staining HR peroxidase-conjugated affinity purified goat anti rabbit immunoglobulin (DAKO, Denmark) was used at a dilution of 1:200. Chromogen substrate (3,3'-diaminobenzidine tetrahydrochloride; DAKO, Denmark) was then applied according to instructions of the manufacturer, and thereafter the sections were counterstained with haematoxylin Mayer RB 69 (Apoteksbolaget AB, Sweden). Negative controls included omission of primary antibodies, substitution of preimmune serum or preabsorption of the antiserum with the synthetic peptide (0.5 mg/ml) that had been used to immunize the rabbit. Between each incubation the slides were washed three times with phosphate buffered saline.

3. Results

The peptides used to immunize the rabbits were chosen on the basis of 1) their probability for high immunogenicity predicted by the Jameson and Wolf antigenic index and 2) their predicted high specificity for the human MC_1 receptor protein determined by comparing the protein sequences of all the melanocortin receptors cloned to date. Two different

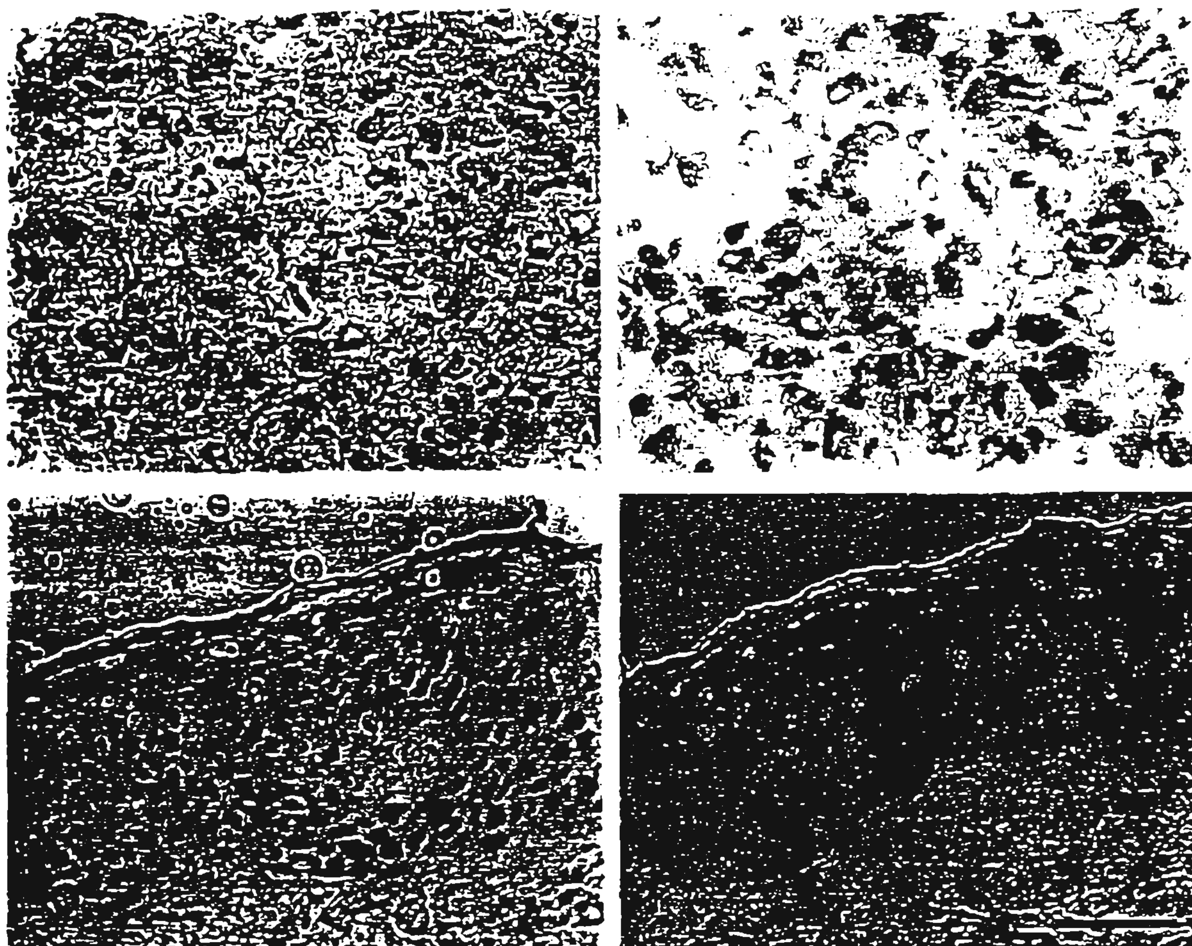


Fig. 5. Peroxidase staining of freeze cut sections of a primary cutaneous human malignant melanoma tumour and the skin located adjacent to the tumour. The specimen was excised from the arm skin of a 43-year-old male and the sections stained with anti M1-Y sera using the peroxidase method. Top left panel: Positive immunostaining can be seen in melanoma cells of the tumour. Note the distinct staining of the area of the cells surrounding their nuclei (indicated by arrows). Top righ panel: Sections of the melanoma tumour stained with preimmune serum. Bottom left panel: Section of the skin located adjacent to the melanoma tumour. A considerable number of scattered cells in the epidermis are stained with the anti-M1-Y serum. Bottom right panel: A parallel section to the section shown in the bottom left panel stained with preimmune serum.

p..tides, M1-Y and M2-Y (Fig. 1), were selected from the N-terminus of the MC_1 receptor and synthesised chemically, conjugated to thyroglobulin and injected into rabbits. Both peptides proved to be highly im-..genic and good production of antibodies resulted i.. all four rabbits that had been injected with either one of the conjugates. Thus, after the first booster injection immunoreactivity was detected in all the sera at dilutions of $1:10^4$ or more, using ELISA plates covered with 70 pmol of respective synthetic peptide (Fig. 2, top panel). By contrast, no ELISA reactivity could be observed for the preimmune sera (data not shown). Both antisera were also demonstrated to be specific for their respective peptide since the ELISA response was dose-dependently inhibited by preincubating the sera with the proper peptide, but not by preincubating them with an irrelevant peptide (Fig. 2, bottom panel).

In order to evaluate the ability of the antisera to bind to the MC_1 receptor, a genetically engineered stable MC_1 receptor expressing COS-7 cell line was immunostained with the sera. The results are shown in Fig. 3. Both M1-Y and M2-Y antisera produced a strong fluorescent pattern on the MC_1 receptor expressing cells which appeared to be mainly located in cytoplasmic or perinuclear areas, although a staining of the cell membrane was also probably present (Fig. 3 left top and bottom panels and Fig. 4, top right panel). (C.f. phase contrast photomicrographs of cells shown in Fig. 4, left top and bottom panels). In contrast, there was no significant staining of a COS-7 cell line that did not express the MC_1 receptor (Fig. 3, right top and bottom panels) or cells stained with antiserum preabsorbed with its respective synthetic peptide (Fig. 4, bottom right panel).

The properties of the antisera were further evaluated using cryostat sections from cutaneous malignant melanoma tumours and normal skin. In the melanoma tumours both the M1-Y and M2-Y antisera could specifically immunostain the tumour cells. The top left

panel of Fig. 5 shows results for the staining of a melanoma tumour with an M1-Y antiserum, using the peroxidase method. As can be seen from the figure a strong reaction is present in the area of the tumour cells that surrounds the cell nuclei, indicating that the staining is presumably located in the cell membrane of the melanoma cells. By contrast, when preimmune serum was used staining of the melanoma cells was absent (Fig. 5, top right panel). Moreover, the malignant melanoma sections were also strongly positively stained when the immunofluorescent staining method was used in conjunction with the anti MC_1 receptor antisera, whereas the preimmune sera gave negative staining (data not shown).

In Fig. 5 (bottom left panel) is shown the staining of a sample of the skin that had been obtained just adjacent to a melanoma tumour, with the M1-Y antisera. As can be seen from the figure scattered cells in the epidermis show a strong positive peroxidase reaction. The cells being stained are arranged in a gradient with most cells being located in the basal layer while fewer cells are stained in the outer layers. By contrast, preimmune serum did not stain the epidermal cells (Fig. 5, right bottom panel). In strong contrast to these results cells of normal human skin did not show any positive staining to the anti MC_1 receptor sera (data not shown). In order to eliminate the possibility that the quenching of endogenous peroxidase of the sections using the hydrogen peroxide pre-incubation could have led to a destruction of the MC_1 receptor antigen in cells of the normal skin, we performed the staining excluding the hydrogen peroxide pre-incubation step, still staining was completely absent in these samples. Moreover, using ethanol instead of acetone as a fixative the tissue did not induce any positive staining of the normal skin samples (data not shown).

4. Discussion

In this study we have prepared antisera against a human MSH receptor. The antisera were raised in rabbits by immunisation with peptides that had been synthesised according to the amino acid sequence predicted from the MC_1 receptor cDNA, that we had previously cloned (Chhajlani and Wikberg, 1992). Three factors were considered in choosing the two peptides used for the immunisations. Firstly, the peptides were selected from the N-terminal extracellular region of the receptor since this part of the amino acid sequence should with high likelihood form an epitope that would be accessible to the antibody. Secondly, the peptides were selected according to their predicted high immunogenicity. It has been suggested that peptides which have a more than 10 residues length, which are hydrophillic and which are derived from the C- and N-terminal regions of proteins tend to be more antigenic (Appel et al., 1990; Harlow and Lane, 1988). Thirdly, the peptides were selected on the criteria that they should be substantially different from any sequences found in other melanocortin receptors. The various other cloned melanocortin receptors (MC_2, MC_3, MC_4 and MC_5 receptor) show between 30-60% homology with the MC_1 receptor, the largest homology being found in transmembrane segments, whereas e.g. the N-terminal sequences show a low degree of homology. Moreover, a complete search in the EMBL database indicate that there are no other known proteins which show substantial similarities with the MC_1 receptor (Chhajlani and Wikberg, 1992).

The two selected peptides of 15 (M1-Y) and 11 (M2-Y) residue length proved to be highly antigenic as high titres were obtained in all rabbits already after the first booster injection. Moreover, the antisera gave no cross-reaction with the irrelevant peptide in the ELISA assay indicating that the sera were specific. In order to evaluate the ability of the antisera to bind to the MC_1 receptor protein they were tested for their ability to immunostain cells that expressed the MC_1 receptor. In these studies we used a genetically engineered COS-7 cell line that showed stable expression of the MC_1 receptor. Thus, whereas the MC_1 receptor expressing COS-7 cells were strongly stained by both the M1-Y and M2-Y antisera, normal COS-7 cells were not at all stained, thus indicating that the antisera did recognize the MC_1 receptor. Moreover, neither preimmune serum nor antiserum pre-adsorbed with its synthetic peptide was able to stain the cells. Our data also indicate that a substantial amount of the specific immunostaining occurred in the intracellular or perinuclear compartments. It may be observed that such a localisation of an MC_1 receptor is quite possible as a number of studies have suggested both an internal and external localisation of MSH-receptors in melanoma cells; the ability of the receptor to become internalised being crucial for the responsiveness of the cells for MSH (Orlow et al., 1990; Chakraborty et al., 1991)

We used the antisera in a preliminary attempt to immunostain MC_1 receptor in human skin and melanoma tumours. Our data clearly show that the antisera could immunostain cells of malignant melanoma tumours. The staining of the melanoma cells was specific because pre-immune sera did not stain these cells. Moreover, scattered cells which were present in the epidermis of the skin localised immediately adjacent to one of the melanoma tumours stained intensely with our antisera. Since malignant melanoma is derived from melanocytes it was of interest to compare this pattern of staining with that of normal skin. In strong contrast to the results of the skin sample of the melanoma patient, the normal skin did not show any specific staining with our antisera. These results

were repeated in samples from two different donors. Moreover, excluding the peroxidase pre-quenching step (see methods for details) did not disclose any immunoperoxidase staining cells in the normal skin. Since melanocytes were clearly present in the normal skin samples, as was evident from the presence of darkly pigmented cells, it seems justified to conclude that MC_1 receptors were not expressed in sufficiently high levels in the normal melanocytes to allow our antisera to immunostain them. It has been reported that cultured human melanocytes express functional MSH receptors (Thody et al., 1993), although the levels of MC_1 receptor mRNA in cultured human melanocytes seems to be lower than the levels present in cultured murine melanocytes or in a melanoma cell line (Cone et al., 1993). The nature of the scattered MC_1 reactive cells found adjacent to the melanoma tumour is at present not entirely clear. Nevertheless since these stained cells were found only in the vicinity of the melanoma tumour it is highly probable that they were related to the melanoma. Moreover, the pathological histological examination showed a malignant melanoma with a continuous intraepidermal growth as well as at places, an intraepidermal invasive growth. It is also interesting to note that the staining pattern of the presumed melanoma cells was is irregular. Thus in the top left panel of Fig. 5 the staining appeared to be predominantly perinuclear plus membranal whereas in the bottom left panel of the same figure the staining appears to be nuclear. The reason for these differences in staining pattern is at present not clear but it could possibly be related to the ability of the MC_1 receptor to migrate between different cellular compartments as was already mentioned above. However, whatever the explanation is for these findings we would like to point out that our observations should be regarded as preliminary. The sole purpose of the studies on melanoma tumours was to show that it was possible to detect the MC_1 receptor in a clinical sample. Thus, further studies will be required to validate our observations and to map out the tissue distribution of the MC_1 receptor in the human body.

In conclusion, we have developed two antipeptide antibodies against the human MSH receptor of the MC_1 type. Both antibodies displayed high titre and specificity for their respective antigen and could specifically immunostain MC_1 receptor expressing cells, as well as the cells of malignant melanoma tumours. These antibodies will be of value in further studies of the organ distribution of the MC_1 receptor and in investigations of the molecular biology of the MC_1 receptor in melanoma.

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A THREE DIMENSIONAL MODEL FOR THE INTERACTION OF MSH WITH THE MELANOCORTIN -1 RECEPTOR

Peteris Prusis*, Per-Anders Frändberg*, Ruta Muceniece[¤], Ivars Kalvinsh[#] and Jarl E.S. Wikberg*+§

*Department of Pharmaceutical Pharmacology Uppsala University, Uppsala, Sweden

[¤]Laboratory of Pharmacology and [#]Department of Medicinal Chemistry, Institute of Organic Synthesis, Riga, Latvia

+Wa Pharm, Umeå, Sweden

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SUMMARY: A model for the interaction of the melanocortin-1 (MC1) receptor with MSH peptide has been constructed. The model was built by homology modelling using bacteriorhodopsin as template. A cyclic analogue of MSH could be docked into a binding pocket located between transmembrane (TM) domains 2, 3 and 6 of the receptor. The most significant receptor to ligand interactions occur between D117 in TM3 of receptor with histidine in cyclic MSH-peptide, H260 in TM6 with glutamic in peptide and D121 in TM3 with arginine in peptide. The model finds support from earlier mutagenesis data. © 1995 Academic Press, Inc.

The 3D modelling of several G-protein coupled receptors (GPCRs) has led to better understanding of the interactions of the receptors with their ligands. However, such modelling was in the past mainly done for the cationic GPRCs (1) and yet only two partial models for peptide hormone GPRCs were reported (2,3).

We and others recently cloned a new family of GPCRs, the melanocortin receptors, that contains 5 known members termed MC1 - MC5 (4-8). All of the melanocortin receptors interact with pro-opiomelanocortin derived peptides such as α-MSH, β-MSH, γ-MSH and ACTH, albeit with differing binding affinities (9).

§Address all correspondence to Jarl Wikberg, Pharmaceutical Pharmacology, Box 571, BMC, S-751 24, Uppsala, Sweden.

Abbreviations:
GPCR, G-protein coupled receptor; MCR, melanocortin receptor; MSH, melanocyte stimulating hormone; TM, transmembrane.

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The melanocortin receptors show high sequence similarity within their group, but the sequence similarity to other GPCRs is comparatively low. Nevertheless the most conserved aminoacids among the GPCRs are also present in the melanocortin receptors and their all over features clearly identify them as belonging to the GPCR class. In a recent paper we reported specific alterations in the ligand binding properties in the MC1 receptor subtype by site directed mutagenesis of specific aminoacids in the third and sixth transmembrane segments, indicating that these aminoacids were part of the ligand binding pocket of the receptor (10). In the present paper we report for the first time a 3D model for a melanocortin receptor.

MATERIALS AND METHODS

Software and hardware. Molecular modelling was done by using the Sybyl (Tripos, St. Louis, Missouri, USA) package and a Sun IPX workstation. Sequence alignments were performed using the MacMolly package on a Macintosh computer (Soft Gene, Germany). Kyte-Doolittle plots were done using the GCG package (11) on a VAX computer.

Sequence analysis. Sequences were obtained from the Brookhaven Protein Data Bank or experimentally by DNA sequence analysis of the cloned receptor as earlier reported (4,5). The sequences of all five MC receptors were aligned using the MacMolly program. The location of transmembrane helices were determined from Kyte-Doolittle hydrophobicity plots. The alignment of MC receptor amino acid sequences to other GPCRs and to bacteriorhodopsin were taken from the literature (12) except for transmembrane segment 5 (TM5) which alignment will be detailed in the Results and Discussion section.

Peptide hormone modelling. For modelling the peptide hormone the cyclic peptide:

Ac-Cys-Glu-His-D-Phe-Arg-Trp-Cys-NMe

was used. Its structure was built using the standard random conformation protein building routine of Sybyl. Molecular dynamics simulations was applied at a temperature of 300K using 25 ps step time to yield 2000 conformations from which the 36 with lowest energy were chosen and energy minimised using the Tripos force field. The molecule with the lowest energy was used in our studies.

Receptor modelling. Modelling was performed using Sybyl with default parameters unless otherwise specified. The seven transmembrane helices of the MC1 receptor were first constructed separately using α-helical conformation. Helices 6 and 7 contains proline residues for which the geometry were fixed resulting in kinked helices. The helices were then superimposed on the helices of bacteriorhodopsin (13).

To preserve the general structure of the receptor its backbone was first constrained and molecular dynamics simulation (100K temperature, 1 fs time step) was applied to yield 1000 conformations, from which 4 were chosen and energy minimised using the Kollman force field (14) The constraint of the backbone was then removed and the models were subjected further energy minimisation. The model with the lowest energy was selected for use in the docking studies.

Ligand docking. The cyclic ligand, obtained as described above, was manually docked to the receptor model and the complex subjected to energy minimisation using the Kollman force field (14).

RESULTS AND DISCUSSION

Modelling of ligand. Modelling of the natural MSH peptide hormones poses big problems because of their flexibility and the lack of any previous 3D structural data. The cyclic peptide used for the present study is related to α-MSH 5-9, which is the central core in α-MSH that earlier studies suggest is binding to the MSH-receptors (15). Earlier studies have also shown that the cyclisation at the 4-10 positions in MSH gives a compound with high affinity for the MSH-receptors (16). Moreover higher activity of both cyclic and linear MSH-peptides may be afforded by substitution of the L-Phe with D-Phe (17,18). While the chosen cyclic MSH-analogue has some restriction due its SS bridge, there still remains a possibility for many conformers. Its structure was therefore built by performing long molecular dynamics simulation and selecting the ones with lowest energy for further energy minimisation. The structure with the lowest energy thus obtained was used in the docking studies as is shown in fig 3.

Sequence alignments. The alignment of all the MC receptor sequences is shown in fig. 1. Most of the conserved amino acids are located within the presumed TM helices. The carboxyterminal, first extracellural and third intracellular loops differs significantly whereas the other extra and intracellular loops as well as the amino-terminal show regions of substantial similarity.

It was previously reported that the MCR transmembrane sequences can be aligned with the sequences of the whole group of GPCRs (12). Although most conserved amino acids among the GPCRs are also present in the MC receptors there are some exceptions; one of the most interesting being the lack of a proline in TM5 for the MC receptors. This fact might suggest a specific role for TM5 in the MC receptors as compared to other GPCRs. Moreover the extreme short extra-cellular loop between TM4 and TM5 in the MC receptors compared to other GPCRs (12) is an interesting observation.

None of the TM regions of the MCRs showed any apparent homology to bacteriorhodopsin, except TM5 which contains a nine amino acid long alignable motif in which up to five amino acids are equal and some others similar. Moreover, four aminoacids on each side of the motif are also similar (fig. 2).

Modelling of receptor and docking of ligand. The final MC1R model selected after the dynamics simulation and energy minimisations showed an all over preserved 7 transmembrane α-helical structure with substantial helice to helice interactions.

Our earlier mutagenesis studies (10) suggested that D117 in TM3 and H260 in TM6 is part of the ligand binding pocket. Moreover, it is plausible that TM2 is also close to the binding region. This is because a natural point mutation has been found in TM2 of the MC1R giving rise to a constitutively activated receptor (19).

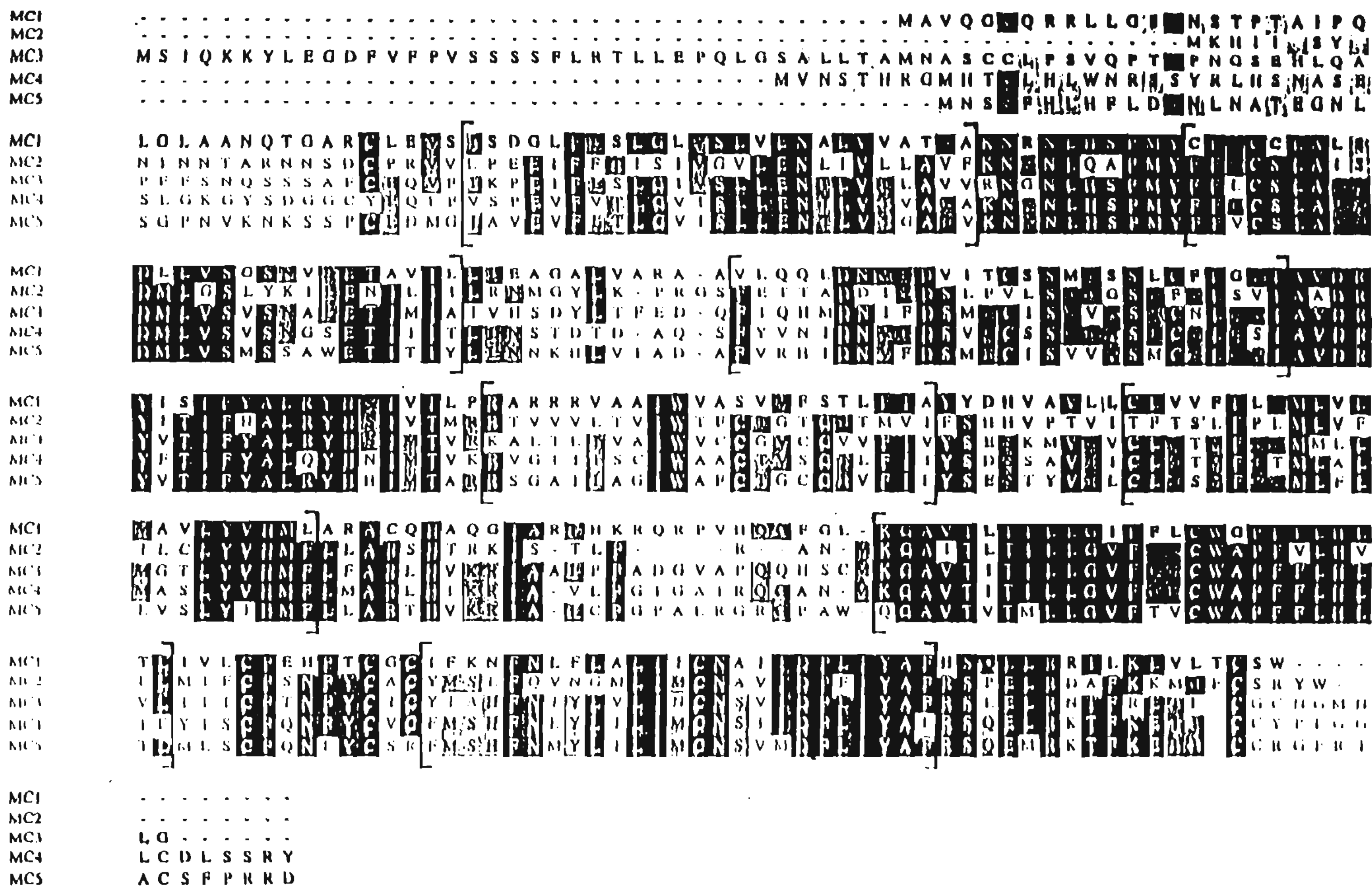

Fig.1. Alignment of MC receptor aminoacid sequences. TM regions used in modelling shown within brackets.

```
BRD  W W   I S T A       Y     L Y       G   T
MC1  C L   V F F L       V       A       H   L
MC2  T F T S L F P       V       L C     H   F
MC3  C L   T M F F       L       G T     H   F
MC4  C L   T M F F T     A       A S     H   F
MC5  C L   S M F F       F     L V S     H   F
```

Fig.2. Alignment of TM5 of MC receptors and bacteriorhodopsin (BRD). The nine amino acid long alignable motif (see text) marked by horizontal bars.

The ligand could be docked into a pocket located between TM2, TM3 and TM6 of the model. Energy minimisation yielded a receptor-ligand complex with several points of interaction between receptor and ligand. The most interesting interactions are the formations of salt bridges between D117 in TM3 with the histidine of the cyclic peptide, between H260 in TM6 of the receptor with the glutamic residue of the ligand, and the D121 in TM3 with the arginine residue of the ligand. There is also an interaction between E55 of TM1 for the receptor and the tryptophan residue of the ligand (fig. 3). In our previous study (10) we found that the D117-->Ala, as well as the H260-->Ala mutation in the MC1R leads to a drop in affinity for the linear α-MSH but not for the analog $[^{125}I]$-Nle^4, D-Phe^7-MSH, a fact that indicates differences in their points of attachment to the MC1R. However, a cyclic peptide analog having the identical core as the peptide used in the present study was also found to loose affinity for the above mentioned mutated receptors in a similar fashion as α-MSH. (In fact the same pattern was also found when the D-Phe of the cyclic analogue was substituted with L-Phe, although the allover affinities became lower; Frändberg et al., unpublished

Fig.3. The model of the interaction of the cyclic MSH analogue with the MC1 receptor. Shown are the backbones of the receptor and ligand (gray) with the side chains of some of the aminoacids (black) having ligand to receptor interactions; from left to right E55-R, F4-L, W6-L, H3-L, D121-R, E2-L, R5-L, H260-R and D117-R. (Code used denotes aminoacid number followed by R for aminoacid of receptor or L for aminoacid of ligand.)

data). Thus these data seem to indicate that the cyclic peptide may have a similar conformation as the natural MSH. The future modelling studies must take into account that different MSH-analogues may bound with slightly different points of attachment to the MC1R.

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EVIDENCE FOR ALTERNATE POINTS OF ATTACHMENT FOR α-MSH AND ITS STEREOISOMER [Nle⁴, D-Phe⁷]-α-MSH AT THE MELANOCORTIN-1 RECEPTOR

Per-Anders Frändberg, Ruta Muceniece, Peteris Prusis, Jarl Wikberg and Vijay Chhajlani

Pharmaceutical Pharmacology Division, Biomedical Centre, Box 591, Uppsala, 751 24 Sweden

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SUMMARY: The molecular basis for the α-melanocyte stimulating hormone (α-MSH) stereoselectivity was studied by examining ligand binding to site specific mutants of the melanocortin 1 receptor (MC1R). The amino acids Asp^{117}, Phe^{179}, His^{209} and His^{260} were targeted for mutation to alanine as they are conserved in the melanocortin receptor family. Expression of the wild type and the MC1R mutants in COS-7 cells revealed that the binding affinities for the α-MSH (L-isomer) was reduced by 267 fold for the $D^{117}\rightarrow A$ mutant and 132 fold for the $H^{260}\rightarrow A$ mutant. In contrast, the binding affinity for the $[Nle^4, D\text{-}Phe^7]$-α-MSH (NDP-MSH; D-isomer) remain unchanged between the wild type and the mutants. Moreover, the mutants also displayed reduction in affinity to L-isomers of all the other melanocortic peptides examined. Thus, the mutation of single amino acids in the third and sixth transmembrane segments results in the display of ligand stereoselectivity of the MC1R. In addition, these data represent the first evidence of the different binding epitopes on a G-protein coupled receptor for a peptide ligand stereoisomers, both of which are shown to be potent agonists. © 1994 Academic Press, Inc.

α-MSH was originally isolated from the pituitary gland (1), and has been shown to induce skin pigmentation (2). Later studies revealed the presence of other melanocortin peptides like β-MSH, γ-MSH and desacetyl-α-MSH (3). Apart from playing a role in skin pigmentation, melanocortin peptides are proposed to have neuromodulatory, behavioural, neurotransmitter and other biological effects (4). Such diverse functions can be attributed to the melanocortin receptor subtypes with their discrete cellular distribution (5, 6, 7, 8, 9).

Abbreviations:
MC1R, melanocortin 1 receptor; MSH, melanocyte stimulating hormone; NDP-MSH, $[Nle^4, D\text{-}Phe^7]$-α-MSH; D, aspartic acid; F, phenylalanine, H, histidine; A, alanine.

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The structure-function relationship of α-MSH has been analysed by studying the biological effects of a number of structurally and stereochemically modified analogues (10, 11, 12). Based on these studies, it was concluded, that the internal six amino acid segment of Met-(Glu/Gly)-His-Phe-Arg-Trp, which is shared by all melanocortic peptide molecules, can independently bring about the melanosome dispersion in the ambiphibian melanophores (13). Earlier studies had also demonstrated, that the heat-alkali treatment of the α-MSH results in a peptide with increased biological activity. This is attributed to the racemization of methionine, and phenylalanine at positions 4 and 7, respectively resulting in the development of a synthetic analogue [Nle^4, D-Phe^7]-α-MSH (NDP-MSH) in which Met at position 4 and of L-Phe at position 7 were replaced by norisoleucine and D-Phe, respectively (14). The NDP-MSH was shown to be 26 times more potent than α-MSH in the adenylate cyclase assay (14). The reason for this increased biological activity was never fully explained, though it was speculated that NDP-MSH due to its altered structure could either irreversibly bind to the receptor, or could irreversibly affect the signal transduction between the receptor and the adenylate cyclase (14).

In this report we demonstrate, that the higher binding affinity of the NDP-MSH as compared to its L-isomer α-MSH, is due to the fact, that these two peptides interact with the melanocortin receptor with atleast two different points of attachment.

MATERIALS AND METHODS

Site directed mutagenesis- The human MC1R cDNA (5) was subcloned in the Hind III site of the pRC/CMV vector (InVitrogen Corp., USA) and subjected to the site directed mutagenesis using the mutagenesis kit for the double stranded plasmid DNA as per manufacturer's conditions (U.S.E. kit, Pharmacia Sweden). The mutagenic primers used were:

(i) GTGACATTGGCCAGCTGC ($D^{117}\rightarrow A$),

(ii) GGTAGGCGATGGCGAGCGTGCTGAA ($F^{179}\rightarrow A$),

(iii) GGGCCAGCATGGCGACGTACAGCAC ($H^{209}\rightarrow A$) and

(iv) GTGAGTGTGAGAGCCAGGAAGAAGGG ($H^{260}\rightarrow A$).

The selection primer used to convert the XbaI site to the NheI in the pRC/CMV vector was: GCTATAGAATAGGGCCCGCTAGCTGCATGCTCGAGCG.

Briefly, the denatured double stranded plasmid DNA was annealed to the mixture of mutagenic and the selection primers and the new strand of DNA was synthesized using the T4 DNA polymerase and sealed with the T4 DNA ligase in a single step. The so modified DNA was used to transform *mut*S E. Coli, the plasmid DNA was prepared and digested with XbaI restriction enzyme, which resulted in the linearization of the wild type DNA while leaving the mutated DNA circular.

Transformation of INVαF' bacteria with the digested DNA yielded colonies 90% of which contained plasmid DNA with the desired mutation as verified by sequencing the cDNA clone. Large scale plasmid preparations were made for the mutated and the wild type DNA using the Qiagen plasmid preparation kit (Qiagen Inc, USA), and used for the expression studies.

Expression of MC1R and radioligand binding- Wild type and the mutant $D^{117}\rightarrow A$ and $H^{260}\rightarrow A$ mutants were transfected into the COS-7 cells using the cationic lipid Lipofectin (Life Technologies Inc. USA) as per the manufacturer's instructions. Cells were harvested 48 hours after transfection, distributed in the 48 well plates and used for radioligand binding. All assays were performed in duplicate. NDP-MSH was labelled with [^{125}I] and purified as described (15). The transfected cells were washed with 0.2 ml of binding buffer (Minimum essential medium with Earle's salts, 25 mM HEPES, pH 7.0, 0.2 % bovine serum albumin, 1 mM 1,10-phenanthroline, 0.5 mg per litre leupeptin and 200 mg per litre bacitracin). The cells were then incubated for 2 hours with 0.2 ml binding buffer containing a fixed concentration of [^{125}I]-NDP-MSH and appropriate concentrations of the unlabelled ligand. After incubation the plates were put on ice, the cells washed with 0.2 ml of ice-cold binding buffer and detached from the plates with 0.4 ml of 0.1 N NaOH. Radioactivity was counted (Packard autogamma, scintillation spectrometer) and data analysed by a iterative, non-linear curve fitting programme, suitable for the radioligand binding analysis. The K_i values were obtained from IC_{50} values using the Cheng and Prusoff equation (16).

RESULTS AND DISCUSSION

The effect of point mutations in the MC1R at four different positions (see fig.1) was examined by comparing the radioligand binding properties of the wild type and the mutated receptors. The point mutations we have created produced four mutant proteins for the MC1R. In three of these mutants charged amino acids were replaced by the nonpolar alanine. In the fourth mutant aromatic phenylalanine was replaced by alanine. The ligand binding properties of the mutated receptors allowed us to identify the amino acids that distinguish the binding pockets of the α-MSH and its D-isomer, NDP-MSH. In order to select residues for mutagenesis the amino acid sequences of the members of the melanocortin receptor family was compared. Based on this comparison the MC1R sequence was analysed for the amino acids which (i) were predicted to be in the transmembrane regions, (ii) had reactive groups and (iii) were conserved among the melanocortin receptor family. Four amino acids namely, D^{117}, F^{179}, H^{209} and H^{260} were selected and mutated to alanine. These four different mutants and the wild type receptors were transiently expressed in the COS-7 cells and the affinity of the melanocortic peptide ligands were determined in [^{125}I]-NDP-MSH competition binding experiments (table 1). The binding to the $D^{117}\rightarrow A$ and $H^{260}\rightarrow A$ mutants were substantially reduced in their affinities for the all L-forms of the melanocortic peptides examined (α-MSH, β-MSH, γ-MSH Nle^4 -αMSH and desacetyl α-MSH), but not at all for the D-form of the peptide, NDP-MSH (fig. 2). For the mutants $F^{179}\rightarrow A$ and $H^{209}\rightarrow A$ the affinity of neither the D- nor the L-forms of the melanocortic peptides were altered, suggesting that they are not involved in the ligand binding.

Several reports have detailed the points of attachments between the nonpeptide ligands and the amino acids in the G-protein coupled receptors of the adrenergic and

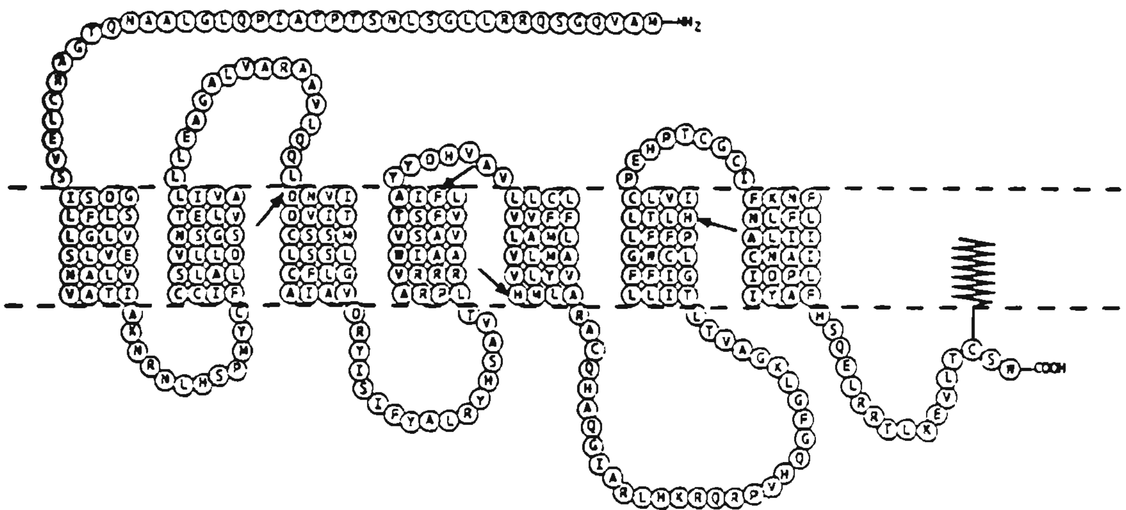

Fig. 1. Putative two dimensional topography of the MC1R showing amino acid sequence and positions of the residue D^{117}, F^{179}, H^{209} and H^{260}, marked by the arrows. Dashed lines denotes the cell membrane boundaries.

serotonergic families (17, 18, 19). The non-peptide antagonists, to the G-protein coupled receptors for small peptide ligands, have been demonstrated to interact with the residues in the transmembrane segments (20, 21). It has also been shown for the neurokinin-1 receptor that the different non-peptide antagonists interact with epitopes located in the transmembrane segments as well as in the extracellular loops of the receptor (22). The alteration in the binding affinity of the L-isomers of the melanocortic peptides to the $D^{117}\rightarrow A$ and $H^{260}\rightarrow A$, reported in this study, demonstrates for the first time the residues involved in the binding to the natural agonistic ligands such as α-MSH. The synthetic, D-isomer NDP-MSH, which has earlier been shown to be a highly potent agonist for the melanocortin receptor (14) has same affinities for the wild type and the mutant receptors ($D^{117}\rightarrow A$ and $H^{260}\rightarrow A$).

Table 1: The K_i values of the melanocortic peptide ligands for the wild type MC1R and the mutated receptors

Ligand	Wild type MC1R K_i (nM)	Mutant $D^{117}\rightarrow A$ K_i (nM)	Mutant $H^{260}\rightarrow A$ K_i (nM)	Mutant $F^{179}\rightarrow A$ K_i (nM)	Mutant $H^{209}\rightarrow A$ K_i (nM)
NDP-MSH	0.021 ± 0.006	0.056 ± 0.005	0.027 ± 0.01	0.058 ± 0.008	0.023 ± 0.005
α-MSH	0.130 ± 0.025	34.8 ± 8.8	17.25 ± 2.7	0.10 ± 0.014	0.040 ± 0.09
Nle^4-α-MSH	0.047 ± 0.011	8.06 ± 0.99	0.8 ± 0.09	n.d.	n.d.
β-MSH	0.882 ± 0.143	357 ± 32	45.8 ± 4.8	n.d.	n.d.
γ-MSH	1.2 ± 0.21	> 30 000	106 ± 10	n.d.	n.d.
desacetyl-α-MSH	0.046 ± 0.005	107 ± 9.3	4.63 ± 0.59	n.d.	n.d.

These results taken together suggests that NDP-MSH and α-MSH binds to the receptor at different amino acid residues. Nevertheless they are still able to bring about the activation of the receptor (14, 23, 24).

In summary, this is the first report describing specific residues of the melanocortin 1 receptor binding site. We also demonstrated for the first time that the stereoisomers of

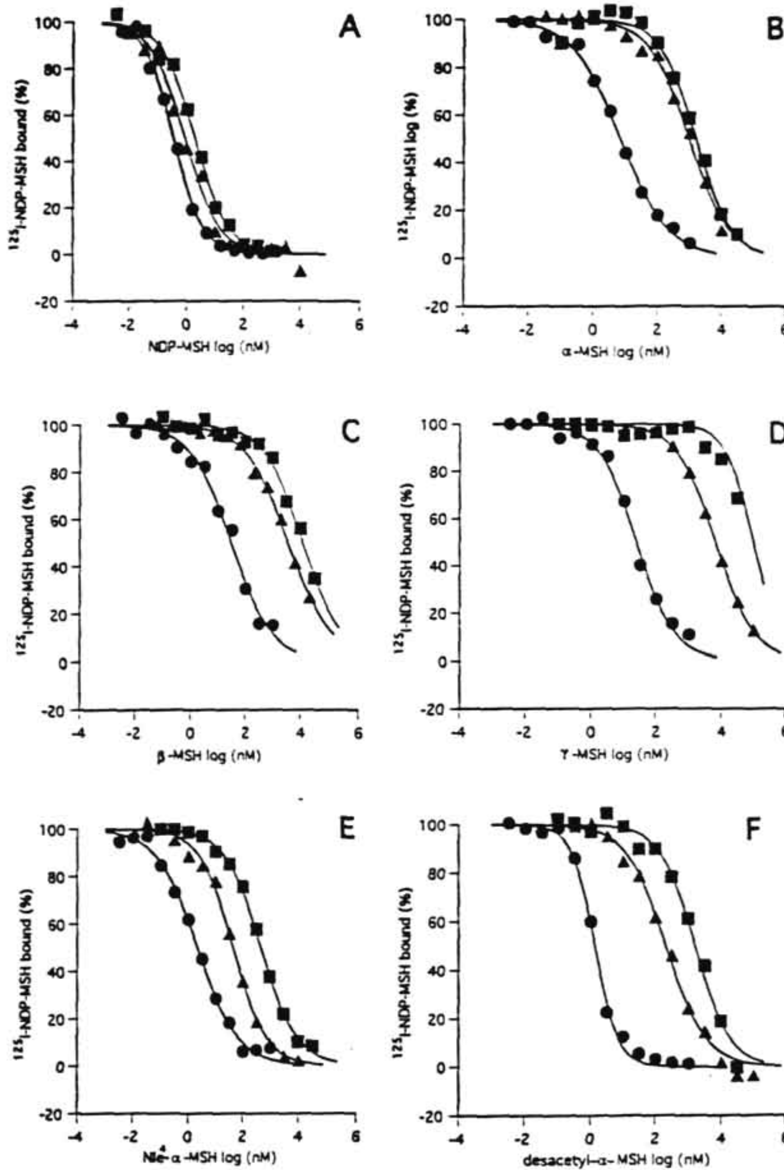

Fig.2. Inhibition of [^{125}I]-NDP-MSH binding by different melanocortin peptides for the wild type MC1R (●) and its mutants $D^{117}\rightarrow A$ (■) and $H^{260}\rightarrow A$ (▲). The competiting ligands were NDP-MSH (A), α-MSH (B), β-MSH (C), γ-MSH (D), Nle^4-α-MSH (E) and desacetyl-α-MSH (F).

a peptide, where both the isomers are agonists, can react with the receptor via different amino acid residues.

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Characterisation of D117A and H260A mutations in the melanocortin 1 receptor

Helgi B. Schiöth[a,*], Ruta Muceniece[b], Michael Szardenings[a], Peteris Prusis[a], Gunnar Lindeberg[c], Shubh D. Sharma[d], Victor J. Hruby[d], Jarl E.S. Wikberg[a]

[a] Department of Pharmaceutical Pharmacology, Uppsala University, Uppsala, Sweden

[b] Laboratory of Pharmacology, Institute of Organic Synthesis, Riga, Latvia

[c] Department of Medicinal and Physiological Chemistry, Uppsala University, Uppsala, Sweden

[d] Department of Chemistry, University of Arizona, Tucson, Arizona, USA

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Abstract

Recent site directed mutagenesis studies on the melanocortin 1 (MC1) receptor have indicated the importance of D117 and H260 amino acid residues for the binding of α-MSH (melanocyte stimulating hormone). Here, we report the testing of 12 cyclic and linear MSH peptides on the D117A and H260A mutant receptors. Moreover, we constructed a double mutant which displayed a major loss in affinity for [Nle^4, D-Phe^7]α-MSH. Our new data of His^6 and Phe^7 substituted MSH peptides are compared with previous results and the hypothesis of putative interactions of D117 and H260 with single amino acids in the MSH peptide. Our conclusions are that the D117A and the H260A mutations may cause conformational changes in the receptor which can not be linked to any specific amino acid in the MSH-peptides. © 1997 Elsevier Science Ireland Ltd.

Keywords: MC1 receptor; MSH; Ligand binding; Mutagenesis

1. Introduction

Molecular cloning has identified five different melanocortin (MC) receptor subtypes, termed MC1-5 [1–5]. The MC receptors belong to the family of G-protein linked receptors and do not share high amino acid homology to any other receptor group. The MC1 receptor binds α-MSH (melanocyte stimulating hormone) with high affinity [6–8] and is the only MC-subtype expressed in melanocytes and melanoma cells, but it has also been detected in rat and human brain along with other subtypes of MC-receptors [9]. The MC2 receptor is the adrenal cortical ACTH (adrenocorticotropic hormone) receptor. This receptor binds ACTH with high affinity, but it shows negligible affinity for MSH peptides [10]. The MC3 receptor, the MC4 receptor and the MC5 receptor are expressed in the brain where they have largely unknown functions, and where they may mediate the diverse central effects caused by exogenously applied melanocortic peptides. The MC3 receptor and the MC5 receptor also have been detected in peripheral tissues (for review see [11]).

By site directed mutagenesis of the MC1 receptor, amino acid residues that may be a part of the receptors binding pocket were identified [12]. Mutation of Asp 117 (D117) in the third transmembrane segment, or of His 260 (H260) in the sixth transmembrane segment to Ala resulted in loss of affinity for α-MSH but not for [Nle^4, D-Phe^7]α-MSH. All natural MSH peptides tested (which have an L-Phe in position 7) lost affinity for both mutants, whereas the D-Phe^7 containing [Nle^4, D-Phe^7]α-MSH was not affected. In another study,

* Corresponding author. Present address: Department of Pharmaceutical Pharmacology, Biomedical Centre, Box 591, 751 24 Uppsala, Sweden. Tel.: +46 18 174160; fax: +46 18 559718; e-mail: helgis@bmc.uu.se

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molecular modelling was applied to gain insight into the receptors 3D structure. This study indicated a putative interaction of the D117 and the H260 in the MC1 receptor with His in position 6 and with Glu in position 5 in the MSH peptides, respectively [13].

The present study was undertaken to shed further light into the interactions of MSH-peptides with the MC1 receptor. We report here the binding of several linear and cyclic MSH peptides to the D117A and H260A mutant MC1 receptors, as well as the properties of a new double mutant clone.

2. Materials and methods

2.1. Chemicals

[Nle[4], D-Phe[7]]α-MSH [14], porcine β-MSH (β-MSHp), Y6-β-MSHp (porcine β-MSH where His in position 6 has been replaced with Tyr; note that in the present paper we use the position numbers shown in Table 2) and [Cys[4], D-Phe[7], Cys[10]]α-MSH(4–13) ((4–13)D) [15,16] were purchased from Saxon Biochemicals GmbH, Germany. Cyclic [Cys[4], D-Phe[7], Cys[10]]α-MSH (1–13) ((1–13)D) and cyclic [Cys[4], L-Phe[7], Cys[10]]α-MSH(1–13) ((1–13)L) were synthesised by Scandinavian Peptide Syntheses. Cyclic [Cys[4], L-Phe[7], Cys[10]]α-MSH(4–10) ((4–10)D), cyclic [Cys[4], D-Nal[7], Cys[10]]α-MSH(4–10) (HS9510), Asn-His-(D-Phe)-Arg-Trp-Gly (NHdFAWG), Met-Asn-His-(D-Phe)-Arg-Trp-Gly (MNHdFAWG) were synthesised in our laboratories using the solid phase approach and purified by HPLC. SHU9119 and Melanotan II (MT II) were synthesised as described previously [17,18]. [Nle[4], D-Phe[7]]α-MSH was radio iodinated by the Chloramine T method and purified by HPLC.

2.2. Construction of receptor clones

The human MC1 receptor gene [1] was cloned into the expression vector pRc CMV (InVitrogen). The mutant MC1 receptor clones D117A (Asp in position 117 mutated to Ala) and H260A (His in position 260 mutated into Ala) were the same as constructed earlier [12]. The double mutant clone was generated by cloning from the single mutant clones. A ca 900 bp fragment was cleaved out of the D260A mutant clone by *Sst* II (*Sac* II) and *Not* I. The *Sst* II site is a unique site between the D117 and H260. This fragment was cloned into the corresponding sites in the D117A mutant and sequenced.

2.3. Expression of receptor clones

For receptor expression COS cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. 80% confluent cultures were transfected with the DNA mixed with liposomes in serum free medium. The liposomes were the commercially available Lipofectin (BRL, USA) or produced according to Campbell (1995) [19]. After transfection the serum-free medium was replaced with the serum containing medium and the cells were cultivated for ca 48 h. Cells were then scraped off, centrifuged and used for radioligand binding.

2.4. Binding studies

The transfected cells were resuspended with binding buffer (Minimum Essential Medium with Earle's salts, 25 mM HEPES, pH 7.0, 0.2% bovine serum albumin, 1 mM 1,10-phenanthroline, 0.5 mg per l leupeptin and 200 mg per l bacitracin) and distributed into 96 well plates (approximately 40 000 cells well) and centrifuged. The binding buffer were immediately replaced with 0.1 ml binding buffer in each well, containing a constant concentration of [^{125}I][Nle[4], D-Phe[7]]α-MSH and appropriate concentrations of an unlabelled ligand. Plates were then incubated for 2 h at 37°C. After incubation, the cells were washed with 0.2 ml of ice-cold binding buffer and detached from the plates with 0.2 ml of 0.1 N NaOH. Radioactivity was counted (Wallac, Wizard automatic gamma counter) and data analyzed with the BindAid software program (Wan System AB, Umeå, Sweden). Data were either analyzed by fitting it to formulas derived from the law of mass-action by the method generally referred to as computer modelling, or by fitting to the four parameter logistic function. K_i-values were calculated by using the Cheng and Prusoff equation [20]. For saturation analysis, 12 concentrations of [^{125}I][Nle[4], D-Phe[7]]α-MSH in the range of 0.02 up to 3 nM were used. Non specific binding was determined in the presence of 3 μM [Nle[4], D-Phe[7]]α-MSH. The K_d value for the double mutants were determined from competition assays with [Nle[4], D-Phe[7]]α-MSH using the law of mass action analysis and sharing the K_d value for [Nle[4], D-Phe[7]]α-MSH and [^{125}I][Nle[4], D-Phe[7]]α-MSH. All the binding assays were performed in duplicate wells and repeated three times. Untransfected COS cells did not show any specific binding to [^{125}I][Nle[4], D-Phe[7]]α-MSH (see Fig. 3).

3. Results

Wild type MC1 receptor and MC1 receptor mutant clones D117A and H260A were expressed in COS cells and tested in binding assays using [^{125}I][Nle[4], D-Phe[7]]α-MSH as radioligand. Saturation curves for the MC1, D117A and H260A are shown in Fig. 1. Competition curves of the 11 MSH-peptides obtained in these tests are shown in Fig. 2. Each curve shows data for a

representative experiment in which each data point is the mean of a duplicate determination. In Table 1, the K_i-values obtained from the computer analysis of these data are shown together with K_d-values for [^{125}I][Nle^4, D-Phe^7]α-MSH obtained from the saturation curves. In Table 2 the structures of the tested peptides are shown, aligned with the sequences of α-MSH and [Nle^4, D-Phe^7]α-MSH. Competition curves for [Nle^4, D-Phe^7]α-MSH on the D117A – H260A double mutant receptor and the wild type MC1 receptor are shown in Fig. 3.

The double mutant clone had very low affinity: for [Nle^4, D-Phe^7]α-MSH the K_d value was 90 ± 16 nM as compared with the 51 ± 6 pM for the wild type MC1 receptor. The low affinity of the double mutant made it impossible to further characterise it with other peptides in competition assays with the same accuracy as for the single mutants.

We also tested the binding of [^{125}I][Nle^4, D-Phe^7]α-MSH to the D117A and H260A mutants by saturation analyses. We observed about 2 fold lower affinity for both the mutants when compared with the wild type clone. This marginal lower affinity was not observed by competition analyses using the [Nle^4, D-Phe^7]α-MSH [12], probably due to a lower accuracy of the competition assay.

A general tendency for the investigated linear peptides was that the D117A caused a higher loss of affinity than the H260A mutation. As can be seen from Table 1, β-MSHp bound with about 200 fold lower affinity to the D117A mutant and 23 fold lower affinity for the H260A mutant, when compared with the wild type MC1 receptor. These results are similar to those reported earlier for the human β-MSH [12]. The Y6-β-MSHp had 280 fold lower affinity for the wild type receptor than the β-MSHp. However, the Y6-β-MSHp had a negligible affinity for the D117A mutant and the H260A mutant. Shorter linear core peptides which have Asn and D-Phe in position 5 and 7, respectively, (i.e. NHdFAWG and MNHdFAWG) had much lower affinity for the mutants and especially for the D117A, which is a pattern similar to that earlier observed for the natural α-, β-, and γ-MSH [12].

Seven cyclic compounds were tested, which did not show this different binding to the two mutants as described above. Five substances are cyclic [Cys^4, Cys^{10}]α-MSH analogues which are cyclisised by generation of a disulphide bridge between the 2 Cys (see Table 2). The (1–13)D and (4–13)D had a very high affinity for the MC1 receptor, whereas the shorter (4–10)D and HS9510 with the bulky amino acid D-Nal^7, had much lower affinity for the wild type MC1 receptor. This was not surprising as it has been reported earlier that the C-terminal tripeptide (Lys-Pro-Val) is very important for the activity of [Cys^4, Cys^{10}]α-MSH analogues in frog and lizard skin bioassays [21]. The (1–13)D, (4–13)D and (1–13)L have all considerable and equally lower affinities for both the mutants, but the (4–10)D binds with similar or only slightly lower affinity to both of the mutants. HS9510 has about eight times lower affinity for the H260A. However, it showed negligible affinity for the D117A mutant since it did not displace [^{125}I][Nle^4, D-Phe^7]α-MSH even in concentrations as high as 100 μM.

SHU9119 and MT II are cyclisised via a lactam bridge between the Asp and Lys side chains (see Table 2). SHU9119 has, like HS9510, the amino acid D-Nal (D-2'-naphthylalanine) in the 7-position. As can be seen from Table 1 the SHU9119 compound showed three fold higher affinity for the H260A mutant compared with the wild type MC1 receptor, whereas the MT II had three fold lower affinity for the H260A. Both MT

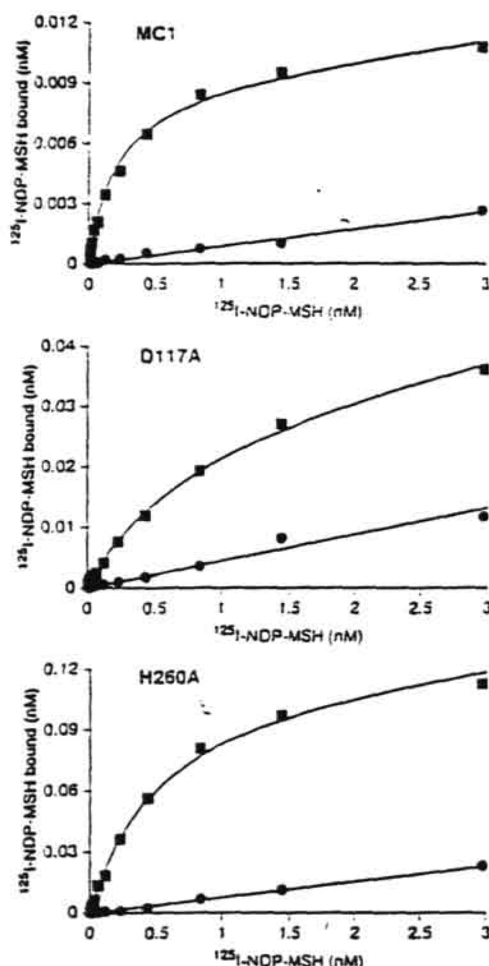

Fig. 1. Saturation curves of [^{125}I][Nle^4, D-Phe^7]α-MSH obtained from transfected COS cell. The figures show total binding (■) and binding in the presence of 3 μM cold [Nle^4, D-Phe^7]α-MSH (●) for wild type MC1 receptor clone, D117A mutant and H260A mutant. Lines represent the computer modelled best fit of the data using an one-site model for the total binding.

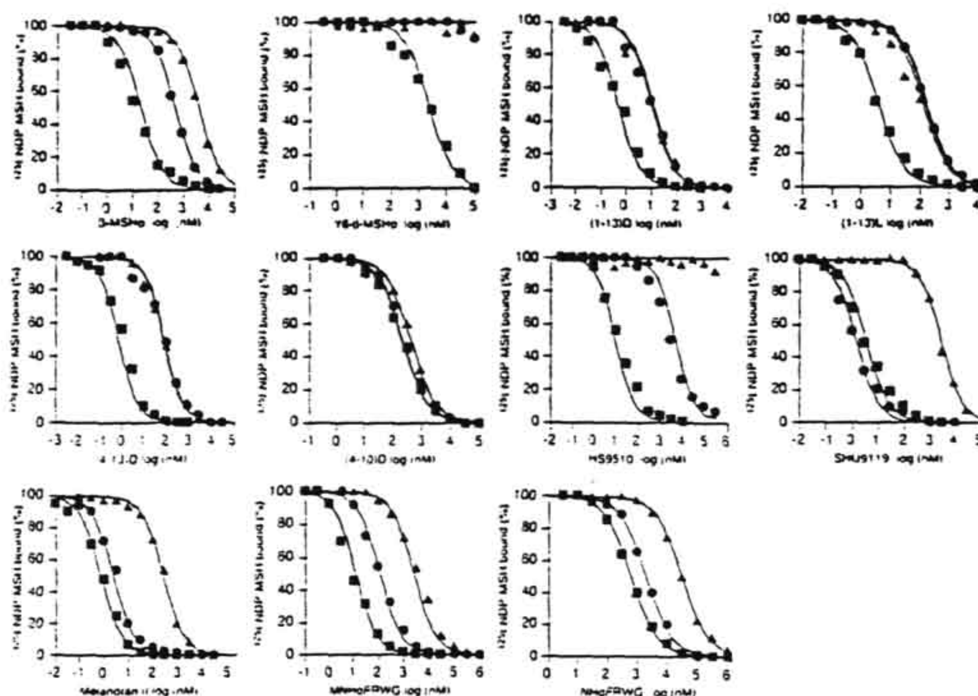

Fig. 2. Competition curves of different MSH analogues obtained on COS cells transfected with the wild type MC1 receptor clone (■), wild type MC1 receptor clone, D117A mutant (▲) or H260A mutant (●) obtained by using a fixed concentration of ~2 nM [^{125}I][Nle^4, D-Phe^7]α-MSH and varying the concentrations of the non-labelled competing peptide. Competing peptides used are indicated on the abscissa for each panel.

II and SHU9119 had much lower affinity for the D117A mutant compared with the wild type. Thus, although the overall affinities of SHU9119 were considerably higher than for HS9510, they showed a similar pattern of affinity changes since they essentially retained their affinities for H260A while losing it for D117A. In this context it may be mentioned that the SHU9119 substance was found to be an antagonist selective for the human MC4 receptors but an agonist for the MC1 receptor [17].

4. Discussion

The hormone binding sites of G-protein coupled receptors for amines, like the catecholamine receptors [22], are comparatively well characterised and a fair understanding of the nature of their interactions with ligands is now available. On the other hand our understanding for the mechanisms underlying ligand binding to G-protein coupled receptors for peptide hormones is much less well developed.

In order to investigate possible interactions of the D117 and the H260 in the MC1 receptor we generated a clone that contained both the D117A and the H260A mutations (D117A + H260A). As can be seen from Fig. 3 the double mutation induced a very drastic reduction of affinity for [Nle^4, D-Phe^7]α-MSH, as measured in competition with [^{125}I][Nle^4, D-Phe^7]α-MSH. These results were unexpected, as the single mutations show only about two fold lower affinity for [^{125}I][Nle^4, D-Phe^7]α-MSH than the wild type. The low affinity of the D117A and H260A mutants might be caused by a rupture of a bond between these two amino acids, if the D117 and H260 were interacting. We might then have expected that the double mutant would have similar affinity to the ligands as the single mutants. The earlier published data [12] may support this as both mutations lead to a similar affinity pattern. The result of the double mutant show otherwise, which might indicate that D117 and H260 are not directly interacting.

Earlier site directed mutagenesis showed that the D117 and the H260 amino acids are important for the binding of MSH peptides to the MC1 receptor. Moreover, based on molecular modelling of the MC1 receptor it was postulated that there might be a direct interaction by a salt bridge formation of the His^6 in the MSH peptide with the D117 of the receptor and the Glu^5 in the MSH peptide with H260 of the receptor [13]. A simple way to test the hypotheses for the His^6/D117 interaction seemed to exchange the His^6 in the MSH peptide to an amino acid capable of only a

Table 1
K_i and K_d values (mean ± S.E.M) obtained from competition and saturation curves using [^{125}I][Nle[4], D-Phe[7]]α-MSH ([^{125}I]NDP) and different MSH analogues on MC1, D117A and H260A transfected COS cells together with relative affinity ratios.

Receptor Ligand	Pos 7	MC1 K_i (nmol/l)	D117A K_i (nmol/l)	H260A K_i (nmol/l)	D117A/MC1	H260A/MC1
[^{125}I]NDP[a]	D-Phe	0.183 ± 0.016	0.478 ± 0.057	0.401 ± 0.023	2.6	2.2
β-MSHp	L-Phe	2.26 ± 0.44	458 ± 53	51.7 ± 3.5	200	23
Y6-β-MSHp	L-Phe	634 ± 99	> 300 000	> 300 000	> 500	> 500
MNHdFRWG	D-Phe	3.57 ± 0.08	2490 ± 1700	62.2 ± 11.6	698	32
NHdFRWG	D-Phe	633 ± 84	31 200 ± 3400	2000 ± 678	49	3.2
(1–13)D	D-Phe	0.037 ± 0.009	0.500 ± 0.100	0.480 ± 0.098	14	13
(1–13)L	L-Phe	0.570 ± 0.110	7.80 ± 1.60	9.55 ± 1.70	14	17
(4–13)D	D-Phe	0.033 ± 0.009	5.10 ± 0.57	4.94 ± 0.45	150	150
(4–10)D	D-Phe	197 ± 32	410 ± 96	217 ± 42	2.1	1.1
HS9510	D-Nal	76.2 ± 7.0	> 100 000	600 ± 278	> 1300	7.9
Melanotan II	D-Phe	0.741 ± 0.147	251 ± 61	2.37 ± 0.710	340	3.2
SHU 9119	D-Nal	0.666 ± 0.174	444 ± 10	0.225 ± 0.055	670	0.34

comparatively weak interaction with D117. This replacement in the ligand should then result in a loss of affinity, comparable to that caused by the D117A mutation, for the natural MSH-peptide. Our data show that replacement of the His[6] in the MSH peptide with Tyr[6] (Y6-β-MSHp) resulted in the expected reduction in affinity for the wild type receptor. However, the Y6-β-MSHp showed negligible affinity for the D117A mutant receptor which was an unexpected finding. In addition, the Y6-β-MSHp peptide failed to bind to the H260A mutant. Thus, the data do not seem to support the hypothesis [13] that there is a direct interaction of the His[6] of the MSH peptides with the D117 in the receptor.

Initial data on the D117A and H260A mutants indicated that the synthetic melanocortic peptide [Nle[4], D-Phe[7]]α-MSH bound with alternate points of attachment to the MC1 receptor, compared with [Nle[4]]α-MSH or the natural melanocortic ligands such as α-MSH. This was because neither of the D117A or H260A mutations affected the affinity of the synthetic ligand whereas the affinities of linear [L-Phe[7]]-MSH-peptides were markedly reduced. In the present study we used cyclic MSH analogues (1–13)D, (4–13)D and (1–13)L, having either a D or an L-Phe at position 7, and we found all these cyclic peptides to behave in a similar fashion since they lost affinity for both the D117A and H260A mutations. Another similar cyclic MSH analogue: (4–10)D had only slightly lower or similar affinity for both the mutants as compared with the wild type. Thus, the new data do not support the hypotheses that [D-Phe[7]]α-MSH analogues bind in a

Table 2
Alignment of α-MSH and [Nle[4],D-Phe[7]]α-MSH (NDP) to the MSH analogues tested in this study

Peptide/position no.	1	2	3	4	5	6	7	8	9	10	11	12	13
α-MSH	Ser	Tyr	Ser	Met	Glu	His	Phe	Arg	Trp	Gly	Lys	Pro	Val
NDP	Ser	Tyr	Ser	Nle	Glu	His	D-Phe	Arg	Trp	Gly	Lys	Pro	Val
β-MSHp	Pro[a]	Tyr	Arg	Met	Glu	His	Phe	Arg	Trp	Gly	Ser	Pro	Pro[b]
Y6-β-MSHp	Pro[a]	Tyr	Arg	Met	Glu	Tyr	Phe	Arg	Trp	Gly	Ser	Pro	Pro[b]
NHdFRWG					Asn	His	D-Phe	Arg	Trp	Gly			
MNHdFRWG				Met	Asn	His	D-Phe	Arg	Trp	Gly			
(1–13)D	Ser	Tyr	Ser	*Cys*	Glu	His	D-Phe	Arg	Trp	*Cys*	Lys	Pro	Val
(1–13)L	Ser	Tyr	Ser	*Cys*	Glu	His	D-Phe	Arg	Trp	*Cys*	Lys	Pro	Val
(4–13)D				*Cys*	Glu	His	D-Phe	Arg	Trp	*Cys*	Lys	Pro	Val
(4–10)D				*Cys*	Glu	His	D-Phe	Arg	Trp	*Cys*			
HS9510				*Cys*	Glu	His	D-Nal[c]	Arg	Trp	*Cys*			
Melanotan II				Nle	*Asp*	His	D-Phe	Arg	Trp	*Lys*			
SHU 9119				Nle	*Asp*	His	D-Nal[c]	Arg	Trp	*Lys*			

All peptides have an acetyl-group on the N-terminus and an amide group on the C-terminus, except MNHdFRWG and NHdFRWG which do not have an acetyl group at the N-end. The amino acid residues which make up the ring closure in the cyclic compounds are shown underlined in italics.

[a]Asp-Glu-Gly of N-terminus is not shown in the table.

[b]Lys-Asp of C-terminus is not shown in the table.

[c]D-Nal = D-2'-naphthylalanine.

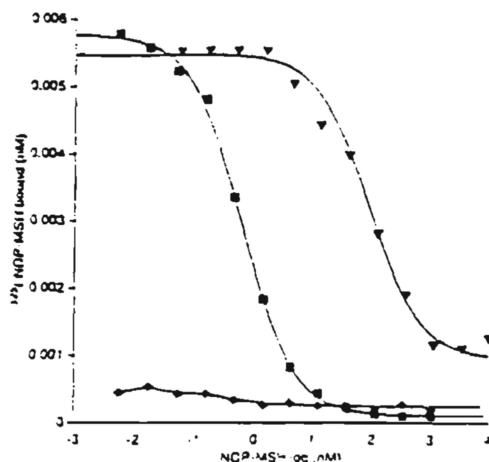

Fig. 3. Competition curves for non-labelled [Nle⁴, D-Phe⁷]α-MSH obtained on COS cells transfected with wild type MC1 receptor clone (■), the D117A – H260A mutant (▼) and non transfected COS cells (◆) using a fixed concentration of ~2 nM [¹²⁵I][Nle⁴, D-Phe⁷]α-MSH for the wild type and non transfected COS cells and ~20 nM [¹²⁵I][Nle⁴, D-Phe⁷]α-MSH for the D117A – H260A mutant.

principally different way to the MC1 receptor compared with [L-Phe⁷]α-MSH analogues as originally suggested in Frändberg et al., 1994 [12].

The cyclic lactam analogues MT II and SHU9119 have high affinities for the MC1 receptor and much lower affinity for the D117A mutant. This property is shared by the HS9510, which like SHU9119 has D-Nal⁷. It is interesting to note that SHU9119 and MT II have an Asp in position 5 which is formally an Asn due to the formation of a lactam bridge with the Lys¹⁰ side chain in the formation of the ring structure of the compound. The fact that SHU9119 retains full affinity for the H260A mutant lends further support to the hypothesis that the H260 in the receptor does not interact with position 5 of the ligand.

Taken together our new data are difficult to reconcile with the original hypothesis of direct interactions of D117 and H260 in the MC1 receptor with His⁶ and Glu⁵, respectively, in the MSH peptides or that there are alternative points of attachment for α-MSH and its stereo isomer [Nle⁴, D-Phe⁷]α-MSH. The common denominator for the D117A and H260A mutations seems to be a drop in affinity for most of the tested peptides. The data indicate that the D117A and the H260A mutations may cause conformational changes in the receptor which can not be linked to any specific amino acid in the MSH-peptides. This is also supported by the double mutant clone which resulted in more than 1000 fold loss in affinity for the [Nle⁴, D-Phe⁷]α-MSH. The D117A mutation greatly affected the binding constants of the bulky D-Nal⁷ compounds and the structurally less restrained linear peptides. Therefore, this mutation might decrease the flexibility of the receptor protein.

Further studies using site directed mutagenesis, modelling of the 3D structure of the MC1 receptor and specific modifications of MSH analogues will be required to achieve a further understanding of these issues.

Acknowledgements

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Alternative translation initiation codon for the human melanocortin MC_3 receptor does not affect the ligand binding

Helgi B. Schiöth [a], Ruta Muceniece [b], Jarl E.S. Wikberg [a,c,*], Michael Szardenings [a]

[a] Department of Pharmaceutical Pharmacology, Biomedical Center, Box 591, Uppsala University, S-75 124 Uppsala, Sweden
[b] Laboratory of Pharmacology, Institute of Organic Synthesis Riga, Latvia
[c] Wa Pharm, Umeå, Sweden

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Abstract

The genomic DNA for the human melanocortin MC_3 receptor indicates an unusually long N-terminus. Two possible translation initiation sites, the one originally proposed and one alternate 111 bp downstream, were mutated. For a third mutant the DNA between these initiation sites was deleted. All mutants were expressed in COS (CV-1 Origin, SV40) cells in the same level, and they bound peptide hormones in the same fashion, as did the wild type clone. The data obtained indicate that both sites can function as the sole translation initiation sites of the human clone and that the proposed N-terminus of the human melanocortin MC_3 receptor is not important for the ligand binding of the receptor.

Keywords: Melanocortin MC_3 receptor; MSH (melanocyte-stimulating hormone); Ligand binding; Mutagenesis

1. Introduction

The melanocortin hormones originate from proopiomelanocortin, which is proteolytically cleaved into three biologically active families of peptides, the adrenocorticotropins, melanocortins and endorphins. Adrenocorticotropin (ACTH), α-MSH (melanocyte-stimulating hormone) and β-endorphin have a well-known role in adrenal cortical function, pigmentation and analgesia. The melanocortins have, additionally to their involvement in pigmentation, a broad array of other physiological functions, which underlying mechanisms are not understood. In the CNS, melanocortins have been shown to influence behaviour, memory, thermoregulation, analgesia and control of cardiovascular systems (Eberle, 1988).

Molecular cloning has identified five melanocortin receptor subtypes, known as MC_1–MC_5 receptors (Chhajlani et al., 1993; Chhajlani and Wikberg, 1992; Gantz et al., 1993a,b; Mountjoy et al., 1992). The melanocortin MC receptors form a family of their own with distinct differences to other G-protein-coupled receptors. Besides other characteristic features, the MC receptors are the smallest G-protein-coupled receptors showing short N and C terminal sequences.

The melanocortin MC_1 receptor has high affinity to α-MSH. It has been detected in human melanocytes and in small numbers in the brains of rats and humans. The melanocortin MC_2 receptor is expressed in the adrenal gland (Mountjoy et al., 1992). The melanocortin MC_3 receptor is expressed in the brain, i.e. predominantly in the arcuate nucleus and in few regions of the brain stem, in addition to placenta and gut tissues (Gantz et al., 1993a). The melanocortin MC_4 receptor is expressed in the brain and the melanocortin MC_5 receptor has, in addition to its expression in the brain, a wide peripheral distribution (for review see Siegrist and Eberle, 1995).

The genomic DNA sequence for the human melanocortin MC_3 receptor revealed a 361 amino acids long open reading frame, which allowed for the assignment of an unusually long N-terminal sequence. Comparison of the human melanocortin MC receptor subtypes shows that methionine in position 38 (Met^{38}) of the melanocortin MC_3 receptors aligns with the Met^1 in the human melanocortin MC_1 receptor. The cloning of the homologous receptors from rat (Roselli-Rehfuss et al., 1993) and mouse (Desarnaud et al., 1994) revealed reading frames of only 323 amino acids for the receptors of both

* Corresponding author. Fax: (46-18) 559-718.

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species. The rat and mouse sequences are lacking the first ATG corresponding to the proposed translation initiation codon of the human melanocortin MC_3 receptor, but they both have translation initiation codons corresponding to Met^{38} of the human clone. The N-terminus of these proposed receptor proteins is therefore 37 amino acids shorter than the human clone. It has been suggested that the start codon of the human melanocortin MC_3 receptor should be assumed to be identical to these other receptors.

Mutagenesis studies (Fründberg et al., 1994) and modelling (Prusis et al., 1995) of the MC-receptors are presently carried out in our laboratory, and we need to know which parts of the receptor proteins are expressed and which are of importance for ligand binding. The length of the N-terminal sequence is also of importance for making specific antibodies against the melanocortin MC_3 receptor. The aim of the present study was therefore to investigate by mutagenesis and binding which parts of the N-terminal sequence of the human melanocortin MC_3 receptor are really essential for the expression and hormone binding of the protein.

2. Materials and methods

2.1. Chemicals

The $[Nle^4,D\text{-}Phe^7]\alpha$-MSH, α-MSH, β-MSH and γ_1-MSH (H_2N-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-NH_2) were purchased from Saxon Biochemicals, Germany. $[Nle^4,D\text{-}Phe^7]\alpha$-MSH was radioiodinated by the Chloramine-T method and purified by HPLC (high-pressure liquid chromatography).

2.2. Mutated and truncated melanocortin MC_3 receptor clones

The mutated and truncated melanocortin MC_3 clones were created with PCR (polymerase chain reaction). The following primers were used in combination with 5′*Hin*dIII linker and 3′*Xba*I linker (mutated positions in bold, the ATG corresponding to Met^1 is underlined). The 5′primer for MC_3-ATG1 was: GGAAGCTT GACTGAGCATC-CAAAAGAAGTATCTGG (the mutation is shown in bold and the position of the first ATG codon is underlined). The 5′primer for MC_3-ATG2 was: GGAAGCTTGAATGAGCATCCAAAAGAAGTATCTG-GAGGGAGATTTTGTCTTTCCTGTGAGCAGCAGCA-GCTTCCTACGGACCCTGCTGGAGCCCCAGCTCGG-ATCAGCCCTTCTGACAGCACTGAATG. The 5′primer for MC_3-MtoM was: GGAAGCTTGAATGAAT-GCTTCGTGCTGC. The 3′primer used for all the clones were; GGTCTAGACTATCCCAAGTTCATGCCG. (All primers are written in 5′ to 3′direction). The PCR products were cloned into the *Hin*dIII and *Xba*I sites of the pRc/CMV vector (Invitrogen) and the inserts and adjacent sequences of the vector DNA used for transfection experiments were confirmed by sequencing.

2.3. Expression of receptor clones

The human melanocortin MC_3 receptor DNA, cloned into the expression vector CMV/neo, was a gift from Dr Ira Gantz (Gantz et al., 1993a). For receptor expression, COS (CV-1 Origin, SV40) cells were grown in Dulbecco's modified Eagle's medium with 10% foetal calf serum. Eighty per cent confluent cultures were transfected with the DNA mixed with liposomes in serum free medium. The liposomes were the commercially available lipofectin (BRL, USA) or produced according to Campbell (1995). After transfection the serum-free medium was replaced with the serum containing medium and the cells were cultivated for ca. 48 h. Cells were then scraped off, centrifuged and used for radioligand binding.

2.4. Binding studies

The transfected cells were washed with binding buffer (Minimum Essential Medium with Earle's salts, 25 mM Hepes, pH 7.0, 0.2% bovine serum albumin, 1 mM 1,10-phenanthroline, 0.5 mg/l leupeptin and 200 mg/l bacitracin) and distributed into 96 well plates. The cells were then incubated for 2 h at 37°C with 0.1 ml binding buffer in each well, containing a constant concentration of $[^{125}I][Nle^4,D\text{-}Phe^7]\alpha$-MSH and appropriate concentrations of an unlabelled ligand. After incubation, the plates were put on ice, the cells washed with 0.1 ml of ice-cold binding buffer and detached from the plates with 0.2 ml of 0.1 N NaOH. Radioactivity was counted (Wallac, Wizard automatic gamma counter) and data analysed with the BindAid software (Wan System AB, Umeå, Sweden). Data were either analysed by fitting it to formulas derived from the law of mass-action by the method generally referred to as computer modelling, or by fitting to the four parameter logistic function. K_i values were calculated by using the Cheng and Prusoff equation. The binding assays were performed in duplicate wells and repeated three times.

3. Results

Three mutants of the human melanocortin MC_3 receptor were created in order to investigate whether the proposed extracellular N-terminal domain of the human melanocortin MC_3 receptor is essential for its hormone binding activity. The ATG codons, corresponding to the first and second methionine in the original sequence (Gantz et al., 1993a), were mutated to CTG resulting in the clones MC_3-ATG1 and MC_3-ATG2, respectively. In a third clone the entire sequence between the first and the second ATG was deleted (MC_3-MtoM). (See schematic presentation of the clones in Fig. 1).

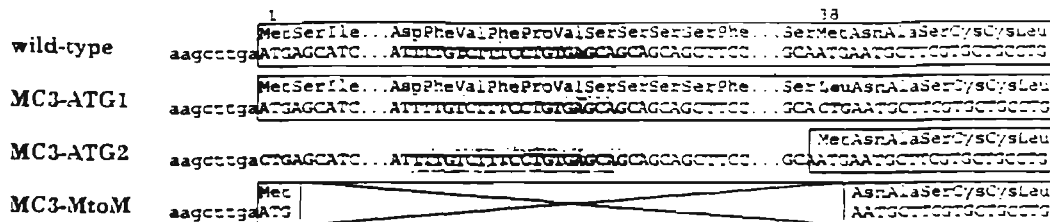

Fig. 1. Comparison of the wild type sequence with the mutant clones described in the text. The 5'-DNA sequence starting from the *Hin*dIII cloning site in the vector pRc/CMV and the likely translated proteins are shown. The sequence of the proposed intron/exon boarder (Desarnaud et al., 1994) is shaded in grey.

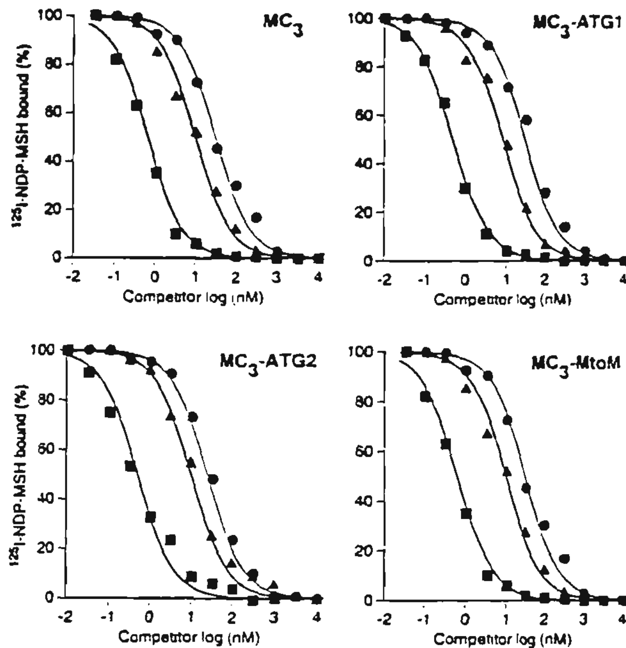

Fig. 2. Competition curves of [Nle^4,D-Phe^7]α-MSH (■), α-MSH (●), and γ_1-MSH (♦) obtained on transfected COS cell using a fixed concentration of ~2 nM [^{125}I][Nle^4,D-Phe^7]α-MSH for the different melanocortin MC_3 receptor clones.

COS cells were transfected with the wild type melanocortin MC_3 receptor and the three mutants. The binding properties of [^{125}I][Nle^4,D-Phe^7]α-MSH for the mutated and the truncated clone were indistinguishable from that of the wild type MC_3 clone. The K_i values are in close agreement with results obtained earlier in our laboratory (Schiöth et al., 1995). The competition curves for [Nle^4,D-Phe^7]α-MSH, α-MSH and γ_1-MSH on transfected COS cells are shown in Fig. 2 for the wild type MC_3, the MC_3-ATG1, MC_3-ATG2 and the MC_3-MtoM, respectively. The K_i values for [Nle^4,D-Phe^7]α-MSH, α-MSH, β-MSH and γ_1-MSH are presented in Table 1. As can be seen from the table the K_i values for different mutants and the wild type receptors were almost the same, and well within the range of the normal variability of radioligand assay used. Moreover, the level of expression indicated by the data analysis was similar for all the four clones investigated (data not shown).

4. Discussion

The alignment of all known melanocortin receptors has already indicated that the proposed N-terminus of the human melanocortin MC_3 receptor and a murine melanocortin MC_5 receptor (Fathi et al., 1995) are unusally long. Both clones contain a possible alternative translation initiation codon, which can be aligned to the initiation codon in clones of the same melanocortin receptor subtype of other species. Recent data also indicate that amino acid residues in the N-terminal loop of the melanocortin MC_1 receptor might be important for the ligand binding (Chhajlani et al., 1996).

Comparison of the sequences surrounding both translation initiation codons reveals strong deviation from what is believed to be a standard eukaryotic translation initiation site (Kozak, 1984). Data from a more recent statistical analysis of known translated sequences (Cavener and Ray, 1991) indicate that the first site (GCAAUGA) occurs in fewer vertebrate sequences than the second (TGAAUGA). Both sequences are underrepresented and this has made it impossible to draw direct conclusions.

In order to investigate the importance of the putative extra long N-terminal chain of the human melanocortin MC_3 receptor we made three mutant clones. The proposed

Table 1
K_i values (means ± S.E.M). obtained from competition curves for MSH peptides on melanocortin MC_3, MC_3-ATG1, MC_3-ATG2 and MC_3-MtoM

Ligand	MC_3 (nmol/l)	MC_3-ATG1 (nmol/l)	MC_3-ATG2 (nmol/l)	MC_3-MtoM (nmol/l)
[Nle^4,D-Phe^7]α-MSH	0.347 ± 0.032	0.289 ± 0.041	0.279 ± 0.075	0.316 ± 0.023
α-MSH	23.1 ± 3.2	35.2 ± 4.9	28.5 ± 4.3	25.8 ± 5.5
β-MSH	32.8 ± 4.7	18.8 ± 1.4	16.6 ± 1.8	31.1 ± 3.5
γ_1-MSH	7.52 ± 0.91	8.68 ± 2.1	12.8 ± 3.7	9.19 ± 3.02

translation initiation codon and the alternate translation initiation codon 111 bp downstream were independently mutated by a point mutation ATG → CTG (MC_3-ATG1 and MC_3-ATG2), while the rest of the sequence was not changed to keep proposed intron/exon borders (Desarnaud et al., 1994). In a third mutant the 111 bp between the first and the second ATG were deleted (MC_3-MtoM). All three mutants were easily expressed in COS cells in similiar levels and readily characterized by radioligand binding. All three clones showed binding properties similiar to those of the original wild type melanocortin MC_3 clone. The data obtained for the MC_3-ATG1 and the MC_3-MtoM mutant is therefore in agreement with previously published data for melanocortin MC_3 receptors from other species. It is thus conceivable that Met^{38} can indeed function as the sole translation initiation site of the human clone. More surprising is the fact that even the MC_3-ATG2 mutant, which does not contain the Met^{38} codon, could be expressed successfully and in amounts similiar to those obtained with the other constructs. The proposed splicing site (Desarnaud et al., 1994) between the first and the second ATG might therefore not be functional for the human melanocortin MC_3 clone, at least in the vector system used for this study. It remains unclear whether both translational products are present when the receptor is expressed in tissues in vivo. Still the similarity of the binding properties suggests that this would have no effect on the pharmacology of this receptor. Our data shows, that the proposed N-terminus of the human melanocortin MC_3 receptor is not important for the ligand binding of the receptor; at least for modelling and mutagenesis studies, only the sequence from Met^{38} needs to be taken into account.

Acknowledgements

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Evidence Indicating That the TM4, EL2, and TM5 of the Melanocortin 3 Receptor Do Not Participate in Ligand Binding

Helgi B. Schiöth,*[1] Ruta Muceniece,*† Michael Szardenings,* Peteris Prusis,* and Jarl E. S. Wikberg*

*Department of Pharmaceutical Pharmacology, Uppsala University, Uppsala, Sweden; and †Laboratory of Pharmacology, Institute of Organic Synthesis, Riga, Latvia

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The TM4, EL2 and TM5 show low amino acid homology within the MC receptor family. Three mutants of the human MC3 receptor were created in order to investigate the participation of these regions in ligand binding. The TM4, EL2 and TM5 were separately changed by multiple mutagenesis so that their amino acid sequences became identical with the human MC1 receptor. The mutants were expressed in COS cells and they bound peptide ligands in the same fashion as the wild type MC3 receptor clone. Our results indicate that the amino acids that were mutated in the MC3 receptor do not affect the binding of MSH peptides. The data provide further evidence, that the mutated regions may not participate at all in ligand binding, as indicated by modelling experiments and homology comparison. © 1996 Academic Press

The roles of the melanocortic peptides, α-MSH and ACTH in pigmentation and steroidoneogeneses are quite well understood. The melanocortins also show a variety of other peripheral and central effects such as antipyretic, neurotrophic, anti-inflammatory, analgesic effects and control of learning, memory and motivation, whose mechanisms of action remain largely unknown [1-2].

By molecular cloning, the genes for five MC receptors were recently isolated [3-7]. Binding experiments of expressed MC1, MC3, MC4 and the MC5 receptor genes have shown that the subtypes bind to the natural melanocortin peptides, α-MSH, β-MSH, γ-MSH and ACTH with specific and unique affinity profiles [8-10]. The MC1 receptor has a high affinity for α-MSH and is expressed in melanoma cells. The MC2 (or ACTH) receptor does not bind the MSH peptides, but binds ACTH with high affinity in contrast to the other MC receptors which bind ACTH with low affinity [11]. The MC3, MC4 and MC5 receptors bind to the MSH peptides with much lower affinity than the MC1 receptor [10]. These subtypes are all expressed in the brain. The MC5 receptor also has a wide peripheral distribution [12].

The MC receptors are G-protein coupled and show topology of 7 transmembrane (TM) receptors. The MC receptor subtypes show considerable amino acid homology: from 38 % between the MC1 and the MC2 up to 60 % between the MC4 and the MC5. The MC receptor family has closest homology to a cannabinoid receptor, to which the MC1 has 32 % amino acid identity.

Very little is known about how the melanocortin peptides bind to the MC receptors in general. Amino acid residues in the TM3 and TM6 [13] and in the extra cellular loops (EL) [14] in the MC1 receptor which may participate in the ligand binding have been identified by site directed mutagenesis. A natural single residue mutation in the TM2 of murine MC1

[1] Address all correspondence to: Helgi B. Schiöth or Jarl E.S. Wikberg, Pharmaceutical Pharmacology, Box 591, BMC, S-751 24 Uppsala, Sweden. Fax: +46-18-559718. E-mail: helgis@bmc.uu.se, jwikberg@bmc.uu.se.

Abbreviations: MSH, melanocyte stimulating hormone; ACTH, adrenocorticotropin; MC, melanocortin; TM, transmembrane; EL, extra cellular loop; PCR, polymer chain reaction; COS, CV-1 Origin, SV40.

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resulted in constitutively active receptor [15]. Molecular modelling and ligand docking using cyclic [Cys[4], D-Phe[7], Cys[10]]α-MSH indicated that this ligand could be docked into a pocket located between the TM2, TM3 and TM6 of the model [16].

The aim of this study was to mutate the TM4, TM5 and EL2 in order to find out whether they participate in ligand binding.

MATERIALS AND METHODS

Chemicals. [Nle[4], D-Phe[7]]α-MSH (NDP-MSH) [17], [Nle[4]]α-MSH and α-MSH were purchased from Bachem, Switzerland. Cyclic [Cys[4], D-Phe[7], Cys[10]]α-MSH (1-13) (cCDC) and cyclic [Cys[4], L-Phe[7], Cys[10]]α-MSH(1-13) (cCLC) were synthesised by Scandinavian Peptide Syntheses. cCDC and cCLC were cyclisised by a disulphide bridge between the two Cys. NDP-MSH was radioiodinated by the Chloramine T method and purified by HPLC.

Mutated MC3 receptor clones. The mutant MC3 receptor clones were made by PCR [18, 19]. Briefly, a fragment of the human MC3 receptor clone [5] was amplified by PCR using downstream primers carrying the mutations and an upstream primer with a *Xba* I linker. These PCR products, or megaprimers, containing the mutations, were purified and used in amplification of the whole receptor gene using a downstream primer with a *HinD*III linker. The PCR products were cloned into the *HinD*III and *Xba*I sites of the pRc/CMV vector (Invitrogen) and the inserts and adjacent sequences of the vector DNA used for transfection experiments were confirmed by sequencing. The 3' end primer used for all the clones was: GGT CTA GAC TAT CCC AAC TTC ATG CCG. (All primers are written in 5' to 3' direction). The 5' end primer used for all the clones was: GCA ACC TTG AAT GAC CAT CCA AAA GAA GTA TCT GG. The mutation primer for MC3-TM4[MC1] was: ACG AAG GCC CTC ACC TTG GTC GCG GCC ATC TGG GTC GCC TCC GTC GTC TTT TCC ACG CTG TTC ATC GCC TAC TCG GAG AGC AAA AT. The mutation primer for MC3-EL2[MC1] was: TG GTG TTC ATC GTC TAC TAT GAT CAC GTA ATC GTC ATT GTG TGC CTC A. The mutation primer for MC3-TM5[MC1] was: TAC TCG GAG AGC AAA GCG GTC CTT CTG TGC CTC GTC GTC TTC TTC CTC GCC ATG CTG GTC CTC ATG GCC GTC CTC TAC GTG CAC ATG TTC CT.

Expression of receptor clones. The human MC1 [3] was cloned into the expression vector pRc/CMV (InVitrogen). The human MC3 receptor DNA, cloned into the expression vector pCMV/neo, was a gift from Dr. Ira Gantz [5]. For receptor expression, COS-1 cells were grown in Dulbecco's modified Eagle's medium with 10 % foetal calf serum. Eighty percent confluent cultures were transfected with the DNA mixed with liposomes in serum free medium (for details see [10]). After transfection, the serum-free medium was replaced with growth medium and the cells were cultivated for about 48 h. Cells were then scraped off, centrifuged, and used for radioligand binding.

Binding studies. The transfected cells were washed with binding buffer [9] and distributed into 96 well plates. The cells were then incubated for 2 h at 37°C with 0.05 ml binding buffer in each well, containing a constant concentration of [^{125}I]NDP-MSH and appropriate concentrations of an unlabelled ligand. After incubation, the cells were washed with 0.2 ml of ice-cold binding buffer and detached from the plates with 0.2 ml of 0.1 N NaOH. Radioactivity was counted (Wallac, Wizard automatic gamma counter) and data analysed with a software package for radioligand binding analyses (Wan System, Umeå, Sweden). Data were either analysed by fitting it to formulas derived from the law of mass-action by the method generally referred to as computer modelling, or by fitting to the four parameter logistic function. K_i-values were calculated by using the Cheng and Prusoff equation [20]. The binding assays were performed in duplicate wells and repeated three times. Untransfected COS-1 cells did not show any specific binding to [^{125}I]NDP-MSH.

RESULTS

Three mutants of the human MC3 receptor were created in order to investigate the participation of TM4, EL2 and TM5 in ligand binding. The TM4, TM5, and EL2 were separately changed so that these amino acid sequences became identical with the corresponding sequences in the human MC1 receptor. 10 amino acids in the TM4, 4 amino acids in the EL2 and 11 amino acids in the TM5 were mutated to the corresponding amino acids in the MC1 to generate the mutants MC3-TM4[MC1], MC3-EL2[MC1], and MC3-TM5[MC1], respectively (see schematic representation of the clones in Fig. 1).

Wild type MC3 receptor and MC3 receptor mutant clones MC3-TM4[MC1], MC3-EL2[MC1] and MC3-TM5[MC1] were expressed in COS cells and tested in binding assays using [^{125}I]NDP-MSH as radioligand. Saturation curves for the MC3, MC3-TM4[MC1], MC3-EL2[MC1] and MC3-TM5[MC1] are shown in Fig. 2. Competition curves of different MSH-peptides obtained in these tests are shown in Fig. 3. In Table 1, the K_i-values obtained from the computer analysis of these data are shown together with the K_d-values for [^{125}I]NDP-MSH obtained from the saturation curves.

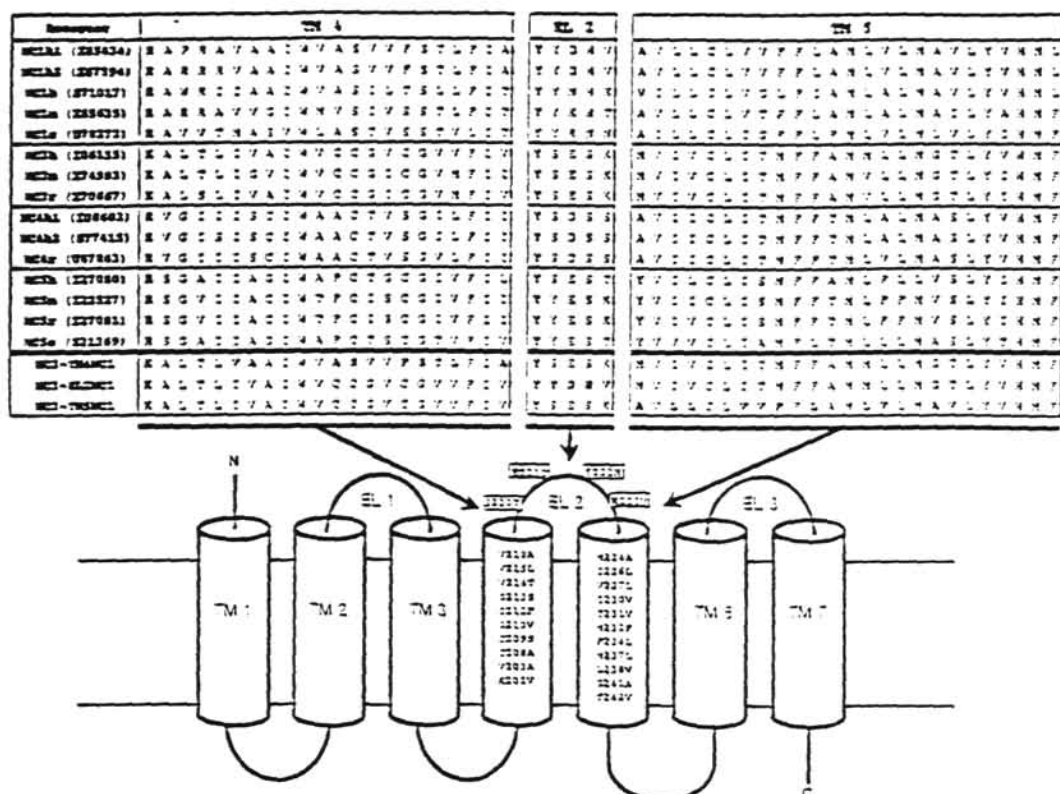

FIG. 1. Top part: Alignment of the amino acid sequences of 15 cloned MC receptors (MC2 not included) with the mutant clones described in the text. Numbers in parentheses denote the accession numbers in EMBL and GenBank Data Libraries. I27080 is identical with U08353 and I22527 is identical with U08354 and X76295 in these regions. The abbreviations for the species are as follows: h: human, b:bovine, m:murine, r:rat, c:chicken, o:ovine. The mutated amino acids in the mutant clones are shown in bold. Bottom part: Two dimensional topography of the MC3 receptor showing the mutations in TM4, EL 2 and TM5.

NDP-MSH has about 4 fold higher affinity for the MC1 receptor compared to the MC3 receptor (see Table 1). α-MSH and [Nle⁴]α-MSH are even more suitable for discrimination between the two receptors as these peptides have about 100 fold higher affinity for the MC1 compared to the MC3 receptor. Cyclic [Cys⁴, Cys¹⁰]α-MSH analogues are known as potent melanotropes in pigmentation assays [21]. It is possible that these peptides bind differently to the receptors than the linear MSH peptides. The cCDC and cCLC have more than 50 fold higher affinity for the MC1 receptor than for the MC3 receptor. As can be seen from Table 1 and Fig. 2 and 3, the K_d values of [^{125}I]NDP-MSH and the K_i values of NDP-MSH, [Nle⁴]α-MSH, α-MSH, cCDC and cCLC of the mutant receptors are indistinguishable from that of the wild type MC3 receptor.

DISCUSSION

Hydropathicity plots of G-protein coupled receptors indicate that they contain hydrophobic domains of 20-25 amino acids in length which represent transmembrane regions. Based on structural similarities with the extensively characterised proteins rhodopsin and bacteriorhodopsin, these regions are predicted to be α-helices and to be oriented to form a ligand binding pocket. For many small ligands like adrenaline, the binding pocket is assumed to be buried between the TM domains. Much less is known about the binding of larger ligands like the

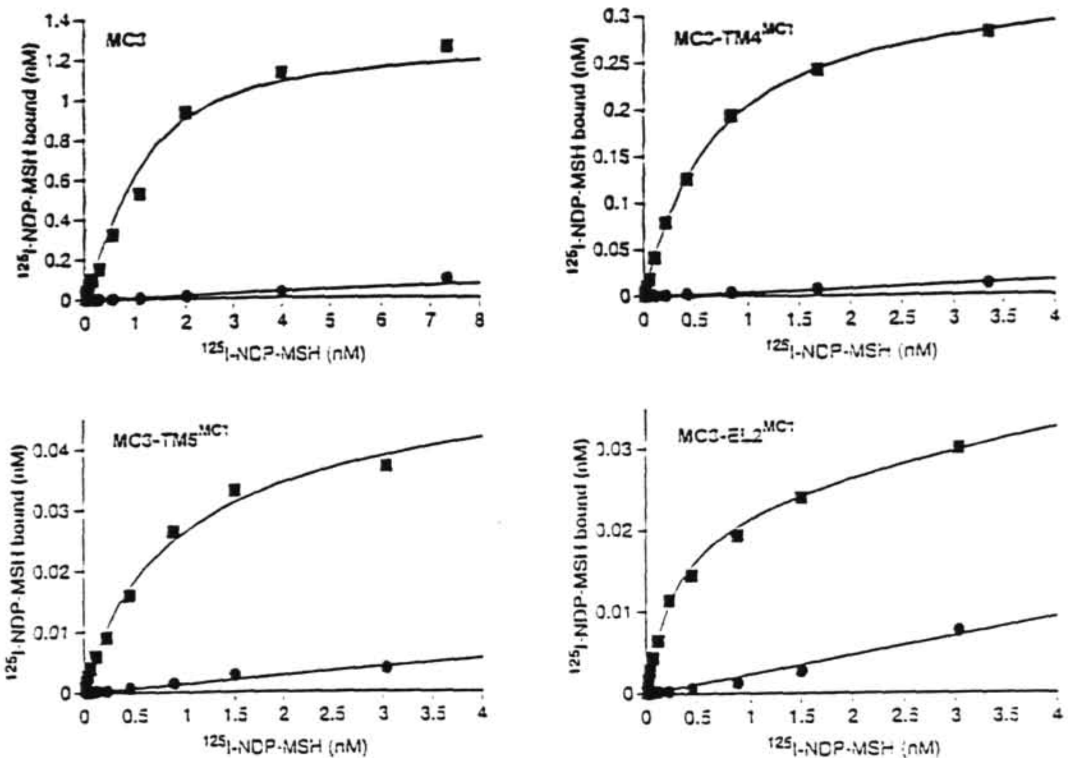

FIG. 2. Saturation curves of [^{125}I]NDP-MSH obtained from transfected COS cells. The figures show total binding (■) and binding in the presence of 3 μM cold NDP-MSH (●) for wild type MC3 receptor clone, MC3-TM4^{MC1}, MC3-TM5^{MC1} and MC3-EL2^{MC1}. Lines represent the computer modelled best fit of the data using a one-site model for total binding.

MSH peptides where the EL may also participate in the ligand binding. Alanine mutations on residues in TM3, TM6, EL2, EL3 and the N-terminal sequence had effects on ligand binding [13,14]. It is not known whether these amino acids have direct contact to the ligand or whether these mutations only cause conformational changes. Multiple alignment of the cloned MC receptors MC1 (human, rat, mouse, bovine), MC3 (human, rat, mouse), MC4 (human, rat) and MC5 (human, rat, mouse, ovine) show low homology of the TM4 and TM5. 12 positions in TM4 are occupied by 4 or more different amino acids. The TM5 has 5 whereas the TM1, TM3 and TM7 have only one, and the TM6 has two and the TM2 has 5 variant positions. The N-terminal and the EL's show low homology (the MC2 was not included in this comparison as it does not bind the MSH peptides). Preliminary molecular modelling of the MC1 receptor indicated an arrangement of the TM domains with the TM4 and TM5 outside of the binding region for the MSH peptide [16].

The MC3 receptor shares 43, 42 and 57 % amino acid identity with the MC1, MC4, and MC5 receptors respectively. Our results show that the binding of the MC3 receptor was not affected by changing the sequence to that of the MC1 in TM4, TM5 and EL2. The data indicate that the amino acids that were mutated in the MC3 do not affect binding of the MSH peptides. It seems quite probable that we would have observed some changes in the ligand binding for these different peptides if the TM4, EL2 or TM5 directly participated in the peptide binding. Our data thus provide further evidence that the mutated regions do not participate at all in ligand binding, as indicated by modelling experiments and homology comparison.

It is interesting to note that the mutation Asp^{184}Ala in the EL2 led to a complete loss

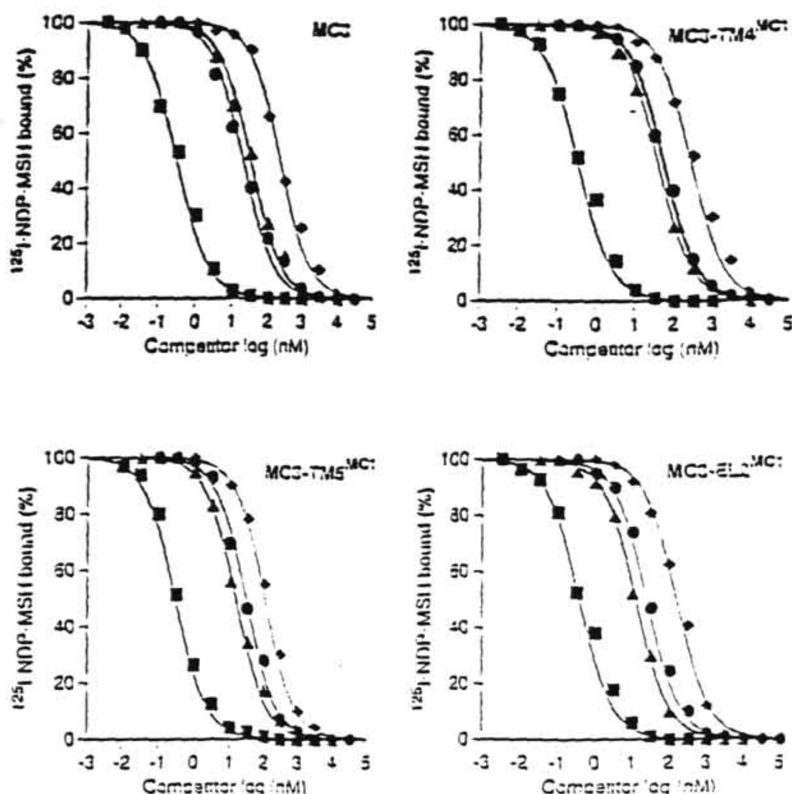

FIG. 3. Competition curves of NDP-MSH (■), α-MSH (●), cCDC (▲) and cCLC (◆) obtained on transfected COS cells using a fixed concentration of ~2 nM [^{125}I]NDP-MSH for the different MC3 receptor clones.

of binding in the MC1 receptor [14]. As can be seen in Fig 1, there is no Asp in the EL2 of the MC3 receptor. If the Asp[184] directly participated in binding to the MSH peptides in the MC1 receptor, we might expect that insertion of Asp into the MC3 or the whole EL2 from the MC1, as in the case for MC3-EL2[MC1], would have effects on the binding. No effect was observed. One plausible explanation for the loss of binding is that the MC1 Asp[184]Ala mutation caused a loss of a hydrophilic residue in the EL2 which influenced its structural functionality. Of course it is possible, although it seems rather unlikely, that

TABLE 1

K_i and K_d[a] Values (mean ± S.E.M.) Obtained from Competition and Saturation Curves Using [^{125}I]NDP-MSH and Different MSH Analogues on Transfected COS-1 Cells

	Receptor				
Ligand	MC3 K_i (nmol/l)	MC3-TM4[MC1] K_i (nmol/l)	MC3-EL2[MC1] K_i (nmol/l)	MC3-TM5[MC1] K_i (nmol/l)	MC1 K_i (nmol/l)
[^{125}I]NDP-MSH[a]	0.358 ± 0.055	0.369 ± 0.04	0.442 ± 0.068	0.404 ± 0.098	0.100 ± 0.009
NDP-MSH	0.304 ± 0.032	0.339 ± 0.044	0.367 ± 0.067	0.301 ± 0.028	0.062 ± 0.009
α-MSH	19.5 ± 3.1	57.9 ± 12.9	26.3 ± 4.8	30.5 ± 6.6	0.228 ± 0.029
[Nle[4]]-α-MSH	27.0 ± 5.0	23.3 ± 4.0	31.8 ± 5.5	34.4 ± 8.9	0.141 ± 0.017
cCDC	23.3 ± 2.9	40.0 ± 4.3	12.9 ± 2.9	13.7 ± 3.6	0.210 ± 0.015
cCLC	226 ± 53	285 ± 63	160 ± 38	107 ± 23	1.32 ± 0.13

the MSH-peptide has an important attachment point at the EL2 in MC1, which the MC3 does not share.

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Deletions of the N-terminal regions of the human melanocortin receptors

Helgi B. Schiöth[a,*], Susanna Petersson[a], Ruta Muceniece[a,b], Michael Szardenings[a], Jarl E.S. Wikberg[a]

[a]*Department of Pharmaceutical Pharmacology, Biomedical Center, Uppsala University, Box 591, 75 124 Uppsala, Sweden*
[b]*Laboratory of Pharmacology, Institute of Organic Synthesis, Riga, Latvia*

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Abstract **The non-homologous N-terminal regions of four human melanocortin (MC) receptors were truncated in order to investigate their putative participation in ligand binding. Eleven constructs were made, where different numbers of residues from the N terminus were deleted. These constructs were used for transient expression experiments in COS cells and analysed by ligand binding. The results show that 27, 25, 28, and 20 amino acids could be deleted from the N terminus of the human MC1, MC3, MC4 and MC5 receptors, respectively, including all potential N-terminal glycosylation sites in the MC1 and the MC4 receptors, without affecting ligand binding or expression levels. The results indicate that the N-terminal regions of the human MC1, MC3, MC4 and MC5 receptors, do not play an important role for the ligand binding properties of these receptors.**

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Key words: Melanocortin (MC) receptor; MSH; Ligand binding; N terminus; Truncation

1. Introduction

Pro-opiomelanocortin (POMC) is mainly expressed in the pituitary, hypothalamus and brain stem, and is post-translationally processed into the melanocortins, i.e. α-MSH (melanocyte stimulating hormone), β-MSH, γ-MSH and ACTH (adrenocorticotropic hormone). Besides the well-known effects of ACTH on steroid production in the adrenal gland and α-MSH on pigmentation, the melanocortins display a broad array of additional physiological actions including effects on behaviour, memory, thermoregulation, pain perception, nerve regeneration, inflammation and blood pressure [1,2].

Five melanocortin receptor subtypes have been cloned [3–7]. The MC1 receptor is expressed in melanocytes and has high affinity for α-MSH. The MC2 receptor is the ACTH receptor in the adrenal gland. The MC2 receptor has a distinct pharmacology from the other MC receptors as it binds ACTH with high affinity but not the MSH peptides [8]. The human MC3 and especially the human MC4 and MC5 receptors bind the MSH peptides with lower affinity than the human MC1 receptor [9,10]. The physiological roles of these receptors are much less established compared to the MC1 and the MC2 receptors. The MC3 receptor is expressed in the brain and also in the periphery where it has been found in the placenta, gut tissues and the heart [5,11]. The MC4 is predominantly found in the brain where it is widely distributed in almost every brain region [6,12]. Recently, it has been shown that disruption of the MC4 receptor results in obesity [13]. The MC5 receptor is also found in the brain, but is more abundantly expressed in peripheral tissues [7,14,15].

Very little is known about how the MSH peptides bind to the MC receptors. By use of site directed mutagenesis amino acids in the N terminal, extracellular loops (EL) [16] and in the transmembrane region (TM) 3 and TM6 [17] in the MC1 receptor have been identified, which may participate in the ligand binding. Multiple mutations in TM4, EL2 and TM5 in the MC3 receptor indicate that these regions may not influence specific ligand binding [18]. Two quite different 3D models of the MC1 receptor which were based on bacteriorhodopsin homology modelling and ligand docking in between the TM regions have been published [19,20]. The sequences of the MC receptor subtypes are highly conserved, particularly in the TM regions whereas the N-terminal regions varies quite a lot between the different subtypes.

The aim of this study was to investigate a putative participation of N-terminal regions of the human MC1, MC3, MC4 and MC5 receptors in ligand binding.

2. Materials and methods

2.1. Chemicals

[Nle[4], D-Phe[7]]α-MSH (NDP-MSH), α-MSH and β-MSH were purchased from Saxon Biochemicals, Germany, or Bachem, Switzerland. NDP-MSH was radioiodinated by the Chloramine T method and purified by HPLC.

2.2. Generation of truncated clones

The truncated clones were made by PCR. Fragments of the wild type MC receptor clones were amplified by PCR, using downstream primers carrying a *Hin*dIII linker and a ATG start codon for various positions in the N-terminal region of the MC receptors and an upstream primer with a *Xba*I linker. These PCR products were purified and cloned into *Hin*dIII and *Xba*I sites of the pRc/CMV vector (Invitrogen) (or the pcDNA3.1/Zeo vector (Invitrogen), see Section 3), and the inserts and adjacent sequences of the vector DNA used for transfection experiments were confirmed by sequencing. The 3′ end primer used for the MC1 clone was: GAC GTC TAG ATT CAC CAG GAG CAT GTC A (all primers are written in 5′ to 3′ direction), for the MC3 clones: GGT CTA GAC TAT CCC AAG TTC ATG CCG, for the MC4 clones: GAC GTC TAG ATT CAA TAT CTG CTA GAC AAG GTC, for the MC5 clones: GAC GTC TAG ATT CAA TCC CTT CTG GGA AAG CT. The 5′ end primer used for the $MC1^{ATG23}$ clone was: GGG AAG CTT CAC ATA TGC AGA CAG GAG CCC GGT, for the $MC3^{ATG44}$ clone: GGA AGC TTG AAT GCC CTC TGT TCA GCC AAC A, for the $MC3^{ATG51}$ clone: GGA AGC TTG AAT GCC TAA TGG CTC GGA GCA C, for the $MC3^{ATG63}$ clone: GGG AAG CTT CAC ATA TGA GCA ACC AGA GCA GCA GCG CCT, for the $MC3^{ATG69}$ clone: GGA AGC TTG AAT GAA TGC TTC GTG CTG C, for the $MC4^{ATG24}$ clone: GGG AAG CTT CAC ATA TGA GCA ATG CCA GTG AGT, for the $MC4^{ATG29}$ clone: GGG AAG CTT CAC ATA TGT CCC TTG GAA AAG GCT A, for the $MC4^{ATG35}$ clone: GGG AAG CTT CTC ATA TGT CTG ATG GAG GGT GCT A, for the $MC5^{ATG14}$ clone: GGG AAG CTT CAC ATA TGA ATG CCA

*Corresponding authors: H.B. Schiöth and J.E.S. Wikberg.
Fax: (46) (18) 559-718.
E-mail: helgis@bmc.uu.se or jarl.wikberg@farmbio.uu.se

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Receptor	N-terminus	TM1	(2)
MC1 (X65634)	MAVQGSQRRLLGSLNSTPTAIPQLGLAANQTGARCLEVS	ISDGLF...	+
MC1-ATG28	MQTGARCLEVS	ISDGLF...	+
MC3 (Z06155)	(1) MNASCCLPSVQPTLPNGSEHLQAPFFSNQSSSAFCEQVF	IKPEVF...	+
MC3-ATG44	MPSVQPTLPNGSEHLQAPFFSNQSSSAFCEQVF	IKPEVF...	+
MC3-ATG51	MPNGSEHLQAPFFSNQSSSAFCEQVF	IKPEVF...	+
MC3-ATG63	MSNQSSSAFCEQVF	IKPEVF...	+
MC3-ATG69	MAFCEQVF	IKPEVF...	−
MC4 (L08603)	MVNSTHRGMHTSLHLWNRSSYRLHSNASESLGKGYSDGGCYEQLF	VSPEVF...	+
MC4-ATG24	MSNASESLGKGYSDGGCYEQLF	VSPEVF...	+
MC4-ATG29	MSLGKGYSDGGCYEQLF	VSPEVF...	+
MC4-ATG35	MDGGCYEQLF	VSPEVF...	−
MC5 (L27080)	MNSSFHLHFLDLNLNATEGNLSGPNVKNKSSPCED	MGIAVF...	+
MC5-ATG24	MNATEGNLSGPNVKNKSSPCED	MGIAVF...	+
MC5-ATG21	MSGPNVKNKSSPCED	MGIAVF...	+
MC5-ATG28	MNKSSPCED	MGIAVF...	−

Fig. 1. Alignment of the N-terminal amino acid sequences of the cloned human MC receptors (MC2 not included) with the mutant clones described in the text. Numbers in parentheses denote the accession numbers in EMBL and GenBank Data Libraries. Borders between the N-terminal region and TM1 are as proposed [19]. Potential N-terminal glycosylation sites are boxed. (1) The human MC3 receptor is displayed from the Met[38] as the preceding sequence may not be expressed [21]. (2) Constructs which did not give rise to specific binding after transfection are denoted by (−) and the others by (+).

CAG AGG GCA, for the $MC5^{ATG21}$ clone: GGG AAG CTT CAC ATA TGT CAG GAC CAA ATG TCA and for the $MC5^{ATG28}$ clone: GGG AAG CTT CAC ATA TGA AGT CTT CAC CAT GTG A.

2.3. Cells and expression

The wild type human MC1 and human MC5 receptor [3,7] were cloned into the expression vector pRc/CMV (Invitrogen). The wild type human MC3 and human MC4 receptor DNA, cloned into the expression vector pCMV/neo, were a gift from Dr. Ira Gantz [5,6]. For receptor expression, COS-1 cells were grown in Dulbecco's modified Eagle's medium with 10% foetal calf serum. Eighty percent confluent cultures were transfected with the DNA mixed with liposomes in serum free medium (for details see [10]). After transfection, the serum-free medium was replaced with growth medium and the cells were cultivated for about 48 h. Cells were then scraped off, centrifuged, and used for radioligand binding.

2.4. Binding studies

The transfected cells were washed with binding buffer [9] and distributed into 96-well micro titer plates (approximately 40 000 cells/well). The cells were then incubated for 2 h at 37°C with 0.05 ml binding buffer in each well, containing the same concentration of [¹²⁵I]NDP-MSH and appropriate concentrations of an unlabelled ligand. After incubation, the cells were washed with 0.2 ml of ice-cold binding buffer and detached from the plates with 0.2 ml of 0.1 M NaOH. Radioactivity was counted (Wallac, Wizard automatic gamma counter) and data analysed with a software package for radioligand binding analyses (Wan System, Umeå, Sweden). Data were analysed by fitting it to formulas derived from the law of mass-action by the method generally referred to as computer modelling. For saturation analysis, 12 concentrations of [¹²⁵I]NDP-MSH in the range of 0.02 up to 20 nM were used. Non-specific binding was determined in the presence of 3 μM NDP-MSH. The binding assays were performed in duplicate wells and repeated three times. Untransfected COS-1 cells did not show any specific binding to [¹²⁵I]NDP-MSH.

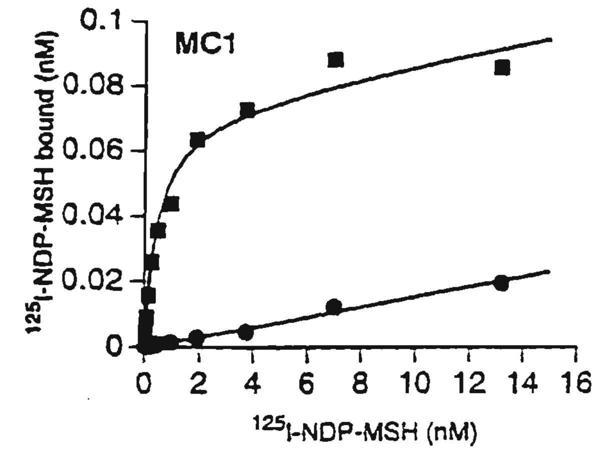
3. Results

Eleven truncated clones of the human MC1, MC3, MC4 and MC5 receptors were created in order to investigate the putative participation of the N terminus in ligand binding (see schematic representation of the clones in Fig. 1). The human wild type MC receptors and truncated clones were used for expression experiments in COS-1 cells which were subsequently tested in binding assays using [¹²⁵I]NDP-MSH as radioligand. The truncated clones expressed at levels similar to the wild type clones, except the $MC3^{ATG69}$, $MC4^{ATG35}$ and $MC5^{ATG28}$ which did not show any specific binding in the COS-1 cells after transfection (data not shown). Saturation curves for the wild type and the $MC1^{ATG28}$, $MC3^{ATG63}$, $MC4^{ATG29}$ and $MC5^{ATG21}$ clones, are shown in Fig. 2. Competition curves of different MSH-peptides for the same receptors are shown in Fig. 3. In Table 1, the K_i values obtained from the computer analysis of these data are shown together with the K_d values for [¹²⁵I]NDP-MSH obtained from the saturation curves.

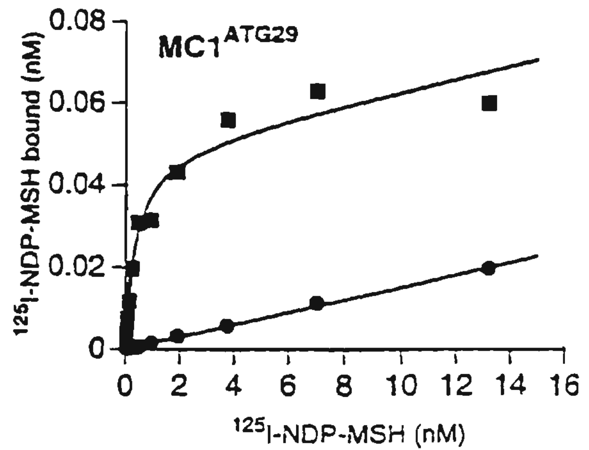
We have earlier shown that the comparatively long cDNA derived N-terminal sequence of the human MC3 receptor up to ATG[38] does not participate in ligand binding [21]. Further truncation of the human MC3, as well as the human MC4 and human MC5 receptors resulted in clones termed $MC3^{ATG44}$, $MC3^{ATG51}$, $MC4^{ATG24}$ and $MC5^{ATG24}$. When these constructs were expressed they bound NDP-MSH (α-MSH) in similar fashion as respective wild type receptor (see Table 1). Deletion of additional amino acids from these receptors and the MC1 receptor, resulted in clones termed $MC1^{ATG28}$, $MC3^{ATG63}$, $MC4^{ATG29}$ and $MC5^{ATG21}$. These clones were expressed and fully characterised by ligand binding using both saturation analysis ([¹²⁵I]NDP-MSH) and competition analysis (NDP-MSH, α-MSH and β-MSH). The results show (Table 1) that the binding constants of the MSH peptides for these truncated receptors are indistinguishable from those of the corresponding wild type receptors. Moreover, the binding constants presented in Table 1 are in close agreement to those found earlier for the wild type MC1, MC3, MC4 and MC5 receptors [9,10,22].

Additional constructs, termed $MC3^{ATG69}$, $MC4^{ATG35}$ and $MC5^{ATG28}$ did not show any specific binding to labelled NDP-MSH when transfected to COS-1 cells. We made two clones of each of these constructs, and we also cloned the $MC4^{ATG35}$ and the $MC5^{ATG28}$ into the pcDNA3.1/Zeo vector (in addition to the pRc/CMV vector) without creating any specific binding in COS-1 cells after transfection.

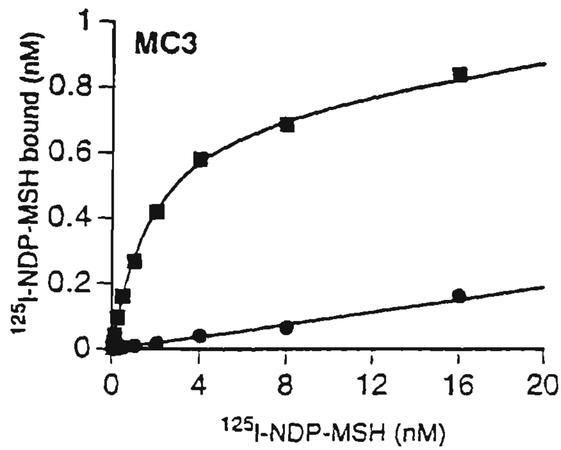
Fig. 2. Saturation curves of [¹²⁵I]NDP-MSH obtained from transfected COS cells. The figures show total binding (■) and binding in the presence of 3 μM unlabeled NDP-MSH (●) for the wild type and $MC1^{ATG28}$, $MC3^{ATG63}$, $MC4^{ATG29}$, $MC5^{ATG21}$ clones. Lines represent the computer modelled best fit of the data using an one-site model.



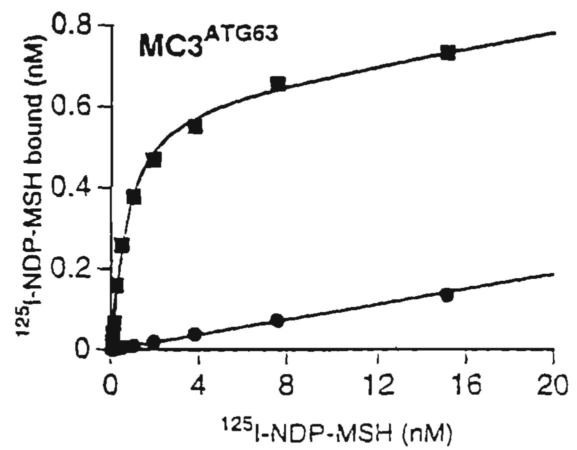
MC1
^{125}I-NDP-MSH bound (nM)
^{125}I-NDP-MSH (nM)



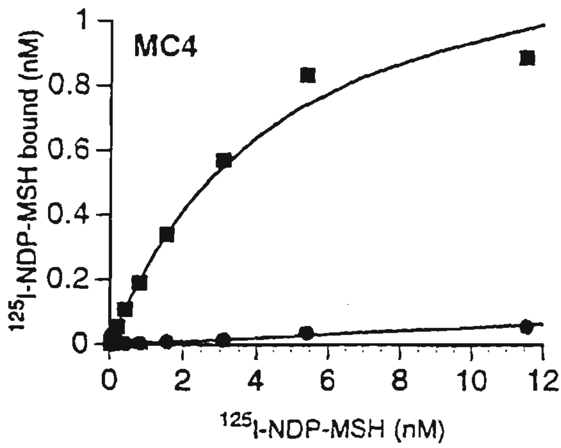
MC1^{ATG29}
^{125}I-NDP-MSH bound (nM)
^{125}I-NDP-MSH (nM)



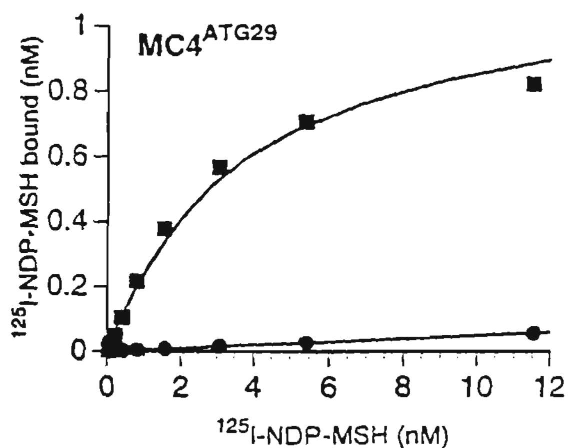
MC3
^{125}I-NDP-MSH bound (nM)
^{125}I-NDP-MSH (nM)



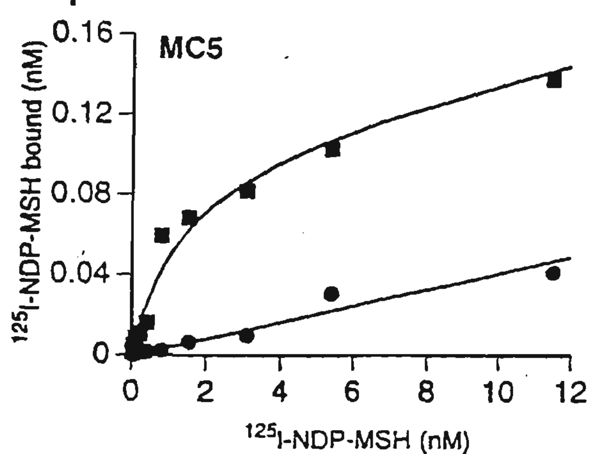
MC3^{ATG63}
^{125}I-NDP-MSH bound (nM)
^{125}I-NDP-MSH (nM)



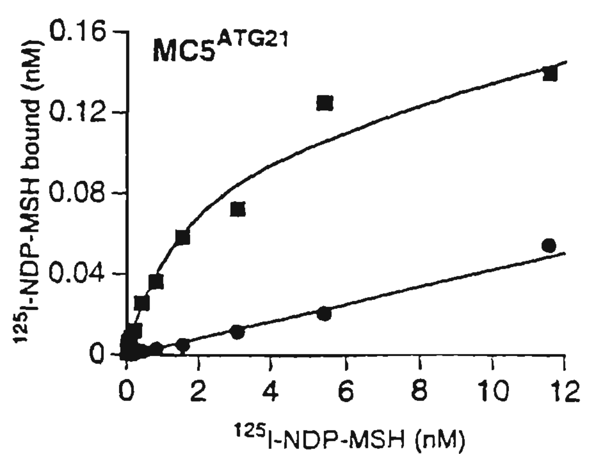
MC4
^{125}I-NDP-MSH bound (nM)
^{125}I-NDP-MSH (nM)



MC4^{ATG29}
^{125}I-NDP-MSH bound (nM)
^{125}I-NDP-MSH (nM)



MC5
^{125}I-NDP-MSH bound (nM)
^{125}I-NDP-MSH (nM)



MC5^{ATG21}
^{125}I-NDP-MSH bound (nM)
^{125}I-NDP-MSH (nM)

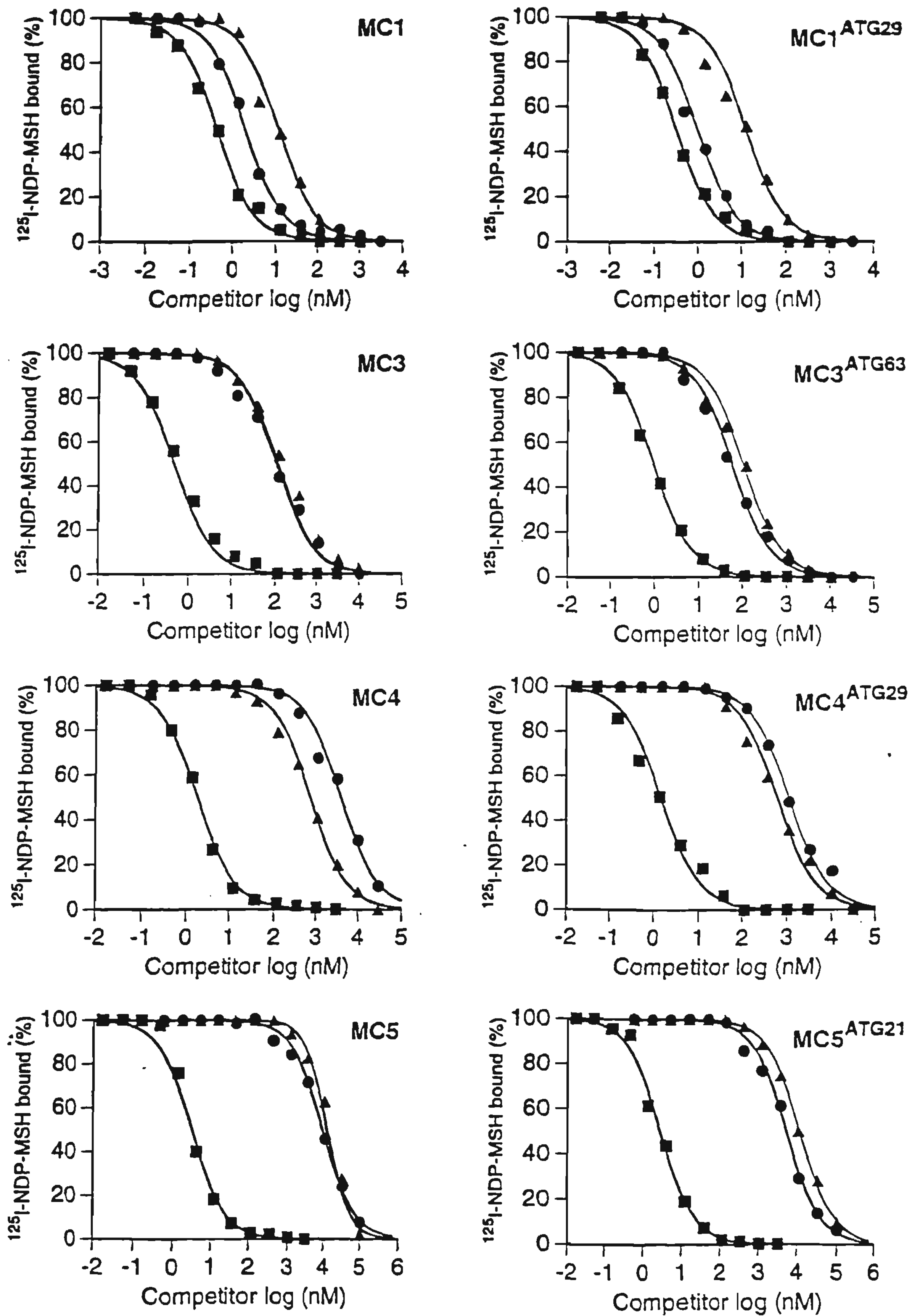


Fig. 3. Competition curves of NDP-MSH (■), α-MSH (●), β-MSH (▲) obtained on transfected COS cells using a fixed concentration of ~2 nM [^{125}I]NDP-MSH for the different MC receptor clones.

Table 1
K_i and K_d (nmol/l) values (mean ± S.E.M) obtained from competition and saturation curves using [125I]NDP-MSH and different MSH analogues on transfected COS-1 cells

Receptor/Ligand	K_d	K_i		
	[125I]NDP-MSH	NDP-MSH	α-MSH	β-MSH
MC1	0.109 ± 0.062	0.046 ± 0.011	0.210 ± 0.089	2.53 ± 0.93
$MC1^{ATG28}$	0.144 ± 0.027	0.050 ± 0.015	0.141 ± 0.090	2.75 ± 0.66
MC3	0.394 ± 0.061	0.493 ± 0.035	53.2 ± 9.62	57.0 ± 12.0
$MC3^{ATG44}$	nd	0.534 ± 0.092	nd	nd
$MC3^{ATG51}$	nd	0.413 ± 0.038	31.2 ± 4.2	nd
$MC3^{ATG63}$	0.435 ± 0.036	0.593 ± 0.044	34.4 ± 5.3	64.3 ± 7.3
MC4	2.29 ± 0.27	1.86 ± 0.35	2030 ± 410	642 ± 150
$MC4^{ATG24}$	nd	1.05 ± 0.10	1730 ± 630	nd
$MC4^{ATG29}$	2.59 ± 0.22	1.28 ± 0.28	957 ± 319	513 ± 150
MC5	2.86 ± 0.71	2.65 ± 0.37	4990 ± 800	13 900 ± 5 600
$MC5^{ATG14}$	nd	1.09 ± 0.21	5570 ± 1730	nd
$MC5^{ATG21}$	1.44 ± 0.23	2.15 ± 0.20	3270 ± 1080	10 100 ± 1 700

nd, not determined.

4. Discussion

For most G-protein coupled receptors, in particular those serving as recognition sites for low molecular weight compounds like adrenaline, the ligand binding pocket is thought to be localised between the TM regions, which are predicted to form α-helices analogous to the extensively characterised bacteriorhodopsin. For other G-protein coupled receptors that bind small peptides, like neurokinins [23], enkephalins [24] or secretin and vasoactive intestinal polypeptide [25] the N-terminal region is known to play an role for the ligand binding. For glycoprotein hormone receptors [26] the N-terminal region can be even more important, such as for the lutropin/chorigonadotropin receptor, where the N-terminal domain alone is sufficient for high affinity hormone binding [27]. Truncations of G-protein coupled receptors have been shown to both increase or decrease ligand affinities [28]. The melanocortin receptor family has the shortest amino acid sequence among the superfamily of G-protein coupled receptors. The human MC1, MC2, MC3, MC4 and MC5 receptors are 317, 297, 361 (or 324 [21]), 333, 325 amino acids long, respectively. They are characterised by having short N- (ca. 25–39 amino acids) and C-terminal regions (ca. 17–21 amino acids) as well as a very small second extracellular loop (ca. 9 amino acids).

Our present results show that 27 amino acids of the human MC1, 25 amino acids of the human MC3 receptor, 28 amino acids of the human MC4 receptor and 20 amino acids from the human MC5 receptor could be deleted from the N termini without affecting the expression or binding of [125I]NDP-MSH, NDP-MSH, α-MSH or β-MSH. Further deletions of the N termini resulted in 'total loss of binding', which may have been caused by misfolding or problems with the membrane integration of the receptor.

Our results show that all potential N-terminal glycosylation sites can be removed from the MC1 and MC4 receptors ($MC1Asn^{15}$, $MC1Asn^{29}$, $MC4Asn^{3}$, $MC4Asn^{17}$ and $MC4Asn^{26}$) as well as two potential glycosylation sites (Asn^{53} and Asn^{39}) from the MC3 receptor and three potential sites from the MC5 receptor (Asn^{2}, Asn^{15} and Asn^{20}) without causing any effects on ligand binding or expression. Our results thus indicate that N-terminal glycosylation is unlikely to play an important role in the folding and membrane targeting of the MC receptors.

It remains unclear whether the non-binding clones ($MC3^{ATG69}$, $MC4^{ATG35}$ and $MC5^{ATG28}$) were expressed and inserted into the cell membrane. It is unlikely that the complete absence of specific ligand binding for these constructs was caused by deletion of residues crucial for ligand binding while all other deletions did not even change the ligand binding properties at all. Structural or expression complications, or influence on the integration of the receptors to the cell membrane are more probable explanations.

The precise border between the extracellular N-terminal part and TM1 is not known. Amino acids proposed to precede TM1, like the conserved Glu ($MC1Glu^{26}$) and positive charges may be important for the structure, folding or ligand binding. Moreover, the conserved Cys ($MC1Cys^{34}$) which may form a disulphide bridge with a corresponding Cys residue in extracellular loop 3 (or even elsewhere in the receptor) may also be important for receptor structure. The observation that the $MC1^{ATG28}$ is giving binding, but not the equally truncated $MC5^{ATG28}$, may indicate important role for amino acids preceding the TM1. A recent analysis of G-protein coupled receptors revealed, that those lacking a leader sequence, like the MC receptors, have a high preference for a positive charge in the N terminus close to TM1[29]. This is in contrast to the more general 'positive inside' rule developed earlier [30] and may indicate a specific role for Lys^{32} and Lys^{28} in the MC4 and the MC5 receptors, respectively. Thus, the loss of a positive charge may play some role in the loss of binding observed, but no such positive charge exists in the MC3 receptor. In the latter case one might speculate that a lack of hydrophilic residues in the N-terminal region, besides the earlier mentioned conserved Glu, might cause problems in membrane insertion and orientation of TM1.

All the MC receptors have a Ser residue close to the N-terminal end ($MC1Ser^{6}$, $MC3Ser^{4}$, $MC4Ser^{4}$ and $MC5Ser^{4}$). This Ser was recently proposed to participate in ligand binding based on the finding that a Ser/Ala exchange in the MC1 receptor resulted in loss in affinity to NDP-MSH and α-MSH [16]. Our data contradict those previous results but at present we do not have any rational explanation to this discrepancy.

In summary, our results indicate that neither the N-terminal regions nor carbohydrates added by glycosylation of these are likely to be essential for ligand binding for the human MC receptors or for their structural fidelity.

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Chimeric Melanocortin MC1 and MC3 Receptors: Identification of Domains Participating in Binding of Melanocyte-Stimulating Hormone Peptides

HELGI B. SCHIÖTH, PHILIP YOOK, RUTA MUCENIECE, JARL E. S. WIKBERG, and MICHAEL SZARDENINGS

Department of Pharmaceutical Pharmacology, Uppsala University, Uppsala, Sweden (H.B.S., P.Y., J.E.S.W., M.S.), and Laboratory of Pharmacology, Institute of Organic Synthesis, Riga, Latvia (R.M.)

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ABSTRACT

The melanocortin receptors MC1 and MC3 are G protein-coupled receptors that have substantial structural similarities and bind melanocyte peptides but with different affinity profiles. We constructed a series of chimeric MC1/MC3 receptors to identify the epitopes that determine their selectivities for natural melanocyte peptides and synthetic analogues. The chimeric constructs were made by a polymerase chain reaction that used identical regions in or just outside transmembranes (TM) 1, 4, and 6 and divided the receptors into four segments. Saturation and competition studies on the expressed chimeric proteins indicate that TM1, TM2, TM3, and TM7 are involved in the subtype-specific binding of melanocyte peptides to these receptors. The results support the hypothesis that TM4 and TM5 may not contribute to the ligand-binding specificity of the MC receptors. This is the first report to describe the subtype-specific hormone-binding domains of the melanocortin receptor family.

The melanocortin peptides are primarily known for their effects on pigmentation in melanocytes (α-MSH) and for regulation of steroid production in the ACTH. They also mediate a variety of other effects with both central and peripheral origin. Effects have been reported on heart rate, blood pressure, lipolysis, learning, memory, behavior, inflammation, pyretic control, analgesia, and nerve regeneration, as well as effects on events surrounding parturition (O'Donahue and Dorsa, 1982; Eberle 1988).

Molecular cloning of the first MC receptor found in melanocytes, MC1, and the ACTH receptor from the adrenal gland, MC2, was followed by identification of three new melanocortin receptors, MC3, MC4, and MC5 (Chhajlani and Wikberg, 1992; Mountjoy *et al.*, 1992; Chhajlani *et al.*, 1993; Gantz *et al.*, 1993a, 1993b). The MC receptors are coupled to G proteins and share considerable amino acid homology.

The physiological roles of the newly discovered MC3, MC4, and MC5 receptors are not fully understood. The MC3 receptor is expressed in the brain and also in the periphery. It has been found in the placenta and gut tissues and has been shown more recently to have relatively high expression in the human heart (Gantz *et al.*, 1993a; Roselli-Refhuss, 1993; Chhajlani 1996). The expression of the MC3 receptor in the heart and its preference for γ-MSH may indicate that it could mediate the effects of γ-MSH regulation of heart rate and blood pressure. The MC4 receptor is predominantly found in the brain, where it is represented at multiple sites in almost every brain region, including the cortex, thalamus, hypothalamus, brain stem, and spinal cord (Gantz *et al.*, 1993b; Mountjoy *et al.*, 1994). Recent findings showing that the agouti peptide is an MC4 receptor antagonist (Lu *et al.*, 1994), that the MSH peptides influence feeding behavior (Fan *et al.*, 1997), and that MC4 receptor knockout mice become fat (Huszar *et al.*, 1997) relate the MC4 receptor to weight homeostasis. The MC5 receptor is found in the brain, and more importantly, it has a wide peripheral distribution, although it has still a much less defined physiological role (Chhajlani *et al.*, 1993; Labbé *et al.*, 1994; Fathi *et al.*, 1995).

The MC1, MC3, MC4, and MC5 receptors have a distinct affinity pattern for the natural melanocortins (α-MSH, β-MSH, γ-MSH, and ACTH) (Schiöth *et al.*, 1995, 1996a), whereas the MC2 receptor binds only to ACTH, not to the MSH peptides (Cammas *et al.*, 1995; Schiöth *et al.*, 1996c). Currently, there are selective substances available for the MC1 and MC2 receptor subtypes (α-MSH and ACTH, respec-

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ABBREVIATIONS: MSH, melanocyte-stimulating hormone; ACTH, adrenocorticotrophic hormone; cCDC, cyclic [Cys^4,D-Phe^7,Cys^{10}]α- melanocyte-stimulating hormone(1–13); cCLC, cyclic [Cys^4,L-Phe^7,Cys^{10}]α- melanocyte-stimulating hormone(1–13); EL, extracellular loop; MC, melanocortin; NDP-MSH; [Nle^4,D-Phe^7]α- melanocyte-stimulating hormone; PCR, polymerase chain reaction; TM, transmembrane.

tively), but there are only a few reports on specific ligands for the other subtypes. However, cyclic lactam analogues (Hruby *et al.*, 1995) and ACTH(4–10) analogues (Adan *et al.*, 1994) were reported to show some selectivity.

The identification of important physiological roles of the MC receptors and the simultaneous lack of potent selective substances have increased interest in defining the ligand-binding regions of these receptors. Residues in the TM3 and TM6 (Frändberg *et al.*, 1994) and in the extracellular loops (Chhajlani *et al.*, 1996) of the MC1 receptor, which may participate in the ligand binding, have been identified by site-directed mutagenesis. Several natural mutations in TM2 and one in the TM7 of the MC1 receptor have been shown to relate to skin and hair or fur color in mice, humans, foxes, and horses (Robbins *et al.*, 1993; Valverde *et al.*, 1995; Marklund *et al.*, 1996; Koppula *et al.*, 1997; Våge *et al.*, 1997). Two different models of the MC1 receptor have been published (Prusis *et al.*, 1995; Haskell-Luevano *et al.*, 1996). In those studies, ligands were docked into the models, and a number of different amino acids were proposed as participants in ligand binding. The aim of this study was to generate MC1/MC3 receptor chimeras to determine the participation of different TM domains in ligand binding.

Materials and Methods

Peptides. NDP-MSH (Sawyer *et al.*, 1980), [Nle^4]α-MSH, α-MSH, and γ1-MSH were from Bachem (Bubendorf, Switzerland). cCDC and cCLC (Sawyer *et al.*, 1982; Knittel *et al.*, 1983) were synthesized by Scandinavian Peptide Syntheses (Kopine, Sweden). cCDC and cCLC were cyclized by a disulfide bridge between the two Cys peptides. NDP-MSH was radioiodinated by the chloramine T method and purified by high performance liquid chromatography. All cell culture media were provided by Life Technologies (Taby, Sweden).

Generation of chimeric MC1/MC3 receptor clones. Chimeras were created by a modification of the 'mega-primer' approach (Landt *et al.*, 1990) using Vent polymerase (Biolabs, Stockholm, Sweden). In the first step, the smaller of the two receptor parts was amplified by PCR with the use of the primers shown in Fig. 1 and the corresponding end primer. Primers P2 and P3 are universal primers, which can be used on both genes; P1 was suitable for the MC1 receptor as a template in the first PCR only. The fragment from the first receptor was purified (Geneclean; Genomed, Bad Oeynhauser, Germany) and used in combination with the other end primer on the gene of the second receptor in a second PCR reaction. An aliquot of the reaction was subjected to horizontal agarose gel electrophoresis, and the band corresponding to the size of the chimeric gene was excised and partially eluted by briefly freezing and centrifuging the agarose piece. An aliquot of the supernatant was used for a third PCR amplification by using the kinased end primers suitable for the chimera. After concatenation with T4-Ligase, to improve efficiency of restriction enzyme treatment (Jung *et al.*, 1993), the gene was prepared by cleavage with *XbaI/Hin*DIII, cloned into pcDNA3.1 (InVitrogen, Oxon, UK), and sequenced. Primers used in this study for the 3′ and 5′ ends of the genes were (5′- 3′ direction) GGT CTA GAC TAT CCC AAG TTC ATG CCG (MC3–3′), GGA AGC TTG AAT GAG CAT CCA AAA GAA GTA TCT GG (MC3–5′), GAC GTC TAG ATT CAC CAG GAG CAT GTC A (MC1–3′), and GGA AGC TTC ACA TAT GGC TGT GCA GGG ATC (MC1–5′).

Expression of receptor clones. The human MC1 receptor (Chhajlani and Wikberg, 1992) was cloned into the expression vector pRc/CMV and the chimeras were cloned into pcDNA3.1 (InVitrogen). The human MC3 receptor DNA, cloned into the expression vector CMVneo, was a generous gift from Dr. Ira Gantz (Gantz *et al.*, 1993a). For receptor expression, COS-1 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Eighty percent confluent cultures were transfected with the DNA mixed with liposomes in serum free medium [for details see Schiöth *et al.* (1996c)]. After transfection, the serum-free medium was replaced with growth medium and the cells were cultivated for about 48 hr. Cells were then scraped off, centrifuged, and used for radioligand binding.

Binding studies. The transfected cells were washed with binding buffer (Schiöth *et al.*, 1995) and distributed into 96-well plates (approximately 40,000 cells/well). The cells were then incubated for 2 hr at 37° with 0.05 ml binding buffer in each well, which contained a constant concentration of [^{125}I]NDP-MSH and appropriate concentrations of an unlabeled ligand. After incubation, the cells were washed with 0.2 ml of ice-cold binding buffer and detached from the plates with 0.2 ml of 0.1 N NaOH. Radioactivity was counted (Wizard automatic gamma counter; Wallac Oy, Turku, Finland) and data were analyzed with a software package for radioligand binding analyses (Wan System, Umea, Sweden). Data were analyzed by using computer modeling to fit them to formulas derived from the law of mass action. For saturation analysis, 12 concentrations of [^{125}I]NDP-MSH ranging from 0.02 to 3 nM were used. Nonspecific binding was determined in the presence of 3 μM NDP-MSH. The binding assays were performed in duplicate wells and repeated three times. Untransfected COS-1 cells did not show any specific binding to [^{125}I]NDP-MSH.

cAMP assay. COS cells expressing the receptors were grown as above. Cells were detached from almost confluent adherent cultures and incubated for 30–60 min at 37° in ordinary growth medium containing 0.5 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine. Aliquots (20 μl) of the hormone dilutions in growth medium were prepared in 96-well plates and placed in a water bath at 37°. For the stimulation, about 3×10^5 cells in 180 μl were quickly added to each hole to obtain immediate mixing. After 15 min, 20 μl of 4.4 M perchloric acid was added, mixed, neutralized after a few minutes by addition of 20 μl base (5 M KOH, 1 M Tris), and centrifuged. The determination of cAMP in the resulting supernatant was carried out as described previously (Nordstedt and Fredholm, 1990). The cAMP assays were performed in duplicate wells and repeated three times.

Results

Eight chimeric clones of the human MC1/MC3 receptor were created to investigate the participation of different domains of the MC1 and the MC3 receptors in ligand binding. The chimeras were made by using short primers based on DNA sequence identity in or just around TM1, TM4, and TM6. Primers and the relevant regions of the receptor genes are shown in Fig. 1. The junctions were chosen primarily on the basis of DNA sequence identities, but care was taken to maximize similarities of the adjacent protein sequences to minimize local structural changes. The second PCR reaction in particular was usually free of nonspecific products and almost all clones sequenced had the expected sequences. Two of the chimeras were made of the MC1 receptor up to TM4 or TM6 with the MC3 receptor making up the rest of the receptor. These were termed 1(4)3 and 1(6)3, respectively. Two complementary chimeras that were also made by using the same set of primers had the amino-terminal segment up to TM4 or TM6 from the MC3 receptor and the carboxyl-terminal end from the MC1 receptor. These were termed 3(4)1 and 3(6)1, respectively. One chimera, termed 1(2)3, had the MC1 receptor sequence only up to TM1. In addition, starting from these chimeras, we made three chimeras with sequences from the MC3 receptor, from TM4 to TM6 [termed 1(4)3(6)1],

TM1 to TM4 [termed 1(2)3(4)1], and TM1 to TM6 [termed 1(2)3(6)1], and the N- and C-termini from the MC1 receptor. A schematic representation of the clones is shown in Fig. 2.

The wild-type human MC3 and MC1 receptors and the chimeras were expressed in COS-1 cells and tested in radioligand-binding assays with $[^{125}I]$NDP-MSH. Saturation curves for the MC1, 1(4)3(6)1, 1(2)3(6)1), 1(4)3, 3(6)1, and the MC3 receptors are shown in Fig. 3. Competition curves of different MSH-peptides with the same receptors are shown in Fig. 4. In Table 1, the K_i values obtained from the computer analysis of these data are shown, together with the K_d values for $[^{125}I]$NDP-MSH obtained from the saturation curves.

NDP-MSH has only 4–5-fold higher affinity for the MC1 receptor than for the MC3 receptor (see Table 1). α-MSH and $[Nle^4]$α-MSH are more suitable for discrimination between the two receptors because these peptides have about 100-fold higher affinity for the MC1 than for the MC3 receptor. Cyclic $[Cys^4, Cys^{10}]$α-MSH analogues are well known as potent melanotropes in pigmentation assays (Knittel *et al.*, 1983). These peptides may bind differently to the receptors than the linear MSH peptides. The cCDC and cCLC have more than 50-fold higher affinity for the MC1 receptor than for the MC3 receptor. γ-MSH, however, has very similar affinity for both of these receptors (3.16 ± 0.78 nM for the MC1 receptor and 7.31 ± 2.51 nM for the MC3 receptor) and therefore was not included in this study.

The results show that the chimeras 1(6)3, 1(4)3, 3(4)1, and 3(6)1 all have intermediate affinities for the MSH analogues that are clearly distinguishable from those of both the MC1 and the MC3 receptors. Almost indistinguishable affinities were found for 1(6)3 and 1(4)3, as well as for 3(4)1 and 3(6)1 and for the two chimeras 1(2)3(4)1 and 1(2)3(6)1. The 1(2)3-receptor affinities are closest to those of the MC3 receptor. The 1(4)3(6)1 receptor displayed affinities that are indistinguishable from those of the MC1 receptor. The 1(2)3(4)1 and 1(2)3(6)1 receptors show affinity profiles that are close to that of the MC1 receptor, although these receptors have lower affinity to the MSH peptides than the MC1 receptor.

COS cells transfected with the wild-type receptors and the chimeras were also stimulated by 10 nM concentration of NDP-MSH. All the cells responded with an increase in the levels of intracellular cAMP in response to NDP-MSH (Fig. 2). The differences in the responses for the different clones may be related to different levels of expression of the receptors.

Discussion

The MC receptors are the smallest G protein-coupled receptors yet cloned. Their characteristic properties are short amino-terminal and carboxyl-terminal ends and a very small second extracellular loop. The MC receptor subtypes share considerable amino acid identity; the identity is lowest between the MC2 and the MC4 receptors (38% identity) and highest between the MC4 and the MC5 receptors (60% identity). The MC1 and the MC3 receptors have 45% amino-acid identity. Generally, the MC receptor subtypes show lowest homology in the intra- and extracellular loops and in TM4 and TM5, and highest homology in TM1, TM3, and TM7. It can not be judged from the sequence data alone whether the larger differences in TM4 and TM5 are the cause of the different specificities of the receptors, or if they were simply less preserved during evolution because they lacked a role in ligand binding.

Construction of chimeric receptors of related G protein-coupled receptors has proven to be a valuable tool to determine binding specificity of receptor domains (Frielle *et al.*, 1988; Kobilka *et al.*, 1988). However, chimeric proteins may be malfunctioning simply because of incompatibility of the different structural elements that are brought together artificially. Because the MC receptors have rather closely related sequences, extra care was taken to use regions of extended sequence similarity for the junction sites of the fusions. This should have reduced local disturbances of the receptor structure. It also improved the probability of obtaining correct PCR products with the mega-primer approach that we were probably first to use for this type of cloning procedure. Other groups have been using overlap extension with similar success (Wang *et al.*, 1995). However, all the chimeric MC1/MC3 receptors constructed in this study could be expressed and

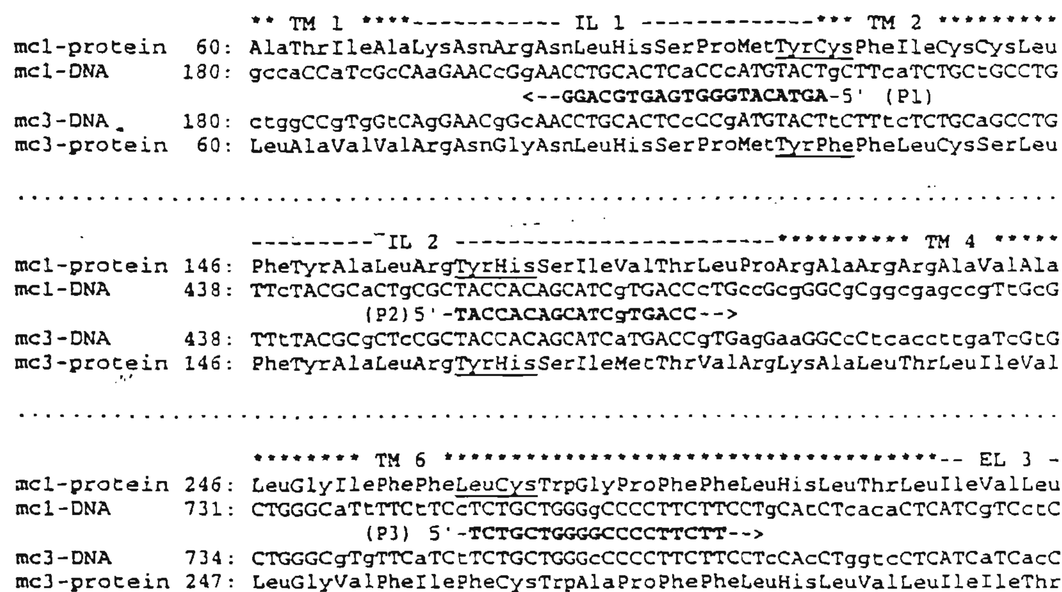

Fig. 1. PCR-primers P1–3 and corresponding sequences in the MC1 and MC3 receptors. The 5′ and 3′ ends of the primers are indicated. *Underlined amino acids*, junction sites between the receptors in the chimeras.

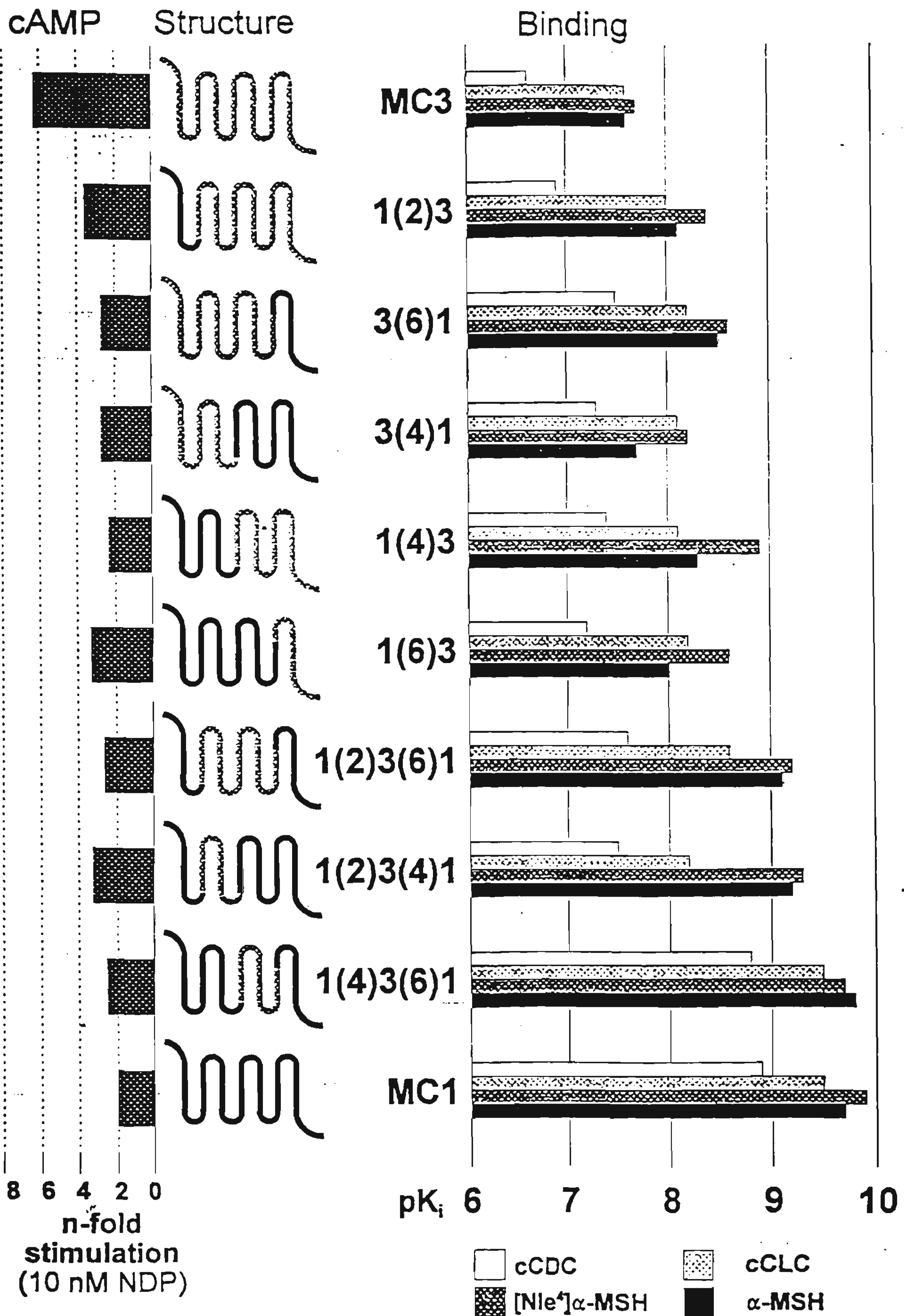

Fig. 2. Schematic representation of the structure of the chimeras aligned with graphical presentation of cAMP response and pK_i values for [Nle[4]]α-MSH, α-MSH, cCDC, and cCLC.

bound by ^{125}I-NDP-MSH with high affinity. Moreover, all the chimeras were shown to be functionally active (Fig. 2).

The MC1/3 receptors were divided into four major segments by taking advantage of identical DNA sequences in or just outside TM1, TM4, and TM6 (see Fig. 1). Our data on the chimeras having the transition in TM6 show that the segment containing the carboxyl terminus with part of the TM6, the entire TM7, and the intracellular carboxyl terminus is important for the specific binding of both the linear and cyclic MSH peptides tested in this study. However, replacement of a central segment, from TM4 to TM6 in the MC1 receptor in the MC3 receptor, which resulted in 1(4)3(6)1, did not seem to affect ligand binding. Moreover, the affinities of 1(4)3 and 1(6)3 to the MSH peptides were indistinguishable, as were the affinities of 3(4)1 and 3(6)1 and the affinities of 1(2)3(4)1 and 1(2)3(6)1. Our data indicate that this central region from TM4 to TM6 is not important for the selective binding of the MSH peptides. The data also show that not only the carboxyl-terminal but also the amino-terminal region of the receptors is important for the selective binding properties. We have recently demonstrated that the amino-terminal regions of MC receptors do not participate in ligand binding; deleting as many as 27 and 28 amino acids from the MC1 and the MC3 receptors did not affect binding (Schiöth *et al.*, 1996d, 1997b). However, our new data on the chimeras having an MC1 to MC3 transition between TM1 and TM2 show that replacement of the TM1 and the amino-terminal region influences the binding. Therefore, residues within TM1 must participate in the selective binding. Moreover, the chimeras 1(2)3(4)1 and 1(2)3(6)1 have affinities that are clearly lower than those of 1(4)3(6)1, which indicates that the end domains of not only TM1 and TM7, but also the TM2/TM3 domain are important for selective binding.

Several natural mutants have been identified that influence the biological function of the MC1 receptor for hair, fur, and skin colors. These include Asp294His in TM7 in the human MC1 receptor (Valverde *et al.*, 1995), Glu92Lys in TM2 in the murine MC1 receptor (Robbins *et al.*, 1993), Ser83Phe in TM2 of the horse MC1 receptor (Marklund *et al.*, 1996), Cys125Arg in the TM3 of the fox MC1 receptor (Våge

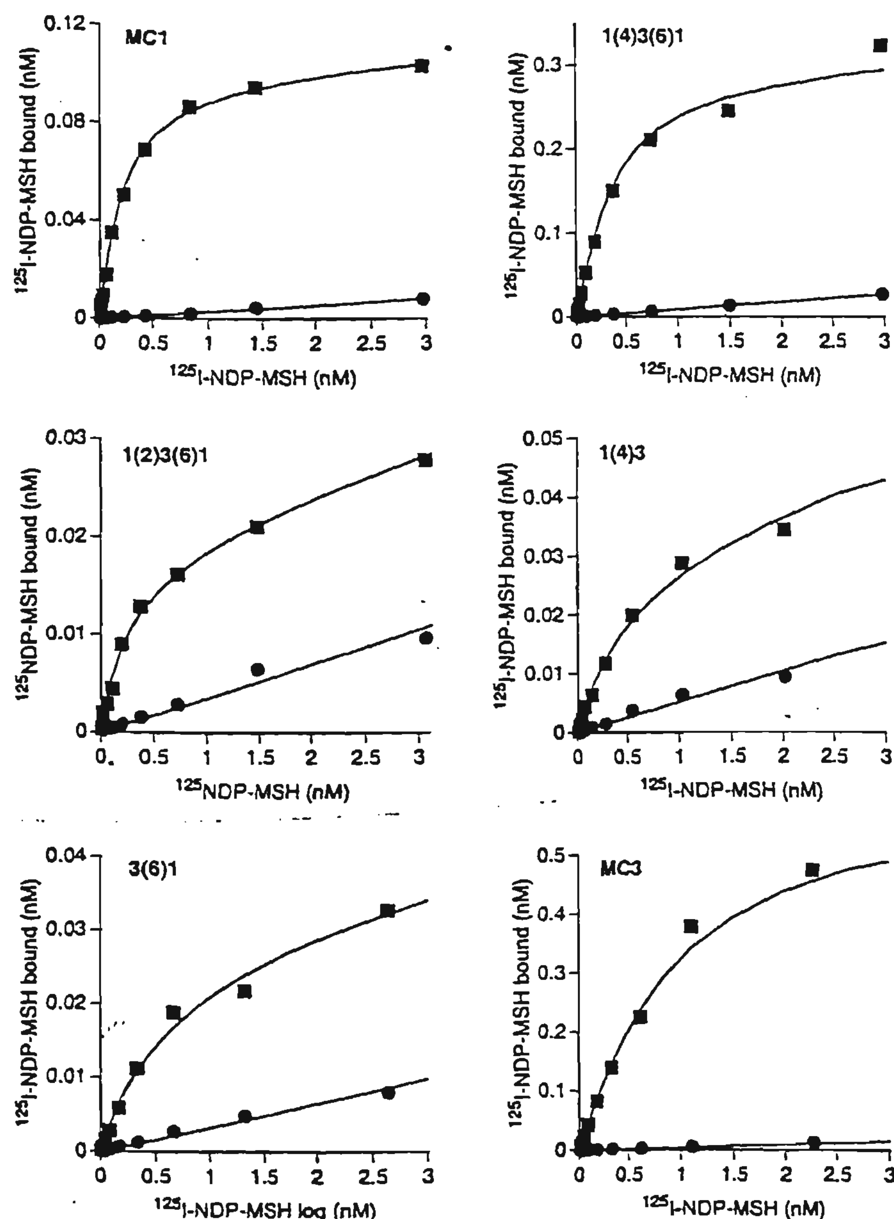

Fig. 3. Saturation curves of [^{125}I]NDP-MSH obtained from transfected COS cells. The figures show total binding (■) and binding in the presence of 3 μM cold NDP-MSH (●) for the MC1, 1(4)3(6)1, 1(2)3(6)1, 1(4)3, 3(6)1, and MC3 receptors. *Lines*, computer-modeled best fit of the data using a one-site model for the total binding.

et al., 1997), Val92Met in TM2 in the human MC1 receptor (Valverde *et al.*, 1995; Xu *et al.*, 1996), and Asp84Glu in TM2 in the human MC1 receptor (Valverde *et al.*, 1995). In a mutagenesis study, His260 (in TM6) and Asp117 (in TM3) were mutated to Ala in the MC1 receptor, which resulted in loss of affinity to α-MSH (Frändberg *et al.*, 1994). Thorough characterization of these mutants indicates that although His260 and Asp117 do not interact with any specific residue in the MSH peptides, these mutations cause conformational changes in the receptor (Schiöth *et al.*, 1997a). We have also shown earlier that multiple mutations in TM4, EL2, and TM5 in the MC3 receptor that transform these regions so that they become identical to the MC1 receptors do not affect ligand binding (Schiöth *et al.*, 1996b). Taken together, these mutant data also support our present interpretation that TM1, TM2, TM3, and TM7 are the most important for ligand binding, whereas TM4 and TM5 may be irrelevant to this aspect of the MC receptors.

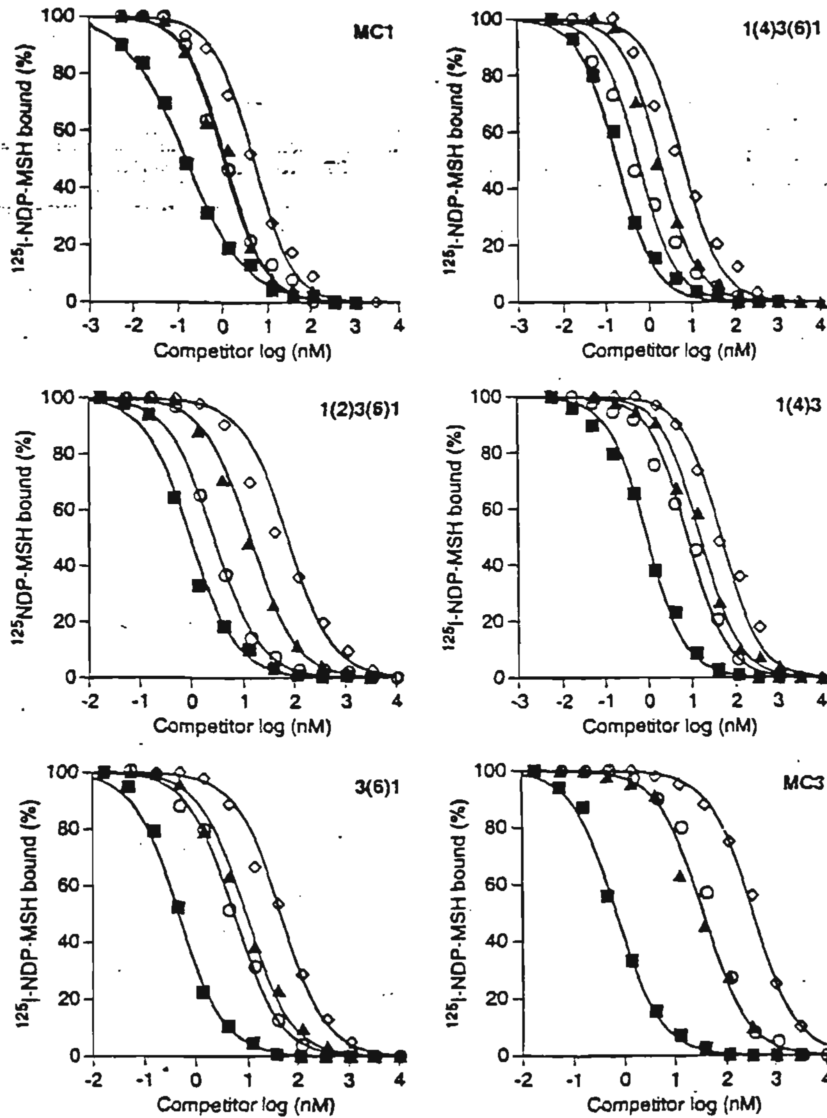

Fig. 4. Competition curves of NDP-MSH (■), α-MSH (○), cCDC (▲), and cCLC (◇) obtained on the MC1, 1(4)3(6)1, 1(2)3(6)1, 1(4)3, 3(6)1, and MC3 receptors by using a fixed concentration of [^{125}I]NDP-MSH.

TABLE 1

K_i values (mean ± standard error) obtained from competition and saturation curves using [^{125}I]NDP-MSH and different MSH analogues on transfected COS-1 cells

Ligand receptor	[^{125}I]NDP-MSH[a]	NDP-MSH	α-MSH	[Nle^4]-α-MSH	cCDC	cCLC
			nmol/L			
MC1	0.121 ± 0.009	0.083 ± 0.034	0.210 ± 0.031	0.115 ± 0.019	0.305 ± 0.044	1.27 ± 0.26
1(4)3(6)1	0.193 ± 0.023	0.048 ± 0.008	0.152 ± 0.067	0.195 ± 0.034	0.337 ± 0.049	1.62 ± 0.41
1(2)3(4)1	0.314 ± 0.042	0.296 ± 0.063	0.642 ± 0.096	0.561 ± 0.098	5.81 ± 1.71	35.1 ± 13.9
1(2)3(6)1	0.341 ± 0.034	0.201 ± 0.024	0.891 ± 0.110	0.700 ± 0.091	2.65 ± 0.28	24.9 ± 2.8
1(6)3	0.341 ± 0.137	0.241 ± 0.040	11.0 ± 2.1	2.28 ± 0.61	6.66 ± 0.49	61.5 ± 11.3
1(4)3	0.380 ± 0.084	0.350 ± 0.158	5.31 ± 1.08	1.20 ± 0.19	8.19 ± 1.31	36.0 ± 7.7
3(4)1	0.386 ± 0.076	0.323 ± 0.023	19.4 ± 3.1	6.28 ± 0.43	8.93 ± 1.09	50.1 ± 4.7
3(6)1	0.313 ± 0.037	0.309 ± 0.027	2.82 ± 0.18	2.25 ± 0.22	5.88 ± 0.79	33.3 ± 6.4
1(2)3	0.327 ± 0.037	0.216 ± 0.033	8.61 ± 0.95	3.86 ± 1.12	10.0 ± 1.2	115 ± 48
MC3	0.564 ± 0.045	0.439 ± 0.038	23.5 ± 5.4	18.4 ± 3.4	23.3 ± 2.9	226 ± 53

[a] K_d values.

The linear and cyclic peptides bind to the different chimeras and wild-type receptors with the same relative order of potency. This is also true for both the linear and cyclic peptides, in which L-Phe is replaced with D-Phe, which indicates that these peptides may not bind in the principally different manner indicated by an earlier report (Frändberg *et al.*, 1994). Because both the MC1 and the MC3 receptors and the chimeras have the same relative potency order to the linear and cyclic peptides, it is tempting to speculate that the binding pocket of both the receptors is conserved.

The primary challenge in the molecular modeling of G protein-coupled receptors for drug design is the orientation of the TM regions with respect to the binding site. Two molecular models have been published describing the human MC1 receptor. Rhodopsin and bacteriorhodopsin were used as a template for both the models, which also took into account the early mutagenesis data. In the first model (Prusis *et al.*, 1995), cCDC was docked into a binding pocket between TM1, TM2, TM3, TM6, and TM7, with putative amino-acid contacts in TM2, TM3, and TM6. In the more recent model (Haskell-Luevano *et al.*, 1996), MTII, a cyclic lactam analogue, and the core tripeptide (D-Phe)-Arg-Trp were docked, and amino acids in all TM regions were identified as possible contact points. The two models are quite different even though they both rely on TM2 and TM3 as important domains for the MSH peptide binding.

It should be taken into account that the construction of chimeric proteins maps only the differences in binding properties between two receptors, but not the properties common to both receptors. Nevertheless, our present and earlier data indicate that the binding site of the MC receptors is formed of two major regions that are conceivably located near one another in a space around a hypothetical center of the receptor. One domain includes TM1, TM2, and TM3. Another domain includes TM7 and perhaps TM6. A third domain consisting of TM4 and TM5 seems not to be involved in the selective binding. This is supported by recent mutagenesis data and the proposed orientation of the TM regions in the first molecular model (Prusis *et al.*, 1995), where the low homologies of TM4 and TM5 led to their placement outside of the peptide-binding pocket.

For amine neurotransmitter receptors, which are much better characterized structurally than other G protein-coupled receptors, the ligand-binding pockets are assumed to be centered between TM3, TM4, and TM5 (Balwin, 1993). Also, the cannabinoid receptors, which are the G protein-coupled receptors with the highest homology to the melanocortin receptors, do have important binding elements centered between TM4 and TM5 (Shire *et al.*, 1996). Most of the neurotransmitter receptors whose ligands are small molecules have a conserved Pro in TM4 or a conserved Cys in EL2 (which can make a disulfide bridge with Cys in EL1). Several peptide binding G protein-coupled receptors also have these conserved amino acids and important ligand interactions in TM4 and TM5. These conserved amino acids are not found in the MC receptors, and TM4 and TM5 do not seem to have an important role for the specific binding of MSH peptides.

The present data and other mutagenesis data for the MC receptors and their structural relationship to other G protein-coupled receptors suggest the conclusion that the MC receptors belong to a group separate from the other receptors mentioned above and that these different groups are distinguished not only by differences in their sequences but also by the mode of interactions with their ligands.

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Send reprint requests to: Dr. Michael Szardenings or Dr. Jarl E. S. Wikberg, Department of Pharmaceutical Pharmacology, Biomedical Center, Box 591, 751 24 Uppsala, Sweden. E-mail: msz@bmc.uu.se or jarl.wikberg@farmbio.uu.se

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Characterisation of melanocortin receptor subtypes by radioligand binding analysis

Helgi B. Schiöth, Ruta Muceniece, Jarl E.S. Wikberg, Vijay Chhajlani *

Department of Pharmaceutical Pharmacology, Box 591, Biomedical Centre, Uppsala 75 124, Sweden

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Abstract

The DNAs encoding three melanocortin receptor subtypes (melanocortin MC_1 receptor, melanocortin MC_3 receptor and melanocortin MC_5 receptor) were expressed individually in COS (CV-1 Origin, SV40) cells to characterise their ligand binding properties. The results indicated that $[^{125}I][Nle^4, D\text{-}Phe^7]\alpha$-MSH (melanocyte stimulating hormone) bound to a single saturable site with K_d values of 85.1 ± 8.0 pmol/l (mean ± S.E.M), 396 ± 65 pmol/l and 5.05 ± 1.00 nmol/l for melanocortin MC_1 receptor, melanocortin MC_3 receptor and melanocortin MC_3 receptor, respectively. The melanocortin MC_1 receptor and the melanocortin MC_5 receptor showed a similar potency order to the melanocortic peptides examined which was markedly different from the potency order of the melanocortin MC_3 receptor. The melanocortin MC_1 receptor and melanocortin MC_5 receptor had a relatively higher affinity for α-MSH than γ-MSH and β-MSH, whereas the melanocortin MC_3 receptor had higher affinity for desacetyl-α-MSH, γ-MSH and β-MSH compared to α-MSH. The inclusion of the endopeptidase inhibitor phosphoramidon to prevent the breakdown of ACTH-(1-39) (adrenocorticotrophic hormone) to α-MSH, decreased ACTH-(1-39) binding affinity showing that ACTH-(1-39) had a much lower affinity for melanocortin MC_1 receptor than reported earlier.

Keywords: Melanocortin receptor subtype; $[^{125}I][Nle^4, D\text{-}Phe^7]\alpha$-MSH ligand binding; MSH (melanocyte stimulating hormone) peptide selectivity; Phosphoramidon

1. Introduction

Proopiomelanocortin (POMC) is primarily expressed in the central and peripheral nervous systems where it is posttranslationally cleaved into several peptide hormones that include melanotropins and adrenocorticotropins (Eipper and Mains, 1980). α-MSH (melanocyte stimulating hormone) was the first MSH peptide hormone discovered (Lerner and Lee, 1955) and was shown to induce skin pigmentation. Later studies have demonstrated the presence of α-MSH along with other proopiomelanocortin peptides such as β-MSH, γ-MSH and other MSH/ACTH (adrenocorticotrophic hormone) fragments in the CNS (central nervous system), where they are proposed to have neuromodulatory, behavioural, neurotransmitter and other biological effects (Bloch et al., 1979; Civelli et al., 1982; De Wied and Jolles, 1982; Tatro, 1990). The recent cloning of melanocortin receptors by us and others (Chhajlani et al., 1993; Chhajlani and Wikberg, 1992; Gantz et al., 1993a,b; Mountjoy et al., 1992), suggests that these various effects may be mediated by different subtypes of MSH receptors. To date 5 different melanocortin receptor subtypes (melanocortin MC_1 receptor, melanocortin MC_2 receptor, melanocortin MC_3 receptor, melanocortin MC_4 receptor and melanocortin MC_5 receptor) [1] have been described. The melanocortin receptors show 25-53% amino acid sequence homology between each other (Chhajlani et

* Corresponding author. Tel.: +46 18 174123; Fax: +46 18 559718.

[1] This nomenclature is the one proposed by the HUGO nomenclature committee. Please note that the melanocortin MC_5 receptor was originally termed by us as melanocortin MC_2 receptor (Chhajlani et al., 1993). However, since the HUGO nomenclature committee decided to reserve the term melanocortin MC_2 receptor for the receptor presumed to represent the ACTH receptor, we decided to agree upon the term melanocortin MC_5 receptor for what we previously called "melanocortin MC_2 receptor", as this receptor was the fifth melanocortin receptor cloned.

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al., 1993)and share a transmembrane topography with other G-protein coupled receptors. The first melanocortin receptor (melanocortin MC_1 receptor) that was cloned is expressed only in the melanocytes and melanoma cells and thus has been postulated to mediate the skin pigmentation effects of MSH peptides.

The melanocortin MC_2 receptor has also been termed the ACTH receptor, a nomenclature that was assigned because of its exclusive localisation in the adrenal gland. The remaining three melanocortin receptors, melanocortin MC_3 receptor, melanocortin MC_4 receptor and, melanocortin MC_5 receptor are all expressed in brain and might be responsible for the neural effects of the melanocortin peptides. None of these three latter receptors have been detected in melanocytes nor in the melanoma cells. In this study we report functional expression of human melanocortin MC_1, melanocortin MC_3 and melanocortin MC_5 receptors in COS (CV-1 Origin, SV40) cells, and their pharmacological characterization using the radioligand binding approach.

2. Materials and methods

2.1. Materials

ORG 2766 peptide was a gift from Organon Internationals, Holland. All the other peptides were purchased from Saxon Biochemicals, Germany. The γ1-MSH (H_2N-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-NH_2), γ2-MSH (H_2N-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH) and, the γ3-MSH (H_2N-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-Arg-Arg-Asn-Gly-Ser-Ser-Ser-Ser-Gly-Val-Gly-Gly-Ala-Ala-Gln-OH) peptides were from Bachem. Phosphoramidon was purchased from Boehringer Mannheim, Germany.

2.2. Expression of cloned melanocortin receptors

The melanocortin MC_1 receptor cDNA was excised from the pcDNA I vector (Chhajlani and Wikberg, 1992) and cloned in the HindIII site of the pRC/CMV vector (InVitrogen, USA). The melanocortin MC_5 receptor DNA was cloned between the HindIII and XbaI sites of the pRC/CMV vector as described earlier (Chhajlani et al., 1993). The melanocortin MC_3 receptor DNA in the expression vector pCMV/neo was a gift from Dr. Ira Gantz (Gantz et al., 1993a).

For receptor expression, COS cells were grown in Dulbeccós modified Eaglés medium with 8% foetal calf serum and non-essential amino acids. Eighty percent confluent cultures (80% of the Petri dish surface was covered with cells) were transfected with 1 μg DNA and 40 μg Lipofectin (BRL, USA) in serum free medium according to the instructions of the Lipofectin manufacturer. Five h after transfection, the serum-free medium was replaced with the serum containing medium and the cells were cultivated for 20 h. Cells were then scraped off, centrifuged, resuspended in serum-containing medium, plated on 48-well plates, and allowed to grow for 24 h before performing the radioligand binding.

2.3. Binding studies

All assays were performed in duplicate. [Nle^4, D-Phe^7]α-MSH (melanocyte stimulating hormone) was labelled with ^{125}I and purified as described before (Tatro and Reichlin, 1987). The transfected cells were washed with 0.2 ml of binding buffer (minimum essential medium with Earle's salts, 25 mM Hepes, pH 7.0, 0.2% bovine serum albumin, 1 mM 1,10-phenanthroline, 0.5 mg per litre leupeptin and 200 mg per litre bacitracin) while being attached to the 48-well plates. The cells were then incubated for 2 h at 37°C with 0.2 ml binding buffer containing a fixed concentration of [^{125}I][Nle^4, D-Phe^7]α-MSH and appropriate concentrations of the unlabelled ligand. After incubation the plates were put on ice, the cells washed with 0.2 ml of ice-cold binding buffer and detached from the plates with 0.4 ml of 0.1 N NaOH. Radioactivity was counted (Packard autogamma, scintillation spectrometer) and data analysed by fitting it to the four parametric logistic function using non-linear least squares regression. The K_i values were obtained from IC_{50} values according to the equation of Cheng and Prusoff (1973).

For saturation analysis, 12 concentrations of [^{125}I][Nle^4, D-Phe^7]α-MSH in the range of 0.02 up to 8 nM were used. Nonspecific binding was determined in the presence of 3 μM [Nle^4, D-Phe^7]α-MSH. The data from saturation experiments were analysed by an iterative, non-linear curve fitting programme suitable for radioligand binding analysis (Bergström and Wikberg, 1986).

For investigating the effect of phosphoramidon on the binding of ACTH-(1-39) and α-MSH, phosphoramidon was added to the binding buffer during incubation and washing steps to a final concentration of 100 μM.

3. Results

In order to characterize the proteins encoded by melanocortin MC_1, melanocortin MC_3 and melanocortin MC_5 receptors pharmacologically, they were transiently expressed from their corresponding DNAs in COS cells. The iodinated α-MSH analogue [^{125}I][Nle^4, D-Phe^7]α-MSH (Tatro and Reichlin

1987)was selected as radioligand because of its high affinity towards the melanocortin receptors (Siegrist et al., 1989)

Saturation curves shown in Figs. 1A–C were obtained by incubating varying concentrations of the [^{125}I][Nle^4, D-Phe^7]α-MSH in the absence and presence of 3 μM unlabelled [Nle^4, D-Phe^7]α-MSH for melanocortin MC_1 receptor, melanocortin MC_3 receptor and melanocortin MC_5 receptor, respectively. The results indicated that [^{125}I][Nle^4, D-Phe^7]α-MSH bound to a single saturable site with a K_d of 85.1 ± 8.0 pmol/l (mean ± S.E.M), 396 ± 65 pmol/l and 5.05 ± 1.00 nmol/l for melanocortin MC_1 receptor, melanocortin MC_3 receptor and melanocortin MC_5 receptor, respectively.

The K_i values obtained from the competition experiments are listed in Table 1, and the competition curves for some of the compounds examined are shown in Figs. 2A–C. As can be seen from Table 1 the potency order for the melanocortin MC_1 receptor was: [Nle^4, D-Phe^7]α-MSH > α-MSH > desacetyl-α-MSH > ACTH-(1–10) > β-MSH > γ3-MSH > ACTH-(1–39) > γ1-MSH > γ2-MSH > ACTH-(4–10). In contrast, the melanocortin MC_3 receptor has a markedly different potency order as compared to the melanocortin MC_1 receptor, the order of potency for melanocortin MC_3 receptor being: [Nle^4, D-Phe^7]α-MSH > desacetyl-α-MSH > γ1-MSH > γ3-MSH > β-MSH > γ2-MSH > α-MSH > ACTH-(1–39) > ACTH-(1–10) > ACTH-(4–10). The melanocortin MC_3 receptor has higher affinity for desacetyl-α-MSH, γ1-MSH, γ2-MSH, γ3-MSH and β-MSH than for α-MSH. The melanocortin MC_5 receptor shows similarities in the potency order to that of melanocortin MC_1 receptor: [Nle^4, D-Phe^7]α-MSH > desacetyl-α-MSH > α-MSH > β-MSH > ACTH-(1–39) > γ1-MSH. However, compared to both the melanocortin MC_1 receptor and melanocortin MC_3 receptor, the melanocortin MC_5 receptor has considerably lower affinity to all the compounds examined. [Nle^4, D-Phe^7]α-MSH is the only compound with K_i value in the nanomolar range whereas all the naturally occurring ligands tested have K_i values in the micromolar range.

ACTH-(22–39) shows no displacement of [^{125}I][Nle^4, D-Phe^7]α-MSH bound to any of the receptors studied. The α-MSH analogue ORG 2677 [Met(O_2)-Glu-His-Phe-D-Lys-Phe-OH] did not show any displacement of [^{125}I][Nle^4, D-Phe^7]α-MSH even at a concentration of 100 mM. Neither H_2N-Ser-Met-Glu-Val-Arg-Gly-Trp-OH (δ-MSH) nor H-Met-Glu-His-Phe-Pro-Gly-Pro-OH did displace [^{125}I][Nle^4, D-Phe^7]α-MSH bound to any of the receptors.

Phosphoramidon is a potent and specific inhibitor of endopeptidase-24.11, which is suggested to degrade the ACTH-(1-39) to the α-MSH (Matsas et al., 1984, Smith et al., 1992). In order to investigate a putative enzymatic degradation of ACTH-(1–39) a 100 μM concentration of phosphoramidon was added to the binding and washing buffers during binding assays. Fig. 3 shows the competition curves for ACTH-(1–39) and α-MSH in the presence and absence of phosphoramidon for melanocortin MC_1 receptor. The binding of α-MSH to melanocortin MC_1 receptor is not influenced by the presence of phosphoramidon, but the curve for ACTH-(1–39) is shifted to the right. For melanocortin MC_1 receptor, the K_i values of α-MSH in the presence and absence of phosphoramidon were 53.5 ± 6.6 pmol/l and 33.4 ± 10.5 pmol/l, respectively. By contrast, the K_i values for ACTH-(1–39) in the presence and absence of phosphoramidon were 2500 ± 470 pmol/l and

Table 1

K_i and K_d values (mean ± S.E.M) obtained from competition and saturation curves, respectively, for MSH/ACTH like peptides on melanocortin MC_1R, melanocortin MC_3R and melanocortin MC_5R transfected COS cells, together with the relative affinity ratios of the melanocortin receptor subtypes

Ligand	MC_1 K_i (nmol/l)	MC_3 K_i (nmol/l)	MC_5 K_i (nmol/l)	MC_3/MC_1	MC_5/MC_1	MC_5/MC_3
[^{125}I] NDP-MSH [a] (3)	0.0851 ± 0.0080	0.396 ± 0.065	5.05 ± 1.00	4.6	59	13
NDP-MSH (10)	0.0231 ± 0.0036	0.224 ± 0.035	2.39 ± 0.10	9.7	100	11
α-MSH (10)	0.0334 ± 0.0105	20.7 ± 3.7	8,240 ± 1,670	620	250,000	400
desacetyl-α-MSH (3)	0.0432 ± 0.0221	3.68 ± 1.35	3,620 ± 770	85	8,400	980
β-MSH (3)	1.17 ± 0.27	13.4 ± 6.4	14,400 ± 1,670	11	12,000	1,100
γ1-MSH (3)	2.68 ± 0.35	7.06 ± 2.90	42,600 ± 6,600	2.6	16,000	6,000
γ2-MSH (3)	11.2 ± 5.4	17.7 ± 1.9	> 100,000	1.6	–	–
γ3-MSH (3)	1.39 ± 0.24	10.9 ± 1.8	> 100,000	7.8	–	–
ACTH-(1–10) (3)	1.08 ± 0.29	145 ± 26	> 100,000	130	–	–
ACTH-(4–10) (3)	106 ± 15	784 ± 135	> 100,000	7.4	–	–
ACTH-(1–39) [b] (3)	2.50 ± 23.9	86.9 ± 23.9	17,000 ± 3,300	35	6800	200
ORG2766 (2)	> 100,000	> 100,000	> 100,000	–	–	–
δ-MSH (2)	> 30,000	> 30,000	> 100,000	–	–	–

[a] K_d values (nmol/l).
[b] Values obtained in presence of phosphoramidon.
Numbers in parentheses equal numbers of times assays were performed.

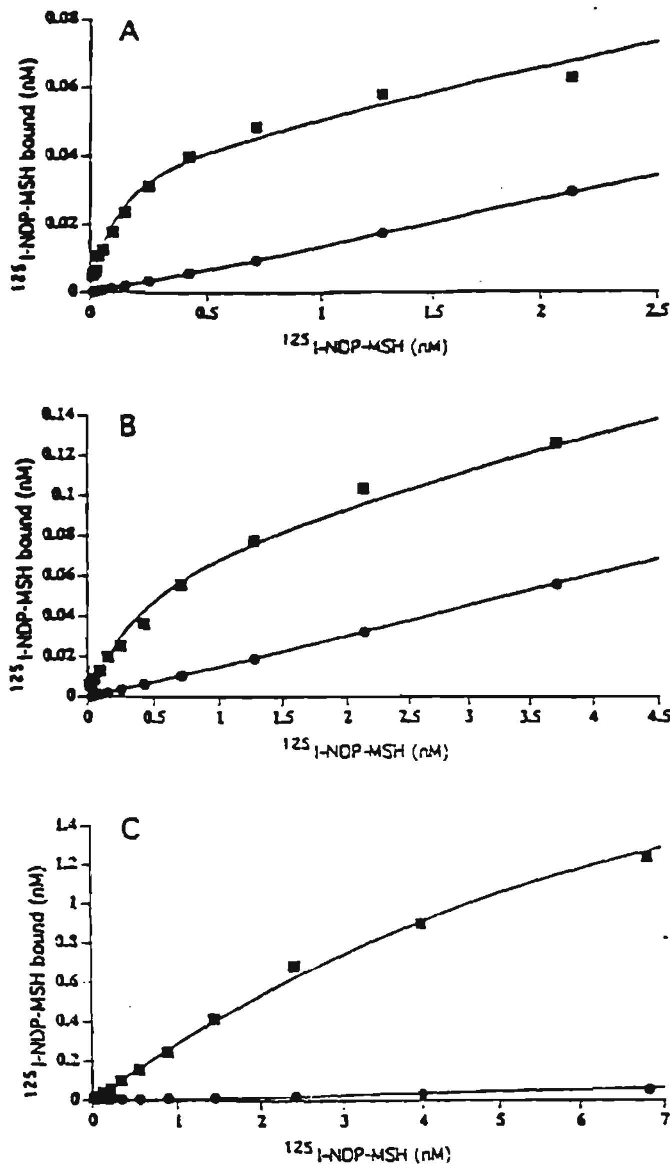

Fig. 1. Saturation curves of [^{125}I][Nle^4, D-Phe^7]α-MSH obtained from transfected COS cell. The figures show total binding (■) and binding in the presence of 3 μM cold [Nle^4, D-Phe^7]α-MSH (•) for: (A) melanocortin MC_1 receptor; (B) melanocortin MC_3 receptor; (C) melanocortin MC_5 receptor. Lines represent the computer modelled best fit of the data using an one-site model for the total binding.

254 ± 66 pmol/l, respectively. Considering the above-mentioned results for melanocortin MC_1 receptor, the competition curves for the ACTH-(1-39) on melanocortin MC_3 receptor and melanocortin MC_5 receptor were performed in the presence of phosphoramidon. The K_i values reported for ACTH-(1-39) in Table 1 are all obtained in the presence of phosphoramidon.

4. Discussion

Extensive studies to correlate the structure and function of the α-MSH have been carried out in relation to its role in peripheral pigmentation. The α-MSH and its structural analogues have been characterised for their effects on e.g. activation of tyrosinase, melanogenesis and melanin dispersion, as well as for their binding to the MSH receptor on the melanoma cells (Eberle, 1988). Binding data for MSH peptides to the receptors expressed on melanoma cells lines (Siegrist et al., 1989; Tatro et al., 1990) are in good agreement with the results reported here for the melanocortin MC_1 receptor. [Nle^4, D-Phe^7]α-MSH is reported to have a K_d value of 62.5 ± 5.2 pmol/l in the human D10 melanoma cells (Siegrist et al., 1989) which is close to the K_d value of 85.1 ± 8.0 pmol/l reported here. This K_d value and the similar potency order for the MSH peptides gives further evidence that the receptor clone of melanocortin MC_1 receptor is

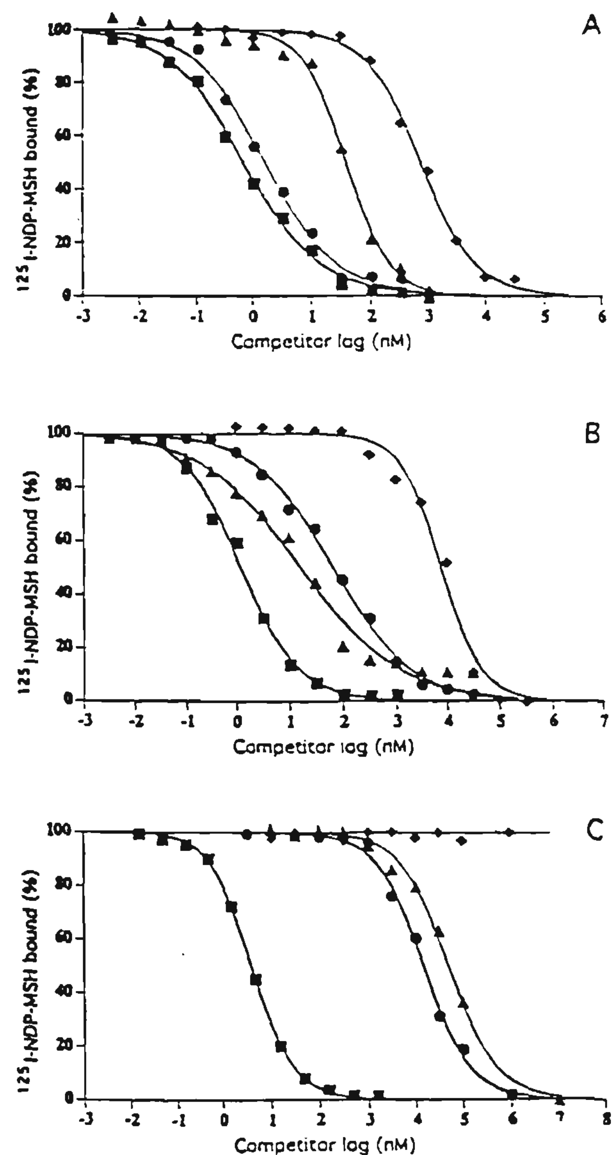

Fig. 2. Competition curves of [Nle^4, D-Phe^7]α-MSH (■), α-MSH (•), γ_1-MSH (▲) and ACTH-(4-10) (♦) obtained on transfected COS cell using a fixed concentration of ~2 nM [^{125}I][Nle^4, D-Phe^7]α-MSH for: (A) melanocortin MC_1 receptor; (B) melanocortin MC_3 receptor; (C) melanocortin MC_5 receptor.

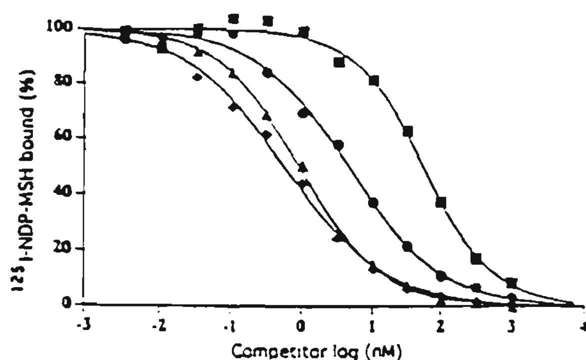

Fig. 3. α-MSH and ACTH-(1-39) in the presence and absence of phosphoramidon. Shown are competition curves of: α-MSH in absence of phosphoramidon (♦), α-MSH in presence of phosphoramidon (▲), ACTH-(1-39) in absence of phosphoramidon (●) and ACTH-(1-39) in presence of phosphoramidon (■) obtained for melanocortin MC_1 receptor transfected COS cells using fixed concentration of ~2 nM [^{125}I][Nle^4, D-Phe^7]α-MSH.

identical to the receptor expressed naturally in the peripheral melanocytic cells. The structure activity data generated earlier can, therefore, most probably be transferred to the genetically expressed melanocortin MC_1 receptor.

α-MSH is the natural ligand showing the strongest binding for the melanocortin MC_1 receptor. Circulating levels of α-MSH in mice have been reported to be in the range 70–140 pM (Thody et al., 1984). K_i value of 33.4 ± 10.5 pmol/l indicates a very high affinity of α-MSH to the melanocortin MC_1 receptor. α-MSH is furthermore selective for the melanocortin MC_1 receptor, as it has 620- and 25 000-fold lower affinities for melanocortin MC_3 receptor and melanocortin MC_5 receptor, respectively. The 22 amino acid long POMC fragment β-MSH shares a seven amino acid long core with α-MSH but has so far no proposed specific physiological function. The affinity of β-MSH for the melanocortin MC_1 receptor is 35 times lower than that of α-MSH, but it is nevertheless still 11- and 12 000-fold more selective for the melanocortin MC_1 receptor compared to the melanocortin MC_3 receptor or melanocortin MC_5 receptor, respectively. Besides indicating the possible physiological preferences of the receptor activation by the peptides, our findings are useful in delineating these receptors in tissue samples.

Desacetyl-α-MSH is posttranslationally processed into α-MSH (Dores et al., 1991; Verburg-van Kemenade et al., 1987). Both the forms occur naturally. The majority of α-MSH originates from the pituitary melanotrophic cells whereas hypothalamus releases the desacetylated form. The desacetyl-α-MSH/α-MSH K_i ratio is 0.4 for the melanocortin MC_5 receptor, 1.3 for the melanocortin MC_1 receptor while it is as high as 15 for the melanocortin MC_3 receptor. Of the naturally occurring peptides, desacetyl-α-MSH has the highest affinity for the melanocortin MC_3 receptor. However, in spite of these observations, desacetyl-α-MSH cannot be called selective for the melanocortin MC_3 receptor as it has a 13-fold lower affinity for the melanocortin MC_3 receptor than for the melanocortin MC_1 receptor.

The third group of MSH peptides processed from the POMC precursor are termed γ-MSH (Nakanishi et al., 1979). These peptides share a tetrapeptide core of His-Phe-Arg-Trp with α-MSH, β-MSH and ACTH. It has been proposed that the γ-MSH might play a specific physiological role in relation to rat melanocortin MC_3 receptor (Roselli-Rehfuss et al., 1993). However, in our hands γ-MSHs are not selective for the melanocortin MC_3 receptor. Thus the K_i values (see Table 1) of the γ-MSH peptides for melanocortin MC_3 receptor are higher than the ones for the melanocortin MC_1 receptor. Moreover, it is not the γ-MSH but the desacetyl-α-MSH which has the highest affinity for the melanocortin MC_3 receptor.

The binding data of the truncated ACTH fragments 1–10 and 4–10 (Table 1) demonstrate that both carboxyl end (Lys-Pro-Val) and the amino end (Ser-Tyr-Ser) are indeed important for the binding to all the receptors studied. ACTH-(1–10) has a distinctly lower affinity than α-MSH and desacetyl-α-MSH for all the receptors studied. The loss of three amino acids at the COOH end of α-MSH, leads to a 33- and 7-fold loss in affinity for the melanocortin MC_1 receptor and melanocortin MC_3 receptor, respectively. This suggests that the amino acid lysine at position 11 of the α-MSH peptide may be contributing to the binding. Moreover, the N-terminal portion of α-MSH, containing the acidic hydrophillic amino acid tyrosine, also seems to be significant for the binding to the receptors as the removal of the first three amino acids in ACTH-(4–10) results in a 98- and 5-fold loss of affinity for the melanocortin MC_1 receptor and melanocortin MC_3 receptor, respectively when compared to the ACTH-(1–10).

The α-MSH/ACTH-(4–9) analogue ORG 2766 which has been reported to be active in several behavioural tests (De Wied and Wolterink, 1988) does not displace [^{125}I][Nle^4, D-Phe^7]α-MSH bound to any of the receptors, even at very high concentrations. ORG 2766 has recently been reported not to displace [^{125}I][Nle^4, D-Phe^7]α-MSH in in vitro binding and autoradiography in several rat brain tissues (Tatro and Entwistle, 1994). These observations indicate that the physiological effects attributed to the ORG 2766 may be mediated through a still unknown receptor.

Endopeptidase-24.11 is present in kidney cells (Fulcher and Kenny, 1983; Kerr and Kenny, 1974) and is highly sensitive to phosphoramidon (Fulcher et al., 1982; Matsas et al., 1984). It has been suggested that inhibition of endopeptidase-24.11 by phosphoramidon blocks the degradation of ACTH-(1–39) into α-MSH

in granulocytes (Smith et al., 1992). The α-MSH corresponds to the first 13 amino acids of the ACTH molecule. Our data show that the presence or absence of phosphoramidon does not influence the binding of the α-MSH to the melanocortin MC_1 receptor. On the other hand, ACTH-(1-39) displays 10-fold lower affinity in the presence of phosphoramidon than in its absence. The higher affinity displayed by ACTH-(1-39) in the absence of phosphoramidon might be due to the fact that it is breaking down to α-MSH during the incubation in the binding assay.

In summary, we have expressed and pharmacologically characterised three human melanocortin receptors in the context of a single cell type. The melanocortin MC_1 receptor shows high and selective affinity for α-MSH and displays binding properties in accordance with those reported for MSH receptors in melanoma cells. The melanocortin MC_3 receptor binds to MSH peptides with high affinity but with another potency order than the melanocortin MC_1 receptor. Most notably, desacetyl-α-MSH and γ1-MSH has higher affinity than α-MSH. The melanocortin MC_5 receptor binds to the MSH peptides with a similar potency order as the melanocortin MC_1 receptor but with markedly lower affinities. We have also observed that the enzyme inhibitor phosphoramidon may block the conversion of ACTH-(1-39) into α-MSH affecting the apparent binding constants of ACTH-(1-39). All the receptors are well expressed in the COS cells and the system demonstrates its usefulness and reliability to characterize binding properties of the melanocortin receptors.

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Characterisation of the Melanocortin 4 Receptor by Radioligand Binding

H. B. Schiöth[1], R. Muceniece[2] and J. E. S. Wikberg[1]

[1]Department of Pharmaceutical Pharmacology, Uppsala University, Uppsala, Sweden and [2]Laboratory of Pharmacology, Institute of Organic Synthesis, Riga, Latvia

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Abstract: The DNA encoding the human melanocortin 4 receptor was expressed in COS (CV-1 origin, SV 40) cells and its radioligand binding properties was tested by using the [^{125}I][Nle^4, D-Phe^7]α-melanocyte stimulating hormone (MSH). The radioligand was found to bind to a single saturable site with a K_d of 3.84±0.57 nmol/l in the MC4 receptor expressing cells. The order of potency of a number of substance competing for the [^{125}I][Nle^4, D-Phe^7]α-MSH binding was the following; [Nle^4, D-Phe^7]α-MSH>[Nle^4]-α-MSH>β-MSH>desacetyl-α-MSH>α-MSH>ACTH (1–39)>ACTH (4–10)>γ1-MSH>γ2-MSH. This order of potency is unique for the melanocortin 4 receptor when compared to our previously published data for the other melanocortin receptor subtypes. Most notably the melanocortin 4 receptor shows highest affinity for β-MSH, among the endogenous MSH-peptides. Furthermore the melanocortin 4 receptor shows very low affinity for the γ-MSH peptides. This distinguishes the melanocortin 4 receptor from the melanocortin 3 receptor, which is the other major central nervous system melanocortin-receptor, as melanocortin 3 receptor shows high affinity for γ-MSH. Our finding might indicate a specific role for β-MSH for the melanocortin 4 receptor.

Proopiomelanocortin is expressed in the pituitary, hypothalamus and brain stem, and is enzymatically processed into the melanocortins, i.e. α-MSH, (melanocyte stimulating hormone) β-MSH, γ-MSH and ACTH (adrenocorticotrophic hormone). Melanocortic peptides binds to specific sites in the brain (Hnatowich *et al.* 1989; Tatro 1990; Lichtensteiger *et al.* 1993) and their central administration influence many systems such as e.g. thermoregulation (Feng *et al.* 1987), behaviour (Garrud *et al.* 1974), and neuroendocrine systems (Wiegant *et al.* 1979).

By the use of molecular cloning, five different subtypes of melanocortin receptors have been identified (Chhajlani *et al.* 1993; Chhajlani & Wikberg, 1992; Gantz *et al.* 1993a & b; Mountjoy *et al.* 1992). The first melanocortin receptor cloned was the melanocortin 1, which proved to be identical to the previously well characterised melanocortin receptor present on melanocytes and melanoma cells. However, recent data also indicate that the melanocortin 1 receptor is expressed in limited areas (periaqueductal gray and preoptic area) of the rat and human brains (Xia *et al.* 1995). The melanocortin 2 receptor is the adrenocortical ACTH receptor. The melanocortin 3 receptor mRNA has been found in distinct areas of the brain, as well as in placental and gut tissues (Gantz *et al.* 1993a; Desarnaud *et al.* 1994, Roselli-Rehfuss *et al.* 1993). The melanocortin 4 has been found in the brain only (Gantz *et al.* 1993b; Mountjoy *et al.* 1994). The melanocortin 5 is expressed in the brain, as well as in several peripheral tissues (Chhajlani *et al.* 1993; Gantz *et al.* 1994; Griffon *et al.* 1994; Labbé *et al.* 1994; Barrett *et al.* 1994; Fathi *et al.* 1995).

We have earlier characterised the human melanocortin 1, melanocortin 3 and melanocortin 5 receptors by using [^{125}I][Nle^4, D-Phe^7]α-MSH radioligand binding. Here, we report the binding properties of the human melanocortin 4 receptor by using the same technique. The results show that the binding properties of the melanocortin 4 receptor is distinct from the other human melanocortin-receptors. Moreover, among the natural MSH-peptides the β-MSH show the highest affinity for the melanocortin 4 receptor possibly indicating a functional role of this peptide for the receptor.

Author for correspondence: Helgi B. Schiöth, Department of Pharmaceutical Pharmacology, Biomedical Center, Box 591, 75 124 Uppsala, Sweden (fax +46 18 559718).

Materials and Methods

Chemicals. The γ1-MSH (H_2N-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-NH_2), γ2-MSH (H_2N-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH) and, the γ3-MSH (H_2N-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-Arg-Arg-Asn-Gly-Ser-Ser-Ser-Ser-Gly-Val-Gly-Gly-Ala-Ala-Gln-OH) peptides were from Bachem. [Nle^4, D-Phe^7]α-MSH was radioiodinated by the Chloramine T method and purified by HPLC. The specific activity of [^{125}I][Nle^4, D-Phe^7]α-MSH was approximately 2000 μCi/nmol. Phosphoramidon was purchased from Boehringer Mannheim GmbH, Germany. ORG 2766 peptide was a gift from Organon Internationals, Holland. All the other peptides were purchased from Saxon Biochemicals GmbH, Germany.

Expression of receptor clones. The genes for the human melanocortin 1 and human melanocortin 5 receptor (Chhajlani and Wikberg 1992; Chhajlani *et al.*, 1993) were cloned into the expression vector pRC/CMV (InVitrogen Corp., U.S.A.). The human melanocortin 3 and human melanocortin 4 receptor DNAs, which had been cloned into the expression vector pCMV/neo, and were gifts from Dr. Ira Gantz (Gantz *et al.* 1993a & b). COS cells were grown in Dulbecco's modified Eagle's medium with 10 % foetal calf serum (Gibco, BRL)

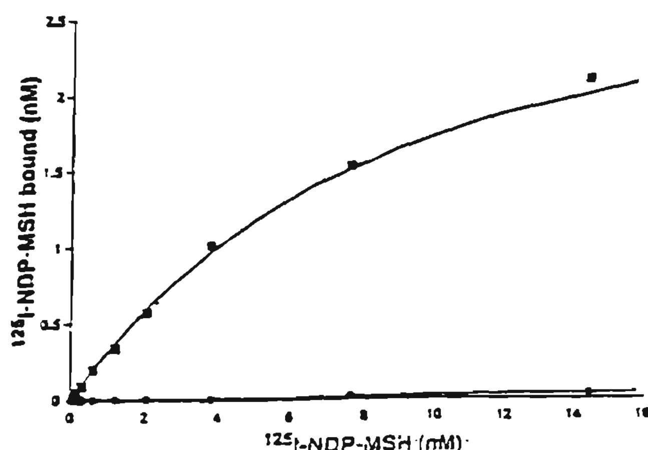

Fig. 1. Saturation curves of [^{125}I][Nle^4, D-Phe^7]α-(MSH) obtained from transfected COS cells. The figures show total binding (■) and binding in the presence of 3 μM cold [Nle^4, D-Phe^7]α-MSH (●) for the melanocortin 4 receptor. Lines represent the computer modelled best fit of the data using an one-site model for the total binding.

and 1X antibiotic/antimycotic solution (Gibco, BRL art. 15240-021). Eighty percent confluent cultures were transfected on petri dishes with the DNA (approximately 1 μg DNA for every 1×10^6 cells) mixed with liposomes in serum free medium. The liposomes used, were either the commercially available Lipofectin (BRL, USA) or produced according to Campbell (1995), both types giving similar results. After transfection the serum-free medium was replaced with the serum containing medium and the cells were cultivated for about 48 hr. Cells were then detached from the petri dishes by incubation in Hank's, balanced salt solution (Gibco, BRL art. 14185-045) with 0.5 mM EDTA for 3 minutes, then scraped off, centrifuged and used for radioligand binding.

Binding studies. The transfected cells were washed with binding buffer (Minimum Essential Medium with Earle's salts, 25 mM HEPES, pH 7.0, 0.2% bovine serum albumin, 1 mM 1,10-phenanthroline, 0.5 mg/l leupeptin and 200 mg/l bacitracin) and distributed into 96 well plates. The cells were then incubated for 2 hr at 37° with 0.1 ml binding buffer in each well, containing [^{125}I][Nle^4, D-Phe^7]α-MSH and appropriate concentrations of an unlabelled ligand. Assays of ACTH (1–39) was performed in the presence of 100 μM concentration phosphoramidon in the binding buffer. Phosphoramidon was used because we have earlier shown that it prevents the breakdown of ACTH(1-39) by endogenous proteases present in the COS cells (Schiöth *et al.* 1995).

After incubation, the plates were placed on ice, the cells washed with 0.1 ml of ice-cold binding buffer and detached from the plates with 0.2 ml of 0.1 N NaOH. Radioactivity was counted by using a Wallac Wizard automatic gamma counter and the data was analysed with use of the BindAid software (Wan System AB, Umeå, Sweden). Data were either analysed by fitting it to formulas derived from the law of mass-action by the method generally referred to as computer modelling, or by fitting to the four parameter logistic function. K_i-values were calculated by using the Cheng & Prusoff (1993) equation. The standard error of the mean is calculated from curve fitting of the experimental points. For saturation analysis, 12 concentrations of [^{125}I][Nle^4, D-Phe^7]α-MSH in the range of 0.02 up to 8 nM were used. Non specific binding was determined in the presence of 3 μM [Nle^4, D-Phe^7]α-MSH.

Results

We transiently expressed the DNA for the melanocortin 4 receptor in COS (CV-1 origin, SV 40) cells and performed binding assays on whole cells in multi well plates. Saturation curves were obtained by incubation with varying concentrations of [^{125}I][Nle^4, D-Phe^7]α-MSH in absence and presence of 3 μM unlabelled [Nle^4, D-Phe^7]α-MSH (fig. 1). Computer modelling analysis of the data indicated that the radioligand bound to a single saturable site with a K_d of 3.84±0.57 nmol/l. Non-specific binding was very low, being less than 5% of the total binding at 10 nmol/l of [^{125}I][Nle^4, D-Phe^7]α-MSH.

The K_i values of MSH and ACTH-peptides obtained from competition experiments are listed in table 1. Given

Table 1.

K_i and K_d values (mean±S.E.M.) obtained from competition and saturation curves, respectively, for melanocortin peptides on melanocortin (MC4) DNA transfected COS cells. Data for the MC1, MC3 and MC5 receptor taken from Schiöth *et al.* (1995) are given together with the relative affinity ratio of these subtypes to the MC4 receptor.

Ligand	MC4 K_i (nmol/l)	MC1 K_i (nmol/l)	MC3 K_i (nmol/l)	MC5 K_i (nmol/l)	MC4/MC1	MC4/MC3	MC4/MC5
[^{125}I] [Nle^4,D-Phe^7]α-MSH^a (6)	3.84±0.57	0.0851±0.0080^c	0.396±0.065^c	5.05±1.00^c	45	9.7	0.76
[Nle^4,D-Phe^7]α-MSH (6)	2.16±0.51	0.0231±0.0036^c	0.224±0.035^c	2.39±0.10^c	94	10	0.90
[Nle^4]-α-MSH (4)	122±28	0.102±0.022	9.85±2.6	4610±790	1200	12	0.026
α-MSH (6)	641±105	0.0334±0.0105^c	20.7±3.7^c	8240±1680^c	19000	31	0.078
desacetyl-α-MSH (4)	569±133	0.0432±0.0221^c	3.68±1.35^c	3620±770^c	13000	150	0.16
β-MSH (6)	376±91	1.17±0.27^c	13.4±6.4^c	14400±1670^c	320	28	0.026
γ1-MSH (4)	29000±1800	2.68±0.35^c	7.06±2.90^c	42600±6600^c	11000	4100	0.63
γ2-MSH (2)	>100000	11.2±5.4^c	17.7±1.9^c	>100000^c	–	–	–
γ3-MSH (2)	33500±5700	1.39±0.24^c	10.9±1.8^c	>100000^c	24000	3100	–
ACTH (4–10) (2)	21300±1400	[illegible]06±15^c	784±135^c	>100000^c	200	27	–
ACTH (1–39)^b (4)	693±38	2.50±0.24^c	86.9±0.25^c	17000±3300^c	280	8.0	0.041
ORG 2766 (2)	>100000	>100000^c	>100000^c	>100000^c	–	–	–
δ-MSH (2)	>100000	>30000^c	>30000^c	>100000^c	–	–	–

^{a} K_d values (nmol/l).
^{b} Values obtained in presence of phosphoramidon.
^{c} Values taken from Schiöth *et al.* (1995).
Numbers in parentheses equal numbers of times assays were performed for the MC4 receptor.

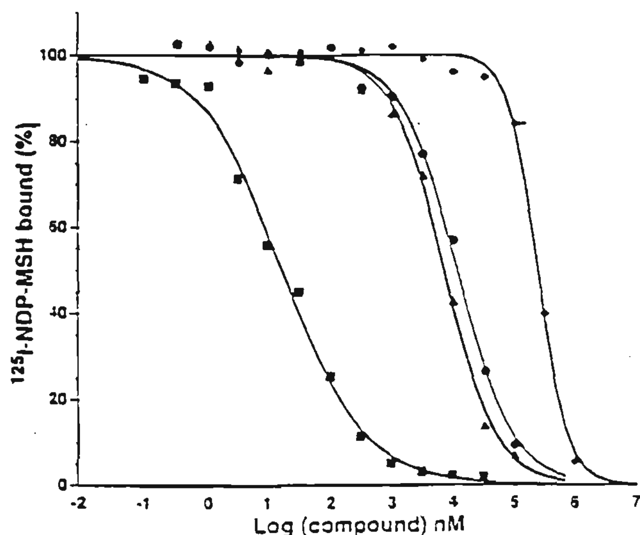

Fig. 2. Competition curves of [Nle^4, D-Phe^7]α-MSH (■), α-MSH (●), β-MSH (▲) and γ1-MSH (◆) obtained on transfected COS cells using a fixed concentration of ~2 nM [^{125}I][Nle^4, D-Phe^7]α-MSH for the melanocortin 4 receptor.

in table 1 are also our earlier published K_i values for the melanocortin 1, melanocortin 3 and melanocortin 5 receptors, which were obtained by using the same experimental methods as in the present study (Schiöth *et al.* 1995). Competition curves for [Nle^4, D-Phe^7]α-MSH, β-MSH, α-MSH and γ1-MSH are shown in fig. 2. From table 1 it can be seen that the potency order of the tested peptides for binding to the melanocortin 4 receptor is: [^{125}I][Nle^4, D-Phe^7]α-MSH >[Nle^4]-α-MSH>β-MSH>desacetyl-α-MSH>α-MSH >ACTH (1–39)>ACTH (4–10)>γ1-MSH>γ2-MSH.

γ2-MSH, the γ-MSH analogue ORG 2677 [Met(O$_2$)-Glu-His-Phe-D-Lys-Phe-OH] or δ-MSH [H$_2$N-Ser-Met-Glu-Val-Arg-Gly-Trp-OH] did not displace [^{125}I][Nle^4, D-Phe^7]α-MSH from the melanocortin 4 receptor in concentrations up to 100 μM. In the present study we also tested the binding of [Nle^4]-α-MSH on the human melanocortin 1, melanocortin 3 and melanocortin 5 receptors expressed in COS cells using the same approach as for our present and previous (Schiöth *et al.* 1995) studies (se table 1 for results).

Discussion

[Nle^4, D-Phe^7]α-MSH was first described as a highly potent α-MSH analogue capable of inducing darkening of frog skin (Sawyer 1980). Since then its radioiodinated form has proven useful for radioligand binding studies of melanocortin receptors, and earlier we used it to characterise the ligand binding properties of the melanocortin 1, melanocortin 3, and melanocortin 5 receptors (Schiöth *et al.* 1995). However, the ligand binding properties of the melanocortin 4 receptor has never before been carefully characterised by the accurate technique of radioligand binding.

The data of our present study show that [Nle^4, D-Phe^7]α-MSH had the highest affinity for the melanocortin 4 receptor, among all the tested MSH-analogues. [Nle^4, D-Phe^7]α-MSH also shows the highest affinities compared to many other MSH-peptide analogues for each of the melanocortin 1, melanocortin 3 and melanocortin 5 receptors (Schiöth *et al.* 1995). Due to these properties as well as due to a high stability [^{125}I] [Nle^4, D-Phe^7]α-MSH is therefore a very suitable radioligand for studying the melanocortin receptors.

Among the endogenous MSH-peptides tested, β-MSH proved to show the highest affinity for the melanocortin 4 receptor, closely followed by α-MSH and ACTH(1-39) which both showed about 2-fold lower affinities than β-MSH. The γ-MSH peptides on the other hand showed very low affinities for the melanocortin 4 receptor. The γ-MSH peptides have much lower affinity for the melanocortin 4 receptor than β and α-MSH have. γ1-MSH has 77 fold and 45 fold lower affinity for the melanocortin 4 than β and α-MSH have, respectively. Furthermore, γ1-MSH has more than 4000 fold higher affinity for the melanocortin 3 receptor than it has for the melanocortin 4 receptor. This affinity difference is even higher for the γ2- and γ3-MSH peptides. This data indicate that γ-MSH has a clear preference for the melanocortin 3 receptor compared to the melanocortin 4 receptor.

In table 2 is summarised the potency orders for various MSH-peptides for the different melanocortin receptor subtypes obtained from the radioligand binding measurements of our present and previous studies. As can be seen the melanocortin 1 and melanocortin 2 receptors show preference for α-MSH and ACTH, respectively, whereas the melanocortin 3 receptor show a slight preference for γ-MSH over β- and α-MSH. The melanocortin 4 receptor on the other hand show slight preference for β-MSH over α-MSH and ACTH (1-39), but it shows very low affinity for γ-MSH, as was already mentioned above. The melanocortin 5 receptor show the same potency order for peptides as the melanocortin 1 receptor. However, as can be seen from table 1 the melanocortin 5 receptor show over all much lower affinities for the peptides as compared to the melanocortin 1 receptor.

Thus, our data show that the pharmacology of the melanocortin 4 receptor is unique compared with the other melanocortin-receptors. It is interesting to note that the melanocortin 1 receptor show preference for α-MSH, the melanocortin 3 receptor for γ-MSH and the melanocortin 4 receptor for β-MSH. Although these properties may be coincidental, it is tempting to speculate that there is a speci-

Table 2.

Ligand preferences of the family melanocortin (MC) receptors.

MC1	NDP>α-MSH>β-MSH>ACTH>γ1-MSH
MC2	ACTH
MC3	NPD>γ1-MSH>β-MSH>α-MSH>ACTH
MC4	NDP>>β-MSH>α-MSH>ACTH>>γ1-MSH
MC5	NDP>>α-MSH>β-MSH>ACTH>γ1-MSH

Double sign (>>) means more than 50 fold affinity difference.

fic role for the different endogenous peptides for each of the three melanocortin receptors.

The melanocortin 4 and the melanocortin 5 receptors have very low affinity to all know endogenous ligands. The low affinity of these receptors to MSH peptides raise doubts whether the melanocortin 4 and melanocortin 5 bind to MSH peptides at physiological concentrations. Circulating levels of α-MSH in humans are much lower than binding constants of the melanocortin 4 and the melanocortin 5 receptors. Although the circulating MSH peptides originate most probably to a large extent from the pituitary, expression of proopiomelanocortin peptides have been detected in a large variety of peripheral tissues such as skin, testes, ovaries, placenta and the gastro-intestinal tract (for review see Eberle 1988). Furthermore, the MSH peptides are present in the neurones of many brain regions (for review see O'Donahue & Dorsa 1982). The physiological relevance of the binding of MSH peptides to the melanocortin 4 and melanocortin 5 receptors remains uncertain but it cannot be excluded that these receptors are expressed at locations where very high concentration of MSH peptides were gained upon release from MSH containing neurones.

The melanocortin 4 and melanocortin 3 receptors appears to be the most abundantly expressed melanocortin receptor subtypes of the central nervous system (Roselli-Rehfuss *et al.* 1993, Mountjoy *et al.* 1994). The melanocortin 3 receptor is predominantly expressed in the arcuate nucleus, as well as in few regions of the brain stem (Roselli-Rehfuss *et al.* 1993). The melanocortin 4 is much more widely distributed in the central nervous system and is represented at multiple sites in almost every brain region, including the cortex, thalamus, hypothalamus, brain stem and spinal cord (Mountjoy *et al.* 1994). Melanotropic peptides induce a number of central and peripheral effects for which there may be some differences in the spectrum of effects induced by different peptides. γ-MSH was reported to induce cardioacceleratory effect (Klein *et al.* 1985), affect cerebral blood flow (De Wildt *et al.* 1995) and to induce pressor and natriuretic effects (Lin *et al.* 1987, Gruber & Callagan 1989). Moreover, α-MSH, but not γ-MSH, was reported to induced grooming behaviour in the rat (O'Donahue *et al.* 1980). Combined with our binding data the melanocortin 4 receptor may therefore be a good candidate for the mediation of grooming behaviour whereas the melanocortin 3 receptor might mediate the cardioacceleratory, cerebral blood flow, pressor and natriuretic effects. Nevertheless, still further studies, as well as the obvious need for the development of highly selective drugs for the melanocortin-receptor subtypes, are warranted to be able to definitely settle these issues.

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MAJOR PHARMACOLOGICAL DISTINCTION OF THE ACTH RECEPTOR FROM OTHER MELANOCORTIN RECEPTORS

Helgi B. Schiöth*, Vijay Chhajlani*, Ruta Muceniece#, Vija Klusa# and Jarl E. S. Wikberg*+

*Department of Pharmaceutical Pharmacology, Uppsala University, Uppsala, Sweden. #Laboratory of Pharmacology, Institute of Organic Synthesis, Riga, Latvia and +Wa Pharm, Umeå, Sweden.

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Summary

The mouse adrenocortical cell line Y1, that expresses ACTH receptors (MC2R), was used to probe the binding of ACTH and MSH peptides by using radio-labelled ACTH (1-39). The Y1 cells were found to bind [^{125}I]-labelled ACTH(1-39) with high affinity (K_d~130 pM). However, none of the melanocortin peptides NDP-MSH, α-MSH, β-MSH or γ1-MSH could compete with the binding of the labelled ACTH(1-39). When other MC receptor subtype DNAs (MC1, MC3 and MC4) were transfected into the Y1 cells, characteristic binding of the [^{125}I]NDP-MSH appeared for each of the receptor subtype, but no specific binding was present in non-transfected cells. This is the first report clearly demonstrating that the ACTH receptor binds only ACTH, but not other melanocortin peptides.

Key Words: melanocortin receptor subtypes, [^{125}I] [Nle^4, D-Phe^7]α-MSH ligand binding, ACTH, Y1

Pro-opiomelanocortin is post-translationally processed into α-MSH (melanocyte stimulating hormone), β-MSH, γ-MSH and ACTH [1]. α-MSH has identical amino acid sequence as the first 13 amino acids in ACTH and all of the MSH peptides share a common core of 4 amino acids that is thought to be of central importance for the binding of MSH peptides to melanocortin-receptors [2]. The role of α-MSH for the melanogenesis in the skin is well established [3], as well as the role of ACTH for steroidogenesis in the adrenal gland [4].

Recently, we and others cloned five receptor genes that encode a family of G-protein coupled receptors showing high amino acid sequence homology [5-9]. These receptors were termed melanocortin receptors 1-5 (MC1R-MC5R). The MC2R was identified as the ACTH receptor as MC2 mRNA has been detected in the adrenal glands and because a positive cAMP response to ACTH was found in cells transfected with the MC2R [6,10]. It has also been shown that MSH peptides can bind to and stimulate MC1, MC3, MC4 and MC5 receptors [5,7,8,11-12]. Although it was shown a quarter of a century ago that [^{125}I]ACTH was capable of labelling receptor sites in the adrenal gland [14] the binding properties of the ACTH receptor has never been thoroughly characterized and compared with other MC receptor subtypes by ligand binding to receptors expressed in the same type of cell.

We have earlier shown that the MC1, MC3 and MC5 receptors bind α-MSH with much higher affinity than they bind ACTH [11]. In this report we show that the native ACTH receptor in a mouse adrenal gland derived cell line, Y1, is capable of binding the ACTH peptide radio-labelled with [^{125}I], but not any of several tested labelled and non-labelled MSH peptides. However, when the

Corresponding author: Helgi B. Schiöth, Pharmaceutical Pharmacology, Box 591, BMC, S-751 24 Uppsala, Sweden, Phone: +46-18-174160, Fax: +46-18-559718.

MC1, MC3 and MC4 receptors were expressed in the Y1 cells, sites capable of binding MSH peptides were found. Our data thus indicate that the ACTH receptor shows a major pharmacological distinction from the MSH receptors.

Materials and methods

Materials: Peptides (ACTH, [Nle⁴, D-Phe⁷]α-MSH (NDP-MSH), [Nle⁴]α-MSH, α-MSH, β-MSH, γ1-MSH) were purchased from Saxon Biochemicals GmbH, Germany. (3-[^{125}I]iodotyrosyl^{23})-ACTH(1-39) was purchased from Amersham. NDP-MSH was radioiodinated by the chloramine T method and purified by HPLC. [Nle^4]α-MSH was labelled with ^{125}I and purified as described before [14]. [^{125}I][Nle^4]α-MSH was checked for its ability to bind to MC1 transfected COS cells as described [11] before use.

Expression of cloned melanocortin receptors: The MC1 and MC5 receptor DNAs in expression vector pRC/CMV (InVitrogen Corp., USA) were cloned by us [5, 9]. The MC3 and MC4 receptor DNA in the expression vector pCMV/neo was a generous gift from Dr. Ira Gantz [7,8]. Y1 cells were grown in Dulbecco's modified Eagle's medium with 10 % foetal calf serum. For receptor expression, eighty percent confluent cultures were transfected with 1 µg DNA and 40 µg Lipofectin (BRL, USA) in serum free medium according to the instructions of the manufacturer. 6 h after transfection the serum-free medium was replaced with the serum containing medium and the cells were cultivated for 48 h. Cells were then scraped off, centrifuged and used for radioligand binding.

Binding studies: The transfected cells were washed with binding buffer (Minimum Essential Medium with Earle's salts, 25 mM HEPES, pH 7.0, 0.2 % bovine serum albumin, 1 mM 1,10-phenanthroline, 0.5 mg per litre leupeptin and 200 mg per litre bacitracin) and distributed into 96-well plates. The cells were then incubated for 2 h at 37°C with 0.1 ml binding buffer in each well, containing a constant concentration of [^{125}I]NDP-MSH, [^{125}I][Nle^4]α-MSH or (3-[^{125}I]iodotyrosyl^{23})ACTH(1-39) and appropriate concentrations of an unlabelled ligand. After incubation, the plates were put on ice, the cells washed with 0.1 ml of ice-cold binding buffer and detached from the plates with 0.2 ml of 0.1 N NaOH. Radioactivity was counted (Wallac, Wizard automatic gamma counter) and data analysed with the BindAid software (Wan System AB, Umeå, Sweden). Data were either analysed by fitting it to formulas derived from the law of mass-action by the method generally referred to as computer modelling, or by fitting to the four parameter logistic function. K_i-values were calculated by using the Cheng and Prusoff equation [15]. The standard error of the mean is calculated from curve fitting of the experimental points. The complete experiments were performed in duplicates.

Results

Y1 cells were incubated with 0.06 nM (3-[^{125}I]iodo-tyrosyl^{23})-ACTH(1-39) in the presence of different concentrations (3.7 pM - 100 nM) of non-labelled ACTH (1-39) and the binding of radioactivity to the cells was measured (fig. 1a). Analysis of the resulting competition curves with computer modelling indicated that the binding occurred to one single high affinity site with a dissociation constant of 132 ± 20 pM (mean ± S.E.M). Attempts were then made to displace the labelled ACTH with α-MSH, β-MSH, γ1-MSH (fig. 1a) or NDP-MSH (data not shown). None of these peptides were capable of preventing the binding of the radio-labelled ACTH to the Y1 cells. Even at concentrations as high as 100 nM the MSH peptides did not cause any displacement of the [^{125}I]ACTH. We then investigated if the Y1 cells could bind radio-labelled NDP-MSH. The Y1 cells were incubated with 1 nM of [^{125}I]NDP-MSH together with varying concentrations (3.7 pM - 100 nM) of cold NDP-MSH (fig. 1b). As can be seen from fig. 1b the level of binding of [^{125}I]NDP-MSH was low. Moreover, increasing concentrations of the non-labelled peptide were not capable of reducing the binding further, thus indicating that the label bound non-specifically to the Y1 cells. Essentially the same results were obtained when cells were incubated with 1 nM [^{125}I][Nle^4]α-MSH in the presence of 3.7 pM - 100 nM of non-labelled [Nle^4]α-MSH (data not shown graphically).

We expressed the MC1, MC3 and the MC4 human receptor DNAs in the Y1 cells. The transfected cells were incubated with 0.4 nM [^{125}I]NDP-MSH in the presence of varying concentrations of cold

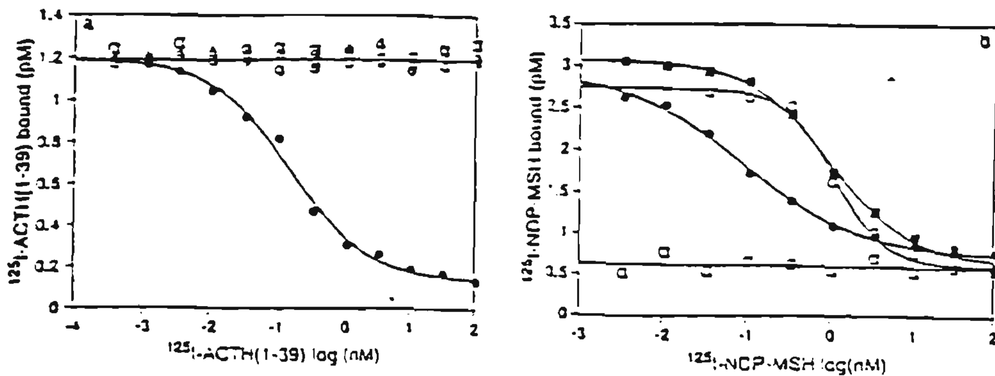

Fig. 1.

a) Effect of ACTH and MSH peptides on [^{125}I]ACTH(1-39) binding to mouse adrenocortical Y1 cells. Y1 cells were incubated with 60 pM of [^{125}I]ACTH(1-39) in the presence of various concentrations of ACTH(1-39) (●), α-MSH (□), β-MSH (O), γ1-MSH (Δ) and NDP-MSH (◇). b) Effect of NDP-MSH on the binding of [^{125}I]NPD-MSH to Y1 cells. Shown are the binding of 0.4 nM [^{125}I]NPD-MSH to native Y1 cells (□), and to Y1 cells transfected with the MC1R (●), MC3R (■) and MC4R (O) genes.

NDP-MSH. As is shown in fig. 1b cells transfected with any of these three MC receptor subtypes showed increased binding of the [^{125}I]NDP-MSH compared to non-transfected cells, and this binding was competed for by non-labelled NDP-MSH in a dose-dependent fashion. The K_i values for NDP-MSH binding was estimated to be 20.0 ± 13 pM, 526 ± 46 pM and 935 ± 94 pM for the MC1, MC3 and the MC4 receptors, respectively. An expression of the MC5 receptor clone in the Y1 cells induced a specific binding to NDP-MSH but as it was only 20-30 % of the total binding, we were not able to determine an accurate K_i value. This was presumably related to the earlier reported low affinity of the MC5 receptor for MSH peptides [11].

Discussion

The gene encoding the mouse MC2 receptor was recently cloned and shown to possess 89 % amino acid sequence homology with the human MC2 receptor [17]. MC2 receptor mRNA can be detected in Y1 cells as well as NCI-H295 human adrenal tumour cells by Northern blotting using the mouse and human MC2 receptor probes, respectively [18]. It was also shown that ACTH can up-regulate the MC2 mRNA levels in both the Y1 mouse adrenal cells and the NCI-H295 cells [18]. Moreover, ACTH was reported to induce a concomitant up-regulation of both ACTH receptor transcripts (probed with Northern blotting using a human MC2-receptor probe) and ACTH receptor numbers (probed with [^{125}I]ACTH(1-39) binding) in the cultured human adrenocortical cells [19]. These data gives strong support that the native ACTH receptor is indeed encoded by the MC2 gene.

The Y1 adrenocortical cell line is of mouse origin and responds to ACTH with an increase in steroid production via the stimulation of ACTH receptors [16]. We used Y1 cells to investigate the binding properties of the native ACTH receptor. The results show that Y1 cells bind specifically to ACTH with high affinity and that the MSH peptides do not affect this binding. Furthermore, labelled MSH peptides do not bind to the Y1 cells in specific manner. These results indicated that there was a marked difference in the properties of the ACTH receptor compared to those reported earlier for the other melanocortin receptors.

Specific binding sites for MSH peptides appear in the Y1 cells after transient transfection of the DNAs for the other melanocortin subtypes. The K_i values presented here, are close to those determined earlier when these receptors were expressed in COS cells: i.e. these K_i's being 23.1 ±

3.6 pM [11], 224 ± 35 pM [11] and 1920 ± 340 pM (Schiöth et al., unpublished observations) for the MC1, MC3 and MC4 receptors, respectively.

The MC2 receptor shows, 38, 42, 46 and 44 % amino acid homology with the MC1, MC3, MC4 and MC5 receptors, respectively. Transmembrane (TM) segments of all of these five melanocortin receptors share an even higher sequence homology, as well as other common characteristics such as an highly conserved asparatic acid in TM segment 3 (corresponding to Asp-117 in the MC1R), and an highly conserved histidine in TM segment 6 (corresponding to His-260 in the MC1R). Our earlier studies using site-directed mutagenesis and molecular modelling of the MC1R receptor 3D structure [2,20] have indicated that these amino acids are involved in the binding of the central core Glu-His-Phe-Arg-Trp [i.e. ACTH(5-9)] found in both MSH and ACTH peptides; a core that is shown to be crucial for melanocortin peptide melanogenetic activity. However, the data of our present study indicate that this core could not have an exclusively dominant role for the binding of the ACTH to the ACTH receptor since none of the MSH peptides showed any appreciable affinity for the receptor. Previous studies have indicated that both the ACTH(5-10) core as well as parts of the C-terminus [i.e. ACTH(15-18)] is crucial for the steroidogenetic activity of ACTH [21]. In view of our present and previous results [22-24] it is therefore tempting to speculate that recognition and activation of the ACTH-receptor is afforded by separated epitopes in the ACTH.

In summary, this is the first report that clearly demonstrates that the ACTH receptor specifically binds ACTH but not other melanocortin peptides. Our finding explains why the MSH peptides are not known to induce steroidogenesis.

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The melanocortin 1, 3, 4 or 5 receptors do not have a binding epitope for ACTH beyond the sequence of α-MSH

H B Schiöth, R Muceniece[1], M Larsson and J E S Wikberg

Department of Pharmaceutical Pharmacology, Uppsala University, Uppsala, Sweden and [1]Laboratory of Pharmacology, Institute of Organic Synthesis, Riga, Latvia

(Requests for offprints should be addressed to H B Schiöth and J E S Wikberg, Department of Pharmaceutical Pharmacology, Biomedical Centre, Box 591, 751 24 Uppsala, Sweden)

Abstract

ACTH(1–39), and several shorter N- and/or C-terminally truncated fragments of ACTH, with and without N-terminal acetylation and/or C-terminal amidation, were tested for binding on a single eukaryotic cell line transiently and independently expressing the melanocortin MC1, MC3, MC4 and MC5 receptors. The results show that none of these MC receptors has specific binding epitopes for the ACTH peptides beyond the amino acid sequence of α-MSH, when tested for their ability to compete with ^{125}I-labelled [Nle^4,D-Phe^7]α-MSH and ACTH. The MC3 receptor favours the natural desacetylated N-terminal end of the ACTH peptides, and it has generally more than 10-fold higher affinity for the ACTH peptides than the MC4 receptor. Considering earlier anatomical localisation data, together with the present data, we suggest that the MC3 receptor is the most likely candidate of the MC receptors to mediate the short-loop negative feedback release of corticotrophin-releasing factor (CRF) caused by ACTH/MSH peptides.

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Introduction

Pro-opiomelanocortin (POMC) is post-translationally processed in a tissue-specific manner to give rise to a variety of biologically active substances including adrenocorticotrophin (ACTH) and the α-, β-, γ-melanocyte stimulating hormone (MSH). The POMC gene is primarily expressed in the pituitary gland, but has also been detected in numerous non-pituitary tissues, including hypothalamus, testes, ovary, placenta, duodenum, liver, kidney, lung, thymus and lymphocytes (Smith & Funder 1988). ACTH stimulates steroidogenesis in the adrenal gland, whereas α-MSH stimulates melanogenesis in melanocytes. Besides these well-known effects, the melanocortins (MC) are reported to have a broad array of other effects, e.g. being neurotrophic, anti-inflammatory, antipyretic, and affecting memory, learning, behaviour and blood pressure (O'Donahue & Dorsa 1982, Eberle 1988).

Five receptors for the melanocortic peptides (ACTH/MSH) have been cloned by us and others (Chhajlani & Wikberg 1992, Mountjoy *et al.* 1992, Chhajlani *et al.* 1993, Gantz *et al.* 1993*a,b*). The MC1 receptor is expressed in melanocytes and binds α-MSH with high affinity. The MC2 (or ACTH) receptor is expressed in the adrenal gland and binds ACTH with high affinity but it does not bind the MSH peptides (Schiöth *et al.* 1996*a*). The MC3 receptor is expressed in the brain (predominantly in the arcuate nucleus, and in few regions of the brain stem), as well as in the periphery where it has been found in the placenta and gut tissues; it is also relatively abundantly expressed in the human heart (Gantz *et al.* 1993*a*, Roselli-Rehfuss *et al.* 1993, Chhajlani 1996). The MC4 receptor is predominately found in the central nervous system, where it is represented in almost every brain region, including the cortex, thalamus, hypothalamus, brain stem and spinal cord (Gantz *et al.* 1993*b*, Mountjoy *et al.* 1994). The MC5 receptor has widespread peripheral tissue distribution but can also be found in the brain (Griffon *et al.* 1994, Labbé *et al.* 1994, Fathi *et al.* 1995).

Corticotrophin-releasing factor (CRF) stimulates release of ACTH from the anterior pituitary gland. Secretion of both CRF and ACTH is inhibited by a long negative feedback effect by the glucocorticoids. The melanocortic peptides are known to mediate a short-loop feedback regulation of CRF production in the hypothalamus (Motta *et al.* 1965, Suda *et al.* 1986) and central administration of α-MSH inhibits CRF release in adrenalectomised rats (Tozawa *et al.* 1994). Recently, MC2 receptor mRNA was shown to be abundant in the adrenal gland but mRNA for the MC2 receptor could be detected neither in the hypothalamus nor in the pituitary (Xia & Wikberg 1996). Early autoradiographic data using ^{125}I-labelled ACTH demonstrated existence of specific binding sites for ACTH in the rat brain including the hypothalamus

(Hnatowich *et al.* 1989). These autoradiographic data may tentatively be explained by assuming that ACTH binds to the newly discovered MC3 or MC4 receptors, as these are most abundantly expressed in the brain.

The objective of the present study was to determine the affinity of ACTH and ACTH fragments for the cloned human MC receptors, with the aim of finding out if any of the different MC receptors have binding epitopes for the ACTH peptides that goes beyond the sequence of α-MSH.

Materials and Methods

Chemicals

ACTH(1–39) (H-Ser¹-Tyr²-Ser³-Met⁴-Glu⁵-His⁶-Phe⁷-Arg⁸-Trp⁹-Gly¹⁰-Lys¹¹-Pro¹²-Val¹³-Gly¹⁴-Lys¹⁵-Lys¹⁶-Arg¹⁷-Arg¹⁸-Pro¹⁹-Val²⁰-Lys²¹-Val²²-Tyr²³-Pro²⁴-Asn²⁵-Gly²⁶-Ala²⁷-Glu²⁸-Asp²⁹-Glu³⁰-Ser³¹-Ala³²-Glu³³-Ala³⁴-Phe³⁵-Pro³⁶-Leu³⁷-Glu³⁸-Phe³⁹-OH) and other peptides were purchased from Saxon Biochemicals GmbH, Hanover, Germany or Bachem, Budendorf, Switzerland, except ACTH(6–24) which was purchased from American Peptide Company, Sunnyvale, CA, USA, diacetyl-α-MSH (Ac-[Ser(Ac)¹]-α-MSH) which was purchased from ICN Biomedicals Inc., Irvine, CA, USA and ACTH(7–16) which was purchased from Neosystem SA, Strasbourg, France. [Nle⁴,D-Phe⁷]α-MSH was radioiodinated by the chloramine T method and purified by HPLC. (3-[^{125}I]iodo-tyrosyl²³)-ACTH(1–39) was purchased from Amersham, Solna, Sweden.

Expression of receptor clones

The human MC1 and MC5 receptors (Chhajlani & Wikberg 1992, Chhajlani *et al.* 1993) were cloned into the expression vector pRc/CMV (InVitrogen, Oxon, UK). The human MC3 and MC4 receptor DNAs, cloned into the expression vector pCMV/neo, were gifts from Dr Ira Gantz (Gantz *et al.* 1993*a*,*b*). For receptor expression, COS-1 (CV-1 Origin, SV40) cells were grown in Dulbecco's modified Eagle's medium with 10% foetal calf serum. Eighty per cent confluent cultures were transfected on 100 mm cell culture dishes with the DNA (approximately 1 µg DNA for every 1 × 10⁶ cells) mixed with liposomes in serum-free medium (for details see Schiöth *et al.* 1996*b*). After transfection, the serum-free medium was replaced with growth medium and the cells were cultivated for about 48 h. Cells were then scraped off, centrifuged, and used for radioligand binding.

Binding studies

The transfected cells were washed with binding buffer (Schiöth *et al.* 1995) and distributed into 96-well plates (approximately 40 000 cells/well). The cells were then incubated for 2 h at 37 °C with 0·05 ml binding buffer in each well, containing a constant concentration of [^{125}I-Tyr²][Nle⁴,D-Phe⁷]α-MSH or (3-[^{125}I]iodo-tyrosyl²³)-ACTH(1–39) and appropriate concentrations of an unlabelled ligand. After incubation, the cells were washed with 0·2 ml of ice-cold binding buffer and detached from the plates with 0·2 ml 0·1 M NaOH. When the binding was determined for ACTH(1–39), phosphoramidon was added to the binding buffer during incubation and washing steps to a final concentration of 100 µM (Schiöth *et al.* 1995). The affinity of ACTH(1–24) was not affected by the presence of phosphoramidon (data not shown) and phosphoramidon was therefore not used for the other peptides. Radioactivity was counted (Wizard automatic gamma counter, Wallac, Turku, Finland) and data analysed with a software package for radioligand binding analyses (Wan System, Umea, Sweden). Data were analysed by fitting them to formulas derived from the law of mass action by the method generally referred to as computer modelling. The K_d value for [^{125}I-Tyr²][Nle⁴,D-Phe⁷]α-MSH for the MC1 (0·0851 ± 0·0080 nmol/l), MC3 (0·396 ± 0·063 nmol/l) and MC5 (5·05 ± 1·00 nmol/l) receptor were taken from Schiöth *et al.* (1995) and for the MC4 (3·34 ± 0·57 nmol/l) from Schiöth *et al.* (1996*a*). Repeated saturation experiments gave similar values (data not shown). The B_{max} for both these studies were close to each other and about 4000–20 000 binding sites/cell for all of the receptor types. Binding constants are essentially independent of the transfection efficiency as the calculation method provides correction of the concentration of free vs bound ligand, minimising problems related to different expression levels. The binding assays were performed in duplicate wells and repeated three times. Untransfected COS-1 cells did not show any specific binding to [^{125}I-Tyr²][Nle⁴,D-Phe⁷]α-MSH or (3-[^{125}I]iodo-tyrosyl²³)-ACTH(1–39).

Results

ACTH(1–39), and several shorter N- and/or C-terminally truncated fragments of ACTH, with and without N-terminal acetylation and/or C-terminal amidation, were tested for binding to the MC1, MC3, MC4 and MC5 receptors. The human DNAs for the MC1, MC3, MC4 and MC5 receptors were transiently and independently expressed in COS-1 cells for competitive receptor binding using [^{125}I-Tyr²][Nle⁴,D-Phe⁷]α-MSH or (3-[^{125}I]iodo-tyrosyl²³)-ACTH(1–39) as radioligands. The expression levels of the different receptor subtypes were similar (data not shown). The K_i values resulting from calculations of the competition curves of binding with [^{125}I-Tyr²][Nle⁴,D-Phe⁷]α-MSH are summarised in Table 1. Competition curves for α-MSH, ACTH(1–14), ACTH(1–17), ACTH(1–24), ACTH(6–

Table 1 K_i values (nmol/l ± S.E.M.) from competition curves for MSH analogues on MC1-, MC3-, MC4- and MC5-transfected COS cells

	Receptor			
	MC1	MC3	MC4	MC5
Ligand				
α-MSH	0·113 ± 0·046	44·7 ± 19·1	357 ± 60	2440 ± 810
Diacetyl-α-MSH	1·96 ± 0·35	72·2 ± 48	2770 ± 1500	5925 ± 1840
Desacetyl-α-MSH	0·279 ± 0·084	13·9 ± 4·8	250 ± 150	1890 ± 750
α-MSH-OH	1·20 ± 0·66	82·9 ± 58	3050 ± 500	18 200 ± 7400
Acetyl-ACTH(1–14)	2·34 ± 0·47	112 ± 30	3680 ± 450	12 400 ± 3700
ACTH(1–14)	0·836 ± 0·33	48·3 ± 9·0	2170 ± 120	13 700 ± 5500
ACTH(1–16)	0·267 ± 0·116	19·0 ± 4·3	698 ± 88	2600 ± 595
Acetyl-ACTH(1–17)	0·205 ± 0·042	42·5 ± 5·5	752 ± 79	5870 ± 1990
ACTH(1–17)	0·230 ± 0·061	14·0 ± 4·5	419 ± 62	4240 ± 1200
ACTH(1–24)	0·209 ± 0·052	27·0 ± 2·5	827 ± 262	2760 ± 780
ACTH(1–39)	3·95 ± 0·67	135 ± 22	2170 ± 120	4920 ± 610
ACTH(6–24)	42·5 ± 5·3	9970 ± 2350	14 700 ± 5400	29 600 ± 18 000
ACTH(7–16)	8550 ± 800	>100 000	>100 000	>100 000
ACTH(7–38)	8770 ± 350	>100 000	>100 000	>100 000

24) and ACTH(1–39) using cells expressing the MC1, MC3, MC4 and MC5 receptor clones are shown in Fig. 1. The competition curve for ACTH(1–39) using (3-[^{125}I]iodo-tyrosyl^{23})-ACTH(1–39) on cells expressing the MC1 receptor is shown in Fig. 2.

The affinity of ACTH(1–39) for the MC1 receptor was determined using either [^{125}I-Tyr^2][Nle^4,D-Phe^7]α-MSH or (3-[^{125}I]iodo-tyrosyl^{23})-ACTH(1–39) as tracer. The results using both tracers were very similar; the K_i values for ACTH(1–39) being 2·95 ± 1·03 nM and 3·95 ± 0·67 nM, respectively. The specific binding for the MC3, MC4 and MC5 receptors was very low when (3-[^{125}I]iodo-tyrosyl^{23})-ACTH(1–39) (at 0·1 nM concentration) was used (probably due to low affinity of ACTH(1–39) (see Table 1)). This made it impossible to use the labelled ACTH for accurate determination of K_i values for these receptors.

α-MSH, which has an amino acid sequence identical to the first 13 amino acids in ACTH, is acetylated at the N-terminus and amidated at the C-terminus. ACTH is neither acetylated nor amidated (i.e. has a free carboxyl group at the C-terminus). The results show that the α-MSH has highest affinity for the MC1 receptor, lower for the MC3 receptor, lower still for the MC4 receptor, and lowest for the MC5 receptor. This property is shared by all the other peptides tested in this study. α-MSH with two acetyl groups in the N-terminal region (diacetyl-α-MSH) showed slightly lower affinity for all the receptors compared with α-MSH. Desacetyl-α-MSH (without an N-terminal acetyl group) had lower affinity than α-MSH for the MC1 receptor, but higher for the MC3 receptor. The MC3 receptor also had higher affinity for the desacetylated forms of ACTH(1–14) and ACTH(1–17), compared with the N-terminal acetylated forms. This property was not shared by the other receptors.

As can be seen from Table 1, α-MSH with a free carboxyl group at the C-terminus (α-MSH-OH), has lower affinity for all the receptors. Extension of the ACTH(1–13) by the Gly^{14} (acetyl-ACTH(1–14)) does not affect this affinity loss, but for the longer fragments (see acetyl-ACTH(1–17)) this effect vanished as the affinities increased. Our data indicate that the amino acid extensions of the ACTH(1–13) fragments up to ACTH(1–24) do not affect the binding to any of the receptors. The longer sequence of the full length ACTH(1–39) lowered the affinity for the MC receptors, an effect which was largest for the MC1 receptor. Truncation of the N-terminus, as in ACTH(6–24) results in much lower affinities for all the subtypes, whereas the MC1>MC3>MC4>MC5 preference order remains the same. ACTH(7–38) and ACTH(7–16) bound with similar affinity to the MC1 receptor but not to the other subtypes. Other ACTH fragments, ACTH(11–24) and ACTH(22–39) did not displace [^{125}I-Tyr^2][Nle^4,D-Phe^7]α-MSH from any of the MC receptors at 100 μM concentration.

Discussion

It is now well established that the MC2 receptor is the adrenal gland ACTH receptor. The ACTH receptor has high affinity for ACTH, but does not bind to the MSH peptides. Despite distinct pharmacology of the MC2 receptor compared with the other MC receptors, all the five cloned MC receptors show considerable amino acid homology. The amino acid identity of the MC2 receptor is highest for the MC4 receptor (46%) and lowest for the MC1 receptor (38%). It is believed that the first 13 amino acids of ACTH are essential for the activation of the

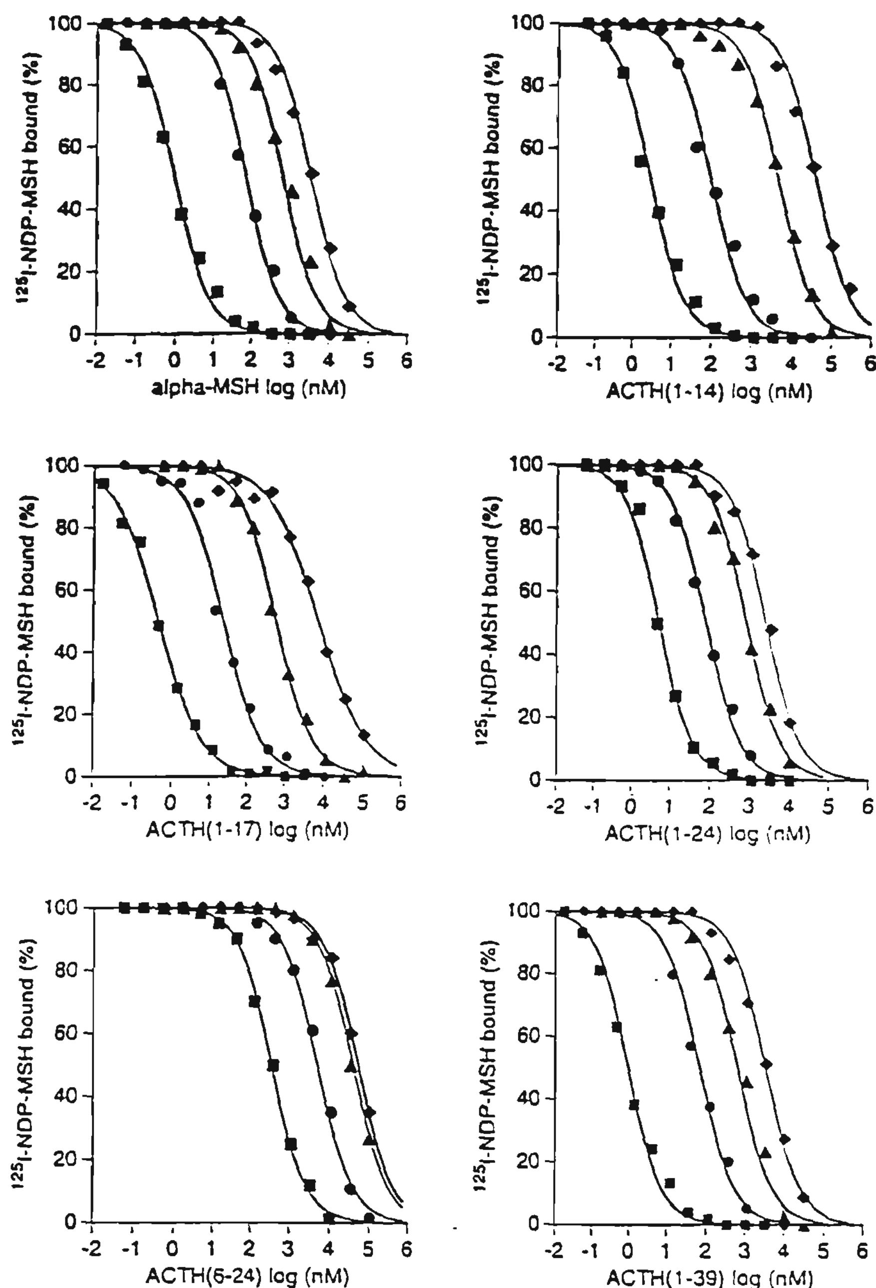

Figure 1 Competition curves of different ACTH/MSH analogues obtained on COS-1 cells transfected with the MC1 (■), MC3 (●), MC4 (▲) or MC5 (◆) receptor clones, obtained by using a fixed concentration of ~2 nM [^{125}I][Nle^{4},D-Phe^{7}]α-MSH (^{125}I-NDP-MSH) and varying concentrations of the non-labelled competing peptide. Competing peptides are indicated on the abscissa of each panel.

receptor, while the amino acids Lys^{15}-Lys^{16}-Arg^{17}-Arg^{18} play a crucial role for the binding of the peptide to the ACTH receptor (Hofmann *et al.* 1970, Schwyzer 1977, Buckley & Ramachandran 1981). The other MC receptors bind to the natural α-, β-, γ-MSH peptides (which all share the common core: His^{6}-Phe^{7}-Arg^{8}-Trp^{9}) with different relative affinities, indicating that the N-, and/or the C-terminus are important for determining the specificity of the binding of these peptides (Adan *et al.* 1994, Miwa *et al.* 1995, Schiöth *et al.* 1995, 1996*b*).

There are three naturally occurring forms of α-MSH: desacetyl-, monoacetyl- and diacetyl-α-MSH. Our data show that N-terminal acetylation has an important role for the binding of the melanocortic peptides to the MC

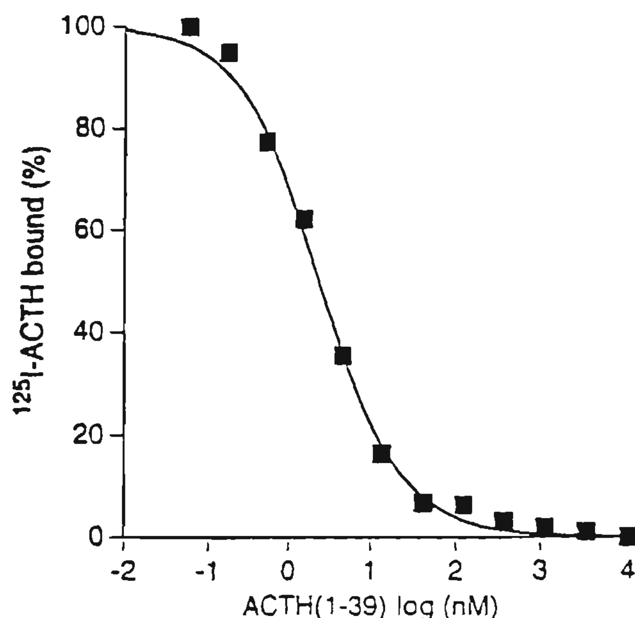

Figure 2 Competition curve of ACTH(1–39) obtained on COS-1 cells transfected with the MC1 receptor clone, obtained by using a fixed concentration of 0·1 nM (3-[^{125}I]iodo-tyrosyl^{23})-ACTH(1–39).

receptors. Diacetylation lowers the affinity of α-MSH for all the MC receptors, while the desacetyled forms of ACTH(1–13), ACTH(1–14) and ACTH(1–17) seem to favour the MC3 receptor, compared with the acetylated peptides. These data thus indicate that the natural desacetylated form of ACTH peptides favours the MC3 receptor even though the affinity differences between acetylated and desacetylated peptides are not very high.

The data indicate that the C-terminal amide form of the ACTH(1–13) is favoured by all the receptors, compared with the free carboxyl form. This effect disappears when the C-terminus is extended by three or more residues. Extension of the ACTH/MSH peptide from 13 to 24 amino acids at the C-terminus seems not to affect binding to any of the receptors. Further extension, to a full length ACTH(1–39), resulted in lower affinity especially for the MC1 receptor, which may indicate that the amino acid sequence 25–39 causes a steric hindrance. When labelled ACTH was used on the MC1 receptor, similar K_i values were obtained for ACTH(1–39) as when labelled [Nle^4,D-Phe^7]α-MSH was used. Moreover, labelled ACTH did not identify an additional site on the MC3, MC4 or MC5 receptors beyond that which could be detected by labelled [Nle^4,D-Phe^7]α-MSH. Taken together, the data show that the MC1, MC3, MC4 or MC5 receptors do not have a specific binding epitope for the sequence of ACTH beyond the α-MSH sequence. We have earlier shown that the MC2 receptor does not bind to the MSH peptides (Schiöth *et al.* 1996*a*) and our present data thus indicate an additional pharmacological distinction between the MC2 receptor and the other MC receptors.

The apparent absence of the MC2 receptors in the hypothalamus, together with the fact that α-MSH can inhibit CRF release indicates that some other MC receptor mediates the short-loop inhibition of CRF release in the hypothalamus. The MC1 receptor is an unlikely candidate, despite the fact that it has higher affinity for both α-MSH and ACTH(1–39) than the other MC receptors. This is because the MC1 receptor has only been found in limited areas (periaqueductal grey) of the rat and human brains (Xia *et al.* 1995). On the other hand the MC4 and the MC5 receptors show very low affinity for all the peptides, something which is specially true for the MC5 receptor. The present study shows that the MC3 receptor has more than 10-fold higher affinity for the ACTH peptides than the MC4 receptor. Earlier anatomical localisation data, which show the presence of both MC3 and MC4 receptors in the hypothalamus, and the present data showing a relatively high affinity of the MC3 receptor for the ACTH peptides, compared with the MC4 receptor, and the preference of the MC3 receptor for the desacetylated peptides, makes the MC3 receptor a good candidate for the mediator of the short-loop regulation of CRF release caused by the melanocortic peptides.

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Short communication

Binding of cyclic and linear MSH core peptides to the melanocortin receptor subtypes

Helgi B. Schiöth [a,*], Ruta Muceniece [b], Monika Larsson [a], Felikss Mutulis [a], Michael Szardenings [a], Peteris Prusis [a], Gunnar Lindeberg [c], Jarl E.S. Wikberg [*,a]

[a] *Department of Pharmaceutical Pharmacology, Biomedical Center, Uppsala University, Box 591, 751 24 Uppsala, Sweden*
[b] *Laboratory of Pharmacology, Institute of Organic Synthesis, Riga, Latvia*
[c] *Department of Medicinal and Physiological Chemistry, Uppsala University, Uppsala, Sweden*

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Abstract

We report here the binding of 5-, 6- and 7-amino-acid-long linear and cyclic core peptides of MSH (melanocyte-stimulating hormone) to cells transiently expressing the human melanocortin MC_1, MC_3, MC_4 and MC_5 receptors. The results show that in contrast to the natural peptides, the core peptides did not differentiate between the melanocortin MC_3 and MC_4 receptors. All tested cyclic peptides had much lower affinities than their corresponding linear homologues. Interestingly, the relative loss of binding due to the cyclisation did not change as the ring size decreased. Therefore, decreasing the ring size does not seem to force the peptide into a more unfavourable conformation.

Keywords: Melanocortin receptor subtype; MSH (melanocyte-stimulating hormone); Ligand binding

1. Introduction

Five different melanocortin receptor subtypes have been identified by us and others by use of molecular cloning (Chhajlani et al., 1993; Chhajlani and Wikberg, 1992; Gantz et al., 1993a,b; Mountjoy et al., 1992). The presence of different subtypes of the melanocortin receptors may explain the variety of effects that are caused by melanocortic peptides (Eberle, 1988). The five melanocortin receptors show unique affinities for the melanocortic peptides (Low et al., 1994; Siegrist and Eberle, 1995; Schiöth et al., 1995, 1996a,b,c). The melanocortin MC_1 receptor shows high affinity for α-MSH (melanocyte-stimulating hormone), but lower affinities for β-MSH, γ-MSH and ACTH (adrenocorticotropin). The melanocortin MC_2 receptor (the ACTH receptor) binds ACTH with high affinity, but it does not bind the MSH peptides. The melanocortin MC_3 receptor shows slightly higher affinity for γ-MSH compared to β-MSH and α-MSH. The melanocortin MC_4 receptor shows slight preference for β-MSH over α-MSH and a very low affinity for γ-MSH. The melanocortin MC_5 receptor, finally, shows the same potency order for the MSH peptides as the melanocortin MC_1 receptor, although (at least for the human case) it binds the peptides with much lower affinities.

Early structure-activity studies using pigment dispersing activity of melanophores (presumed melanocortin MC_1 receptor mediated effect) identified the His-Phe-Arg-Trp as the core sequence for melanotropic activity (Eberle, 1988). This core is shared by the natural melanocortic peptides: α-MSH, β-MSH, γ-MSH and ACTH.

Currently, there are selective substances available for the MC_1 receptor subtype, like α-MSH and [Nle^4,D-Phe^7]α-MSH, but there are only few reports on specific analogues for the other subtypes. Cyclic lactam analogues (Hruby et al., 1995) and ACTH-(4–10) analogues (Adan et al., 1994b) were reported to show certain selectivity. More basic knowledge is needed about the binding of the different receptor subtypes to different regions and conformations of the MSH peptides to elucidate the subtype specific properties and to allow construction of selective compounds. Docking of long peptides into molecular models of the melanocortin receptors is simplified by adding a constraint (e.g. by formation of a ring) into the peptide ligand. A cyclic [Cys^4,D-Phe^7,Cys^{10}]α-MSH was used for

* Corresponding authors. Fax: (46-18) 559-718; e-mail: helgis@bmc.uu.se; jwikberg@bmc.uu.se

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ligand docking to a preliminary 3-dimensional model of the melanocortin MC_1 receptor (Prusis et al., 1995). Binding data for small cyclic MSH analogues should facilitate the interpretation of results from ligand docking experiments.

The aim of the present study was therefore to synthesise new cyclic and linear analogues based on the core MSH peptide and test the subtype specific binding using the human melanocortin MC_1, MC_3, MC_4 and MC_5 receptors transiently expressed in an eukaryotic cell line.

2. Materials and methods

2.1. Chemicals

[Nle^4,D-Phe^7]α-MSH (Sawyer et al., 1980) was purchased from Saxon Biochemicals, Germany. [Nle^4,D-Phe^7]α-MSH was radioiodinated by the chloramine T method and purified by HPLC (high performance liquid chromatography). His-(D-Phe)-Arg-Trp-Gly-NH_2 (HdFAWG), Asn-His-(D-Phe)-Arg-Trp-Gly-NH_2 (NHdFAWG), Met-Asn-His-(D-Phe)-Arg-Trp-Gly-NH_2 (MNHdFAWG) and their corresponding cyclic homologues, i.e., $HdFAWG_c$, $NHdFAWG_c$ and $MNHdFAWG_c$, were synthesised in our laboratories using the solid phase approach and purified by HPLC. The correct molecular weights of the peptides were confirmed by mass spectrometry. His-Phe-Arg-Trp-OH (HFAW) was purchased from Bachem, Switzerland. In the text, we use the position numbering of α-MSH: Ser^1-Tyr^2-Ser^3-Met^4-Glu^5-His^6-Phe^7-Arg^8-Trp^9-Gly^{10}-Lys^{11}-Pro^{12}-Val^{13}.

2.2. Expression of receptor clones

The human melanocortin MC_1 and human melanocortin MC_5 receptors (Chhajlani and Wikberg, 1992; Chhajlani et al., 1993) were cloned into the expression vector pRc/CMV (InVitrogen). The human melanocortin MC_3 and human melanocortin MC_4 receptors, cloned into the expression vector pCMV/neo, were a gift from Dr. Ira Gantz (Gantz et al., 1993a,b). For receptor expression COS-1 (CV-1 Origin, SV40) cells were grown in Dulbecco's modified Eagle's medium with 10% foetal calf serum. Eighty percent confluent cultures were transfected with the DNA mixed with liposomes in serum-free medium (for details, see Schiöth et al., 1996b). After transfection, the serum-free medium was replaced with the serum-containing medium and the cells were cultivated for about 48 h. Cells were then scraped off, centrifuged, and used for radioligand binding.

2.3. Binding studies

The transfected cells were washed with binding buffer (see Schiöth et al., 1995) and distributed into 96-well plates. The cells were then incubated for 2 h at 37°C with 0.05 ml binding buffer in each well containing a constant concentration of [^{125}I][Nle^4,D-Phe^7]α-MSH and appropriate concentrations of an unlabelled ligand. After incubation the cells were washed with 0.2 ml of ice-cold binding buffer and detached from the plates with 0.2 ml of 0.1 M NaOH. Radioactivity was counted (Wallac, Wizard automatic gamma counter) and data analysed with BindAid, a software package (Wan System, Umeå, Sweden). Data were either analysed by fitting them to formulas derived from the law of mass action by the method generally referred to as computer modelling, or by fitting to the four-parameter logistic function. K_i values were calculated by using the Cheng and Prusoff equation. The K_d values for [^{125}I][Nle^4,D-Phe^7]α-MSH for the melanocortin MC_1, melanocortin MC_3 and melanocortin MC_5 receptors were taken from Schiöth et al. (1995) and for melanocortin MC_4 from Schiöth et al. (1996b). The binding assays were performed in duplicate wells and repeated three times. Untransfected COS-1 cells did not show any specific binding to [^{125}I][Nle^4,D-Phe^7]α-MSH.

3. Results

In order to probe the binding of the core of the MSH peptides to the melanocortin receptor we synthesised and evaluated three linear peptides, and three corresponding cyclic homologues. The smallest peptide was 5 amino-acids long that included the main MSH core: His^6-(D-Phe^7)-Arg^8-Trp^9 and Gly^{10}. The other peptides had in addition Asn^5 or Met^4-Asn^5 at the N-terminus. We used the D-isomer of Phe^7 as these peptides have higher affinity compared to L-Phe-containing compounds, as high affinity was a prerequisite to obtain reliable data. It may be pointed out here that [Nle^4,D-Phe^7]α-MSH is a peptide that shows the highest affinities of all ligands for all the melanocortin receptor subtypes, except for the melanocortin MC_2 receptors. According to earlier structure-activity data (Eberle, 1988), and reports of alanine scanning of the MSH peptide on the murine melanocortin MC_1 and the rat melanocortin MC_3 receptors (Sahm et al., 1994a,b), the Glu^5 and the Gly^{10} on each side of the MSH peptide main core are not very important for ligand binding. We included the Gly^{10} but replaced Glu^5 with Asn^5 to facilitate the synthesis of the $NHdFAWG_c$ and $MNHdFAWG_c$. The ring closure of the cyclic peptides was created by ordinary peptide bonds.

The K_i values for the linear and cyclic peptides were evaluated in competition with [^{125}I][Nle^4,D-Phe^7]α-MSH on melanocortin MC_1, MC_3, MC_4 and MC_5 receptor clones and the data obtained are given in Table 1. In Table 1 is also given the K_i value for [Nle^4,D-Phe^7]α-MSH obtained from our previous studies for comparison. The assays were performed using the same approach as we used earlier for the evaluation of several natural melanocortic peptides (Schiöth et al., 1995, 1996b). Com-

Table 1
K_i values (mean ± S.E.M.) obtained from competition curves, for MSH analogues on human melanocortin MC_1, MC_3, MC_4 and MC_5 receptor transfected COS-1 cells together with relative affinity ratios

Ligand	K_i (nmol/l)				MC_3/MC_1	MC_4/MC_1	MC_5/MC_1
	MC_1	MC_3	MC_4	MC_5			
[^{125}I]NDP-MSH [a]	0.0851 ± 0.0080 [b]	0.396 ± 0.065 [b]	3.84 ± 0.57 [c]	5.05 ± 1.00 [b]	4.6	45	59
MNHdFRWG	2.79 ± 0.08	350 ± 120	285 ± 34	962 ± 250	130	100	340
MNHdFRWG$_c$	355 ± 65	14600 ± 2700	8400 ± 1000	58900 ± 3200	41	24	170
NHdFRWG	399 ± 78	7220 ± 870	6110 ± 1300	24100 ± 12000	18	15	60
NHdFRWG$_c$	2730 ± 270	103000 ± 17000	73300 ± 13000	208000 ± 39000	38	27	76
HdFRWG	292 ± 53	7760 ± 1900	7170 ± 980	61200 ± 28000	27	25	210
HdFRWG$_c$	3210 ± 320	113000 ± 21000	71400 ± 9300	207000 ± 45000	35	22	64
HFRW	69300 ± 19000	> 300000	> 300000	> 300000	–	–	–
MNHdFRWG$_c$/MNHdFRWG	130	42	30	61	0.31	0.24	0.50
NHdFRWG$_c$/NHdFRWG	6.8	14	12	8.6	2.1	1.8	1.3
HdFRWG$_c$/HdFRWG	11	15	10	3.4	1.3	0.88	0.30

[a] K_d values. Data taken from [b] Schiöth et al. (1995) and [c] Schiöth et al. (1996b).

petition curves for MNHdFRWG, MNHdFRWG$_c$, NHdFRWG, NHdFRWG$_c$, HdFRWG and HdFRWG$_c$ are shown in Fig. 1.

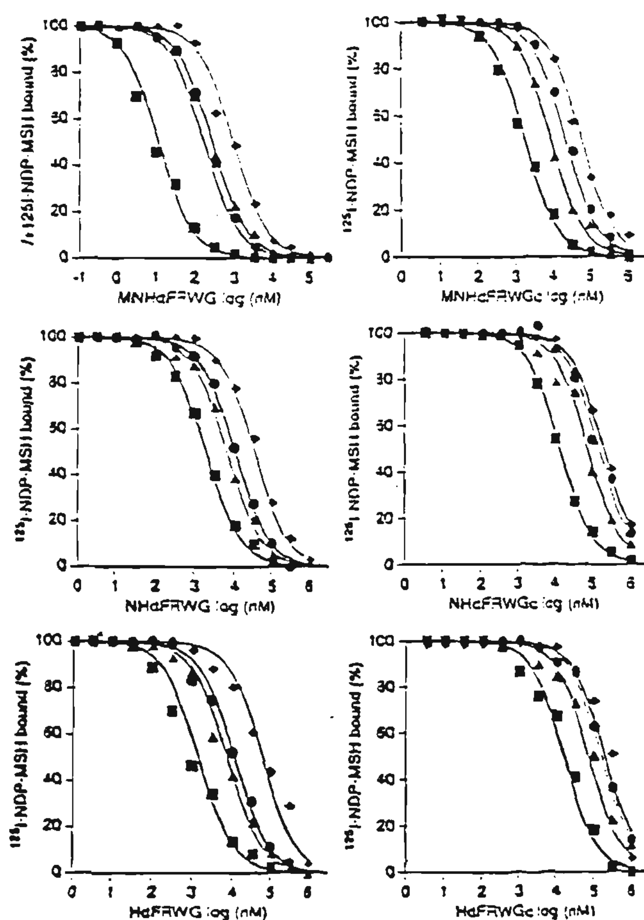

Fig. 1. Competition curves of MNHdFRWG, MNHdFRWG$_c$, NHdFRWG, NHdFRWG$_c$, HdFRWG, HdFRWG$_c$ on COS-1 cells transfected with the MC_1 (■), MC_3 (●), MC_4 (▼), or MC_5 (◆) receptor obtained by using a fixed concentration of ~ 2 nM [^{125}I][Nle^4,D-Phe^7]α-MSH and varying concentrations of the non-labelled competing peptide. Competing peptides used are indicated on the abscissa for each panel.

As can be seen from Table 1 the melanocortin MC_1 receptor shows the highest affinity for all the tested ligands. The melanocortin MC_3 receptor shows 18–130-fold lower affinity for the ligands compared to the melanocortin MC_1 receptor. The melanocortin MC_4 receptor shows similar or slightly higher affinities for the ligands, compared to the melanocortin MC_3 receptor. The melanocortin MC_5 receptor shows overall lower affinities for all the tested peptides.

Thus, the melanocortin MC_3 and the melanocortin MC_4 receptors show very similar affinities to the core peptides. This contrasts quite strongly to our recent results that show that the melanocortin MC_3 receptor has a 10-fold higher affinity for [Nle^4,D-Phe^7]α-MSH, a 30-fold higher affinity for α-MSH and an about 6000-fold higher affinity for γ-MSH, compared with the melanocortin MC_4 receptor.

In all cases, the cyclic peptides have lower affinities than the corresponding linear homologues. The difference is largest for the 7-amino-acid-long cyclic and linear peptides, especially in the case of the melanocortin MC_1 receptor. The affinity differences between the 6- and 5-amino-acid-long cyclic and linear peptides are in the same order of magnitude for all the receptors. Moreover, the 5- and the 6-amino-acid-long peptides show very similar affinities. Thus, the addition of the Asn^5 does not affect the affinities of the peptide for the different receptors.

In addition to the tests described above we also evaluated the binding of the α-MSH main core HFRW, which has an L-Phe^7, instead of the D-Phe^7 that was used for all the other core peptides evaluated in this study. As expected, HFRW showed much lower affinities for all the melanocortin receptors compared to the D-Phe^7 peptides.

4. Discussion

Each of the melanocortin receptor subtypes binds the natural melanocortic peptides with specific affinity. It is

not known how different these receptors bind the core of the MSH peptide (i.e. His[5]-Phe[7]-Arg[8]-Trp[9]). There is evidence that the C- and/or the N-terminal side chains may play an important role in determining the subtype specific binding, as the γ-MSH has much higher affinity for the MC_3 than for the MC_4 receptor (Adan et al., 1994a; Miwa et al., 1995; Schiöth et al., 1995, 1996b). Elaboration of the MSH core residues resulted in specific antagonists for the different subtypes (Adan et al., 1994b; Hruby et al., 1995). Cyclic MSH analogues were synthesised by Hruby and co-workers more than 15 years ago. A classical example of these compounds is the disulfide bridged cyclic [Cys[4],Cys[10]]α-MSH which shows high potency and prolonged biological activity (Sawyer et al., 1982). It was proposed that a β turn or other reversed chain structure of the main core could contribute to the bioactivity of the MSH peptide.

Our present data show that the melanocortin MC_1 receptor shows the highest affinities, the melanocortin MC_3 and MC_4 receptors intermediate affinities, and the melanocortin MC_5 receptor low affinities for the core MSH peptides. These data thus clearly indicate that differences in core peptide binding may also have a role for the high, intermediate and low affinities of the melanocortin receptor subtypes for the natural MSH peptides. However, it was a very interesting observation that the melanocortin MC_3 and MC_4 receptors show very similar affinities to all the evaluated core peptides, and these data may thus indicate that the C- and/or N-terminal parts of the natural MSH peptides are the sites responsible for the selectivities of α-, β- and γ-MSH for the melanocortin MC_3 and MC_4 receptors.

That Met[4] along with the His[5], Phe[7], Arg[8] and Trp[9] are the most important amino acids for receptor binding in the MSH peptide is supported by our present data which show that the addition of Met[4] to the linear NHdFRWG leads to a more than 100-fold higher affinity for the melanocortin MC_1 receptor, and about 20-fold higher affinity for the other subtypes. The importance of the Met[4] was shown in early structure-activity studies (Eberle, 1988), as well as by alanine scanning of the α-MSH peptide (Sahm et al., 1994a,b).

The cyclic analogues of the present study show lower affinities for the melanocortin receptor subtypes compared to their linear homologues. This may be due to constraint in the cyclic peptides or that free N- and/or C-termini are important for the binding. It is notable that the differences in affinities between the cyclic and linear peptides (displayed as relative ratios in Table 1), are not decreased as the rings become larger. One might have anticipated that an increase in the size of the ring would result in greater flexibility and allow the peptide to take a more optimal binding conformation. However, cyclisation of the 7-amino-acid-long core peptide led to a relatively larger loss of binding affinities than was observed for the 6- and 5-amino-acid-long peptides (Table 1). The ring closure in the $MNHdFRWG_c$ is based on a peptide bond which connects the Met[4] to Gly[10]. As can be observed in Table 1 the addition of a Met[4] leads in the linear case to a drastic increase in affinities. One plausible explanation for the low affinity of the cyclic $MNHdFRWG_c$ might therefore be that cyclisation forces the peptide to an unfavourable conformation for Met[4] interactions with the receptors. Another very interesting observation was that the cyclic 6- and 5-amino-acid-long peptides show virtually identical affinities for the respective melanocortin receptor subtype. Besides indicating that Asn[5] does not have any important role in the binding of the ligand (cf., also the results for 5- and 6-amino-acid-long linear peptides), this shows that the decreasing ring size does not force the peptide into unfavourable conformations for binding to the receptors. Nevertheless, still further synthesis of MSH peptides, molecular modelling of receptor structure and mutagenesis studies will be necessary to achieve full insight into the mechanisms for the binding of peptides to the different melanocortin receptor subtypes.

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Discovery of novel melanocortin$_4$ receptor selective MSH analogues

[1,4]Helgi B. Schiöth, [1,2]Felikss Mutulis, [1,3]Ruta Muceniece, [1]Peteris Prusis & [1,4]Jarl E.S. Wikberg

[1]Department of Pharmaceutical Pharmacology, Uppsala University, Uppsala, Sweden, [2]Department of Medicinal Chemistry and [3]Laboratory of Pharmacology, Institute of Organic Synthesis, Riga; Latvia

1 We synthesized a novel series of cyclic melanocyte stimulating hormone (MSH) analogues and tested their binding properties on cells transiently expressing the human melanocortin$_1$ (MC$_1$), MC$_3$, MC$_4$ and MC$_5$ receptors.

2 We discovered that compounds with 26 membered rings of [Cys^4,D-Nal^7,Cys^{11}]α-MSH(4–11) displayed specific MC$_4$ receptor selectivity. The preference order of the different MC receptor subtypes for the novel [Cys^{4}D-Nal^{7}Cys^{11}]α-MSH(4–11) analogues are distinct from all other known MSH analogues, particularly as they bind the MC$_4$ receptor with high and the MC$_1$ receptor with low relative affinities.

3 HS964 and HS014 have 12 and 17 fold MC$_4$/MC$_3$ receptor selectivity, respectively, which is much higher than for the previously described cyclic lactam and [Cys^4,Cys^{10}]α-MSH analogues SHU9119 and HS9510.

4 HS964 is the first substance showing higher affinity for the MC$_5$ receptor than the MC$_1$ receptor.

5 HS014, which was the most potent and selective MC$_4$ receptor ligand (K_i 3.2 nM, which is ~300 fold higher affinity than for α-MSH), was also demonstrated to antagonize α-MSH stimulation of cyclic AMP in MC$_4$ receptor transfected cells.

6 We found that a compound with a 29 membered ring of [Cys^3,Nle^{10},D-Nal^7,Cys^{11}]α-MSH(3–11) (HS010) had the highest affinity for the MC$_3$ receptor.

7 This is the first study to describe ligands that are truly MC$_4$ selective and a ligand having a high affinity for the MC$_3$ receptor. The novel compounds may be of use in clarifying the physiological roles of the MC$_3$, MC$_4$ and MC$_5$ receptors.

Keywords: Melanocortin (MC) receptor subtypes; MSH (melanocyte stimulating hormone); cyclic MSH analogues; ligand binding; cyclic AMP; HS964; HS014

Introduction

The melanocortic peptides, ACTH (adrenocorticotropin) and MSH (melanocyte stimulating hormone), are primarily known for their role in the regulation of adrenal steroid production (Simpson & Waterman, 1988), and skin pigmentation (Eberle, 1988). The MSH and ACTH peptides also induce a variety of central and peripheral effects. The melanocortins have, for example, been shown to affect memory, behaviour, inflammation, pyretic control, pain perception, blood pressure, nerve growth and regeneration, and to influence events surrounding parturition (Eberle, 1988; O'Donahue & Dorsa, 1982).

Molecular cloning of five melanocortin receptor subtypes (MC$_1$–MC$_5$) (Chhajlani & Wikberg, 1992; Mountjoy *et al.*, 1992; Gantz *et al.*, 1993a,b; Chhajlani *et al.*, 1993) has provided tools for systematic studies of the molecular mechanisms underlying the above mentioned effects. The natural hormones are not known to be subtype selective, except that α-MSH is selective for the MC$_1$ receptor and ACTH is selective for the MC$_2$ receptor. The MC$_2$ receptor is distinguishable from the other MC receptor subtypes in that it does not bind to the MSH peptides. The MC$_3$ receptor has a slight preference for the γ-MSH, and the MC$_4$ receptor for β-MSH. Still these natural hormones are not selective for these MC receptor subtypes as they both have highest affinity for the MC$_1$ receptor (Schiöth *et al.*, 1995; 1996a,b; Siegrist & Eberle, 1995).

The physiological roles of the newly discovered receptors are not fully known but it has been speculated that they might participate in eliciting the various peripheral and central effects of melanocortins. The MC$_3$ receptor is mainly expressed in the brain, but it is also present in the periphery where it has been found in the placenta, gut tissues and the human heart (Gantz *et al.*, 1993a; Chhajlani, 1996). The MC$_4$ receptor is found only in the central nervous system, where it is widely distributed, including in the cortex, thalamus, hypothalamus, brain stem and spinal cord (Gantz *et al.*, 1993b; Mountjoy *et al.*, 1994). Recent findings with knock-out techniques (Huszar *et al.*, 1997) and i.c.v. injections of the cyclic MSH analogues SHU9119 and MTII (Fan *et al.*, 1997) relate the MC$_4$ receptor to feeding behaviour and weight homeostasis. The MC$_5$ receptor is also found in the brain but, more importantly, it has a wide peripheral distribution, although its physiological role is still much less well defined (Labbé *et al.*, 1994; Barrett *et al.*, 1994).

The demand for substances that discriminate between the newly discovered MC receptors is high as such compounds would help to clarify the physiological roles of these receptors. MC$_4$ receptor-selective substances might also have a potential in the treatment of eating disorders (e.g. overweight, anorexia and bulimia) where good pharmacological remedies are presently not available.

In this study, we designed and synthesized a novel series of cyclic MSH analogues and tested these on cells expressing the cloned human MC$_1$, MC$_3$, MC$_4$ and MC$_5$ receptors.

[4]Authors for correspondence at: Department of Pharmaceutical Pharmacology, Biomedical Center, Box 591, 751 24 Uppsala, Sweden.

Methods

Peptide synthesis

The peptides tested in this study (except NDP-MSH) were synthesized by use of the solid phase approach and purified by high performance liquid chromatography (h.p.l.c). The correct molecular weights of the peptides were confirmed by mass spectrometry. The peptide sequences were assembled with a Pioneer peptide synthesis system (PerSeptive Biosystems). Fmoc (9-fluorenylmethoxycarbonyl)-amino acid derivatives were used in coupling steps. When OPfp (pentafluorophenyl) esters were used the synthesis cycle was as follows: (a) the Fmoc group was removed by 20% piperidine in DMF (N,N-dimethylformamide) (5 min); (b) to form a new peptide bound side chain protected Fmoc-amino acid OPfp ester (4 eq.) and HOAt (1-hydroxy-7-azabenzotriazole) (4 eq.) were dissolved in DMF and circulated through the reaction column for 30–60 min; (c) to cap residual amino groups the support was treated with 0.3 M Ac_2O (acetic anhydride) in DMF for 5 min. If free acids were used only step (b) was different: side chain protected Fmoc-amino acid (4 eq.), HATU (O-[7-azabenzotriazol-1-yl]-1,1,3,3-tetramethyluronium hexafluorophosphate) (4 eq.) and DIEA (N,N-diisopropylethylamine) (4 eq.) were applied. For the deprotection a reagent mixture (TFA (trifluoroacetic acid)-phenol-anisole-1,2-ethanedithiol-water, 92:2:2:2:2) was used for 2.5 h. The S-S bond was formed by dissolving the product in a minimal amount of DMSO (dimethylsulphoxide) and heating at 65°C under argon for 36 h. The raw peptides were purified by h.p.l.c. (10 × 250 mm Vydac RP C18, 90A 201HS1010 column eluted with 20–30% MeCN (acetonitrile) in water + 0.1% TFA and detection at 240 nm).

Expression of receptor clones

The human MC_1 and human MC_5 receptor (Chhajlani & Wikberg, 1992; Chhajlani *et al.*, 1993) were cloned into the expression vector pRc/CMV (InVitrogen). The human MC_3 and human MC_4 receptor DNAs, cloned into the expression vector pCMV/neo, were gifts from Dr Ira Gantz (Gantz *et al.*, 1993a,b). For receptor expression, COS-1 (*C*V-1 *O*rigin, *S*V40) cells were grown in Dulbecco's modified Eagle's medium with 10% foetal calf serum. Eight percent confluent cultures were transfected on 100 mm cell culture dishes with the DNA (approximately 1 μg DNA for every 1×10^6 cells) mixed with liposomes in serum free medium (for details see Schiöth *et al.*, 1996b). After transfection, the serum-free medium was replaced with growth medium and the cells were cultivated for about 48 h. Cells were then scraped off, centrifuged and used for radioligand binding.

Binding studies

The transfected cells were washed with binding buffer (see Schiöth *et al.*, 1995) and distributed into 96 well plates. The cells were then incubated for 2 h at 37°C (Siegrist *et al.*, 1989) with 0.05 ml binding buffer in each well, containing a constant concentration of [^{125}I]-NDP-MSH and appropriate concentrations of an unlabelled ligand. After incubation the cells were washed with 0.2 ml of ice-cold binding buffer and detached from the plates with 0.2 ml 0.1 N NaOH. Radioactivity was counted (Wallac, Wizard automatic gamma counter) and data analysed with a software package for radio ligand binding analyses (Wan System, Umeå, Sweden). Data were analysed by fitting it to formulae derived from the law of mass-action by the method generally referred to as computer modelling. The binding assays were performed in duplicate wells and repeated three times. Untransfected COS-1 cells did not show any specific binding to [^{125}I]-NDP-MSH. The non specific binding was less than 5% of the total binding at 2 nM [^{125}I]-NDP-MSH when determined in presence of 3 μM unlabelled NDP-MSH.

Cyclic AMP assay

The transfected cells and the melanoma B16 cells (cultivated in the same media as the COS cells (see above)) were harvested and incubated for 30 min at 37°C with 0.05 ml serum free Dulbecco's modified Eagle's medium in each tube, containing 0.5 mM IBMX (isobutylmethylxantine) and appropriate concentrations of α-MSH or HS014. After incubation with the indicated drugs, cyclic AMP (adenosine 3':5'-cyclic monophosphate) was extracted with perchloric acid at final concentration 0.4 M. After centrifugation, the protein free supernatants were neutralized with 5 M KOH/1 M Tris (tris-(hydroxymethyl)aminomethane). Then 0.05 ml of the neutralized cyclic AMP extract or a cyclic AMP standard (dissolved in distilled water) was added to a 96 well microtitre plate. The content of cyclic AMP was then estimated essentially according to Nordstedt & Fredholm (1990), by adding to each well [^{3}H]-cyclic AMP (0.14 pmol, approximately 11,000 c.p.m., specific activity 54 Ci $mmol^{-1}$, Amersham) and bovine adrenal binding protein and incubating at 4°C for 150 min. Standards containing unlabelled cyclic AMP were also assayed concomitantly with the samples. The incubates were thereafter harvested by filtration on Whatman GF/B filters by a semiautomatic Brandel cell harvester. Each filter was rinsed with 3 ml 50 mM Tris/HCl pH 7.4. The filters were punched out and put into scintillation vials with scintillation fluid and counted. The cyclic AMP assays were performed in duplicate wells and repeated three times.

Chemicals

[Nle^4,D-Phe^7]α-MSH (NDP-MSH) (Sawyer *et al.*, 1980) (Bachem, Switzerland) was radio iodinated by the chloramine T method and purified by h.p.l.c. β-Cyclohexyl-D-alanine (D-Cha), p-benzoyl-D-phenylalanine (D-Bpa) and β-(2-naphthyl)-D-alanine (D-Nal) were purchased from Bachem, Switzerland. All other amino acid derivatives and chemicals for peptide synthesis (unless specified otherwise) were purchased from PerSeptive Biosystems (Kebo, Sweden). All medium and serum for cell cultivation were purchased from Gibco-BRL (Life Technologies, Sweden). All other chemicals were purchased from Sigma-Aldrich (Sweden), unless specified otherwise.

Results

We designed and synthesized a new series of cyclic MSH analogues which have a disulphide bridge between Cys residues in position 4 and 11. This ring includes an extra Gly in position 10 as compared to the earlier known cyclic [Cys^4,Cys^{10}]α-MSH analogues. This ring includes 8 amino acids or totally 26 atom members when the slide chains of Cys are accounted. The structure of the novel substances are aligned with α-MSH, NDP-MSH, [Cys^4,Cys^{10}]α-MSH(1–13), HS9510, MTII and SHU9119 in Table 1. The human DNAs for the MC_1, MC_3, MC_4 and MC_5 receptors were transiently and independently expressed in COS-1 cells for competitive receptor binding with [^{125}I]-NDP-MSH as radioligand. The expression levels of the different receptor subtypes were similar

data not shown). The K_i values for the different peptides resulting from calculations of the competition curves of binding with [^{125}I]-NDP-MSH are summarized in Table 2. For comparison, we also included in Table 2 the K_i values for α-MSH, NDP-MSH, [Cys^4,D-Phe^7,Cys^{10}]α-MSH(1–13), HS9510, MTII and SHU9119, the values of which we recently demonstrated (Schiöth *et al.*, 1997a,b) by use of the same method as in the present study. Competition curves for HS963, HS964, HS007, HS010, HS012 and HS014 obtained on cells expressing the human MC_1, MC_3, MC_4 and MC_5 receptors are shown in Figure 2.

HS963, HS964, HS005 and HS006 are all [Cys^4,X^7,Cys^{11}]α-MSH(4–11) analogues and differ only in position 7. HS964, which contains D-Nal^7 (β-(2-naphthyl)-D-alanine) (Figure 1), exhibited the highest affinity for the MC_4 receptor and showed 12, 63 and 7 fold selectivity compared to the MC_1, MC_3 and MC_5 receptors, respectively. Moreover, HS964 showed a preferential order $MC_4 > MC_5 > MC_3 > MC_1$, which is a preference order not shared by any other known melanocortic peptide. HS963, which contains D-Phe^7, proved not to be MC_4 receptor-selective. It showed a 44 fold lower affinity for the MC_4 receptor than HS964 and also had much lower affinities for the MC_3 and the MC_5 receptors, but higher affinity for the MC_1 receptor. HS005, which contains

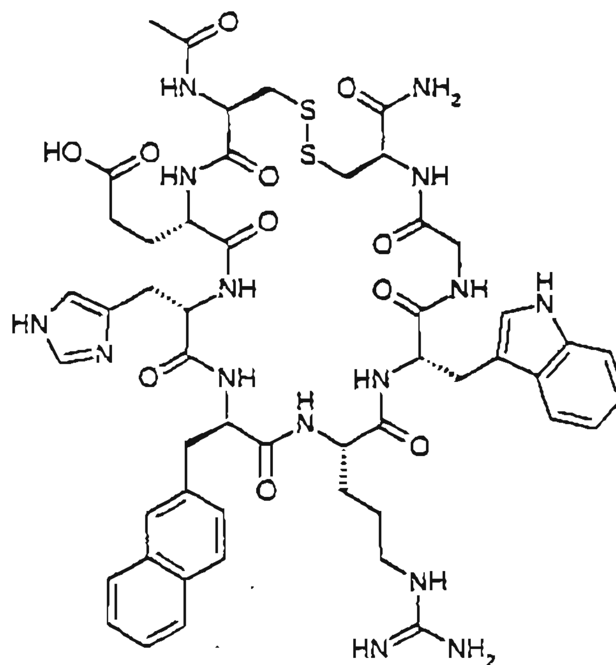

Figure 1 Structure of the MC_4 receptor-selective analogue: HS964 (cyclic [Cys^4,D-Nal^7,Cys^{11}]α-MSH(4–11)).

Table 1 Alignment of α-MSH, NDP-MSH, cyclic [Cys^4,Cys^{10}]α-MSH(1–13), HS9510, MTII and SHU9119 to the new analogues evaluated in this study

Peptide	*Position* 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
α-MSH	Ser	Tyr	Ser	Met	Glu	His	Phe	Arg	Trp	Gly	Lys	Pro	Val		
NDP-MSH	Ser	Tyr	Ser	Nle	Glu	His	D-Phe	Arg	Trp	Gly	Lys	Pro	Val		
HS9510				*Cys*	Glu	His	D-Nal	Arg	Trp	*Cys*					
MTII				Nle	*Asp*	His	D-Phe	Arg	Trp	*Lys*					
SHU9119				Nle	*Asp*	His	D-Nal	Arg	Trp	*Lys*					
HS964				*Cys*	Glu	His	D-Nal	Arg	Trp	Gly	*Cys*				
HS963				*Cys*	Glu	His	D-Phe	Arg	Trp	Gly	*Cys*				
HS005				*Cys*	Glu	His	D-Cha	Arg	Trp	Gly	*Cys*				
HS006				*Cys*	Glu	His	D-Bpa	Arg	Trp	Gly	*Cys*				
HS007				*Cys*	Arg	His	D-Nal	Arg	Trp	Gly	*Cys*				
HS009				*Cys*	Glu	His	D-Nal	Arg	Trp	Gly	*Cys*				
HS011				*Cys*	Glu	Ala	D-Nal	Arg	Trp	Asp	*Cys*				
HS010			*Cys*	Nle	Glu	His	D-Nal	Arg	Trp	Gly	*Cys*				
HS012			Nle	*Cys*	Glu	His	D-Nal	Arg	Trp	Gly	*Cys*				
HS014				*Cys*	Glu	His	D-Nal	Arg	Trp	Gly	*Cys*	Pro	Pro	Lys	Asp

All peptides have an acetyl-group on the N-terminus and an amide group on the C-terminus. The amino acid residues which make up the ring closure in the cyclic compounds are shown underlined in italics.

Table 2 K_i values of MSH analogues obtained from computer analysis of competition curves on human MC_1, MC_3, MC_4 and MC_5 receptor transfected COS cells

Ligand	MC_1	MC_3	MC_4	MC_5
α-MSH*	0.230 ± 0.089	31.5 ± 3.9	900 ± 97	7160 ± 860
NDP-MSH*	0.109 ± 0.010	0.469 ± 0.038	2.93 ± 0.34	5.50 ± 0.11
MTII*	0.686 ± 0.109	34.1 ± 4.4	6.60 ± 0.82	46.1 ± 7.9
SHU9119*	0.714 ± 0.161	1.20 ± 0.30	0.360 ± 0.059	1.12 ± 0.31
HS9510*	148 ± 48	216 ± 47	37.0 ± 5.2	5150 ± 550
HS964	1460 ± 440	281 ± 40	23.2 ± 4.4	164 ± 27
HS963	468 ± 109	3120 ± 610	1780 ± 220	6040 ± 700
HS005	10000 ± 4700	5880 ± 1280	1000 ± 230	7770 ± 1240
HS006	6490 ± 1940	6440 ± 1110	2760 ± 480	3440 ± 1210
HS007	200 ± 72	8.14 ± 3.55	10.1 ± 4.1	42.7 ± 9.4
HS009	69200 ± 36000	68800 ± 13000	5170 ± 920	91400 ± 24600
HS011	2030 ± 1060	17.1 ± 2.9	4.01 ± 1.48	405 ± 35
HS010	170 ± 38	48.5 ± 15.5	179 ± 58	253 ± 91
HS012	396 ± 170	140 ± 22	33.2 ± 6.3	120 ± 25
HS014	108 ± 62	54.4 ± 37.6	3.16 ± 1.16	694 ± 237

Data are presented as means ± s.e.mean ($n = 3$). *Data from Schiöth *et al.* (1997), by courtesy of the publisher.

D-Cha[7] (β-cyclohexyl-D-alanine), had much lower affinity than HS964 for all the MC receptors. Still, HS005 is MC_4 receptor-selective, as it had about 6 fold higher affinity for the MC_4 receptor than the MC_3 receptor. HS006, which contains D-Bpa[7] (p-benzoyl-D-phenylalanine), also had the highest relative affinity for the MC_4 receptor, although it showed much lower all over affinities than HS964 for all the MC receptors. HS006 showed about 2 fold selectivity over the MC_3 receptor, although it had 120 fold lower affinity for the MC_4 receptor compared to HS964.

Three of the peptides (HS007, HS009 and HS011) are based on HS964, but differ in positions 5, 6 and 10. HS007,

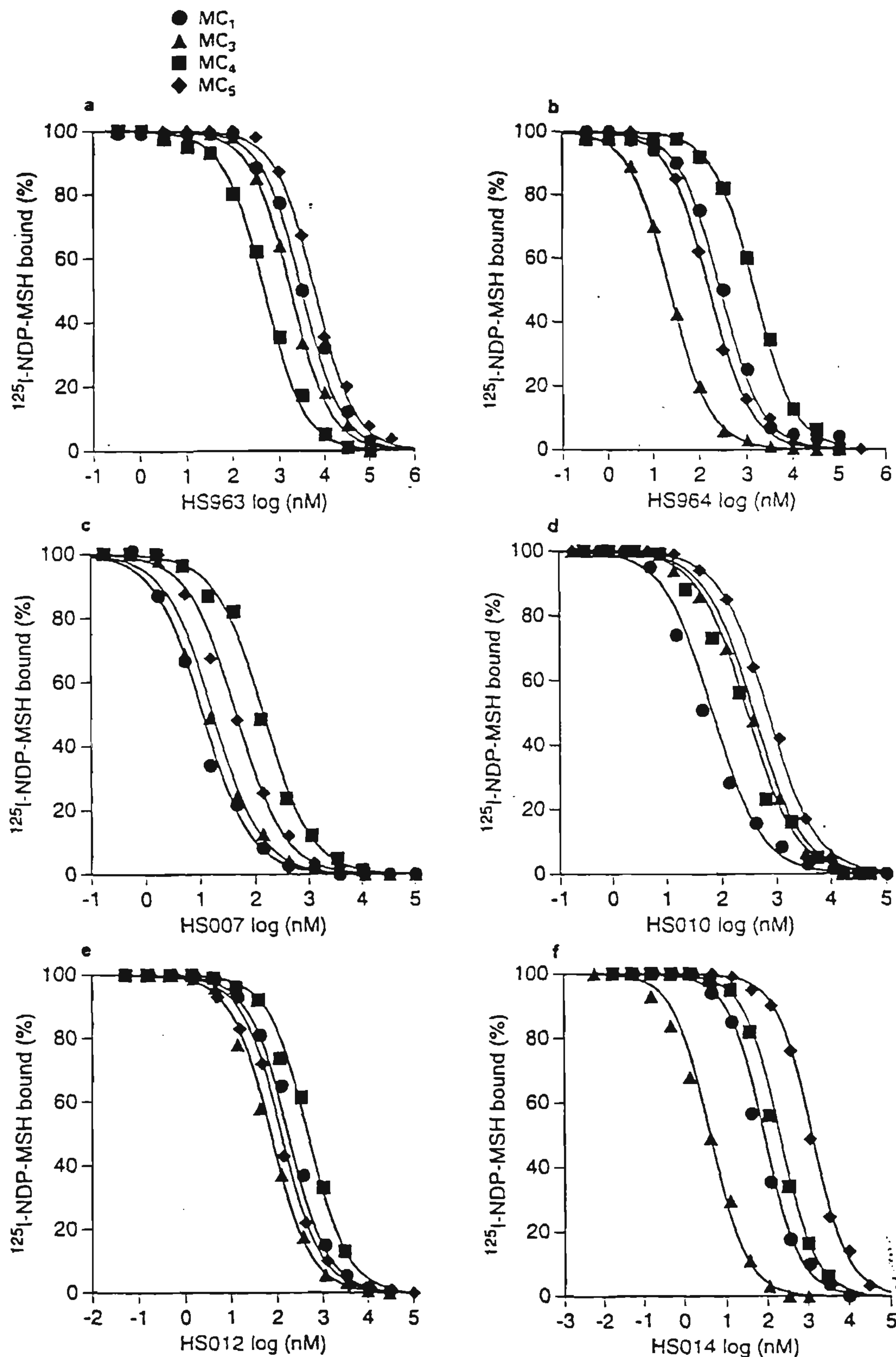

Figure 2 Competition curves for (a) HS963, (b) HS964, (c) HS007, (d) HS010, (e) HS012 and (f) HS014 obtained on COS-1 cells transfected with the MC_1, MC_3, MC_4 or MC_5 receptor clones, obtained by using a fixed concentration of [^{125}I]-NDP-MSH and varying concentrations of the unlabelled competing peptide. Competing peptides used are indicated on abscissa scale. Each experiment was performed in duplicate and repeated three times.

which the acidic hydrophilic Glu^5 is replaced by the basic hydrophilic Arg^5, had about 2 fold higher affinity for the MC_4 receptor than HS964. Interestingly, HS007 had a 35 fold higher affinity for the MC_3 receptor than HS964. Thus, HS007 had similar or slightly higher affinity for the MC_3 receptor than the MC_4 receptor. HS009, where the non polar Gly^{10} is replaced with the acidic hydrophilic Asp^{10}, had 13 fold selectivity for the MC_4 receptor when compared to the MC_3 receptor, but it showed 220 fold lower affinity for the MC_4 receptor than HS964, as well as much lower affinity for the other MC receptor subtypes. In HS011, the basic hydrophilic His^6 is replaced by less polar and slightly hydrophobic Ala^6. HS011 had a similar affinity profile to that of HS964, although it had 16 and 6 fold higher affinity for the MC_3 and MC_4 receptors, respectively, and slightly lower affinities for the MC_1 and MC_5 receptors compared to HS964.

Further expansion of the ring size was attempted in HS010 ($[Cys^3,Nle^4,D\text{-}Nal^7,Cys^{11}]\alpha$-MSH(3–11)) where Nle^4 is added into the ring. This lowered the affinity for the MC_4 receptor but increased it for the MC_3 receptor. HS010 had indeed highest affinity for the MC_3 receptor when compared to its affinity for the other evaluated MC receptors; HS010 showed 4 fold MC_3/MC_1, 4 fold MC_3/MC_4 and 5 fold MC_3/MC_5 selectivity, respectively.

Two peptides were made, which have either C- or N-terminal extensions to the core cyclic structure of HS964. HS012 has Nle^3 on the N-terminal end and HS014 has a 4 amino acid C-terminus which is identical to that of β-MSH. HS012 had very similar affinity profile to HS964 but slightly lower MC_4 receptor selectivity. HS014 had about 7 fold higher affinity for the MC_4 receptor, 14 fold higher affinity for the MC_1 receptor and 5 fold higher affinity for the MC_3 receptor compared to HS964, but similar affinity for the MC_5 receptor. It is also the most MC_4 receptor-selective of all the evaluated compounds, its selectivity for the MC_4 receptor being 34, 17 and 220 fold higher than that for the MC_1, MC_3 and MC_5 receptors, respectively.

We tested the cyclic AMP response of α-MSH and HS014 in COS-1 cells expressing the human MC_1, MC_3, MC_4 and MC_5 receptors (see Figure 3) and in murine B16 melanoma cells (see Figure 4). As can be seen from the figures α-MSH stimulated accumulation of cyclic AMP in all the cell types. For the MC_3 and MC_4 receptor expressing cells HS014, in concentrations up to 100 μM, did not affect the cyclic AMP levels. Instead 1 μM HS014 was found to block completely the cyclic AMP increasing action of α-MSH (Figure 3). In the murine B16 melanoma cells (presumed MC_1 receptor effect) (Figure 4), and in the COS cells transiently expressing MC_1 and MC_5 receptors (Figure 3) the addition of HS014 caused a dose-dependent

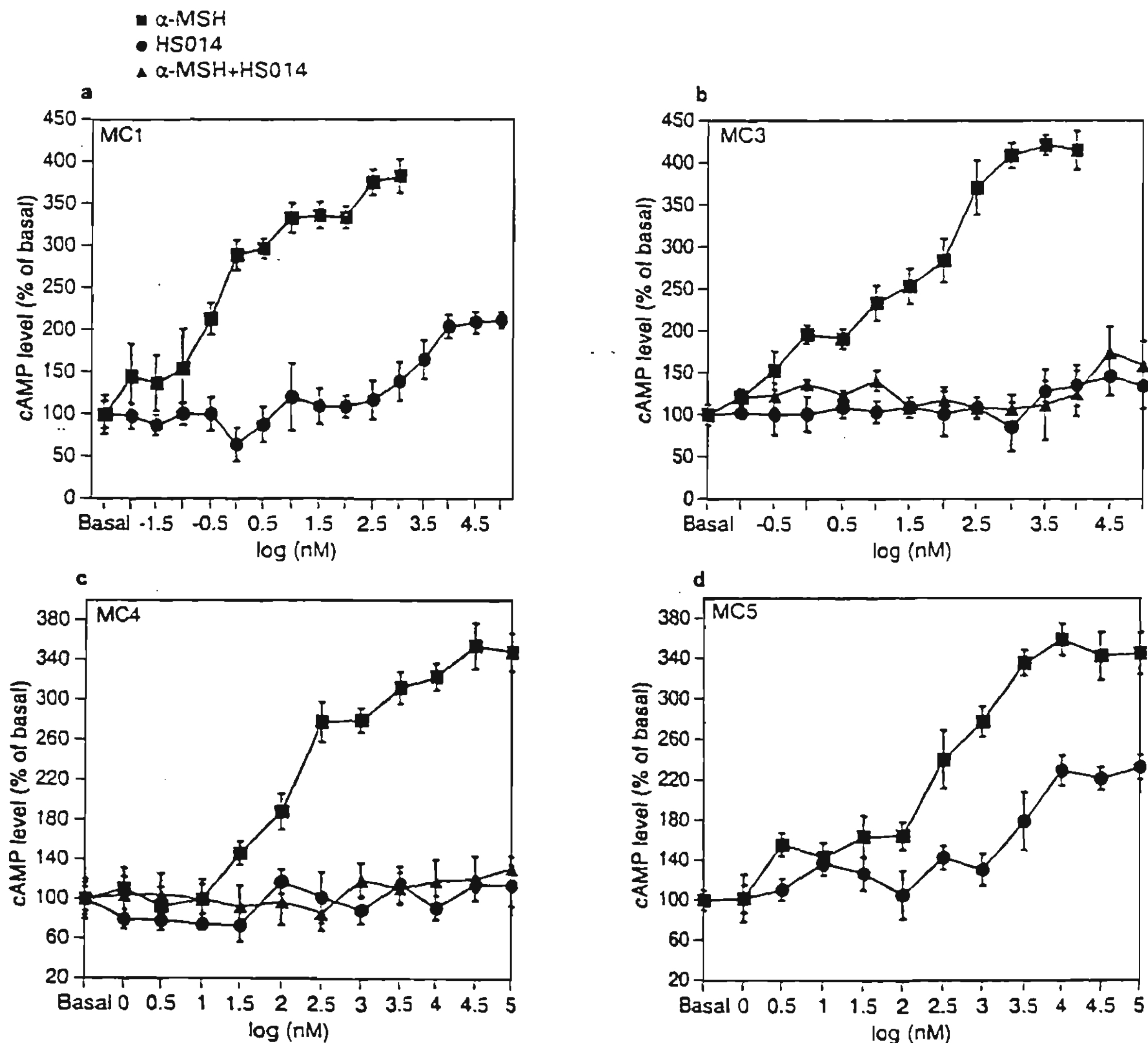

Figure 3 Generation of cyclic AMP in response to α-MSH, HS014 or α-MSH + 1 μM HS014 for the (a) MC_1, (b) MC_3, (c) MC_4 and (d) MC_5 receptors in transfected COS-1 cells. Each point represents the mean and vertical lines show s.e.mean ($n=6$).

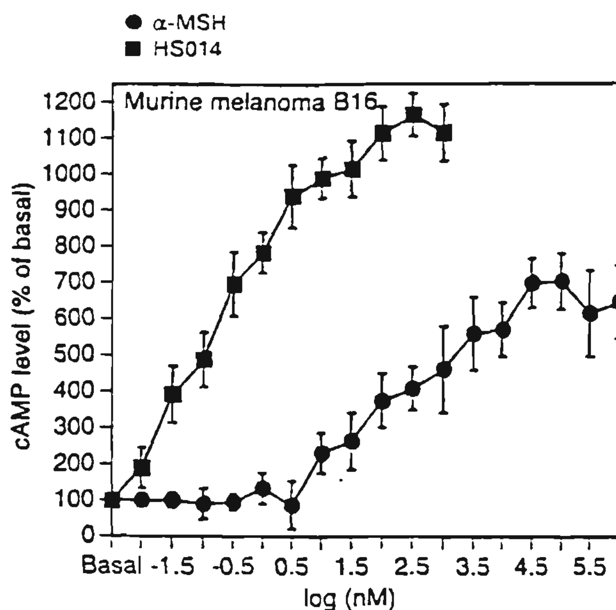

Figure 4 Generation of cyclic AMP in response to α-MSH and HS014 for murine B16 melanoma cells. Each point represents the mean and vertical lines show s.e.mean ($n = 6$).

increase in the cyclic AMP levels. However, the increase induced by HS014 did not, in these cases, reach the same maximum levels as did α-MSH. Moreover, HS014 appeared also to be less potent than α-MSH in increasing cyclic AMP via the MC_1 and MC_5 receptors.

Discussion

A large amount of data have for a long time been accumulated about the structure-activity relationships of different MSH analogues from tests on the biological responses of frog and lizard melanophores, and on melanoma cells, systems which are presumed to involve the MC_1 receptor. The most potent linear MSH analogue obtained from these earlier studies was the α-MSH analogue NDP-MSH (Sawyer *et al.*, 1980). Later, it was shown that cyclic [Cys^4,Cys^{10}]α-MSH analogues are also very potent and may be more long acting and more stable against enzymatic degradation than the linear MSH analogues (Sawyer *et al.*, 1982; Knittel *et al.*, 1983). Further development led to the invention of cyclic lactam analogues, like MTII and later SHU9119 (Al-Obeidi *et al.*, 1989; Hruby *et al.*, 1995). The lactam analogues differ from [Cys^4,Cys^{10}]α-MSH as their ring structure is made of a lactam bridge between the Asp and Lys side chains instead of a disulphide bridge between two Cys residues. The ring of both the [Cys^4,Cys^{10}]α-MSH and the lactams have 23 members. NDP-MSH, cyclic [Cys^4,Cys^{10}]α-MSH analogues and MTII, which are all very potent in melanophore assays, were developed before the cloning and identification of the MC receptor subtypes. In a more recent study, we found that cyclic [Cys^4,Cys^{10}]α-MSH(4–10) analogues favour binding to the MC_4 receptor (Schiöth *et al.*, 1997a). Moreover, the replacement of D-Phe^7 by D-Nal^7 in [Cys^4,Cys^{10}]α-MSH(4–10), resulted in a novel compound, HS9510, with increased selectivity for the MC_4 receptor. In an earlier study the cyclic lactam analogue SHU9119, which also contains D-Nal^7, was found to show some selectivity for the MC_4 receptor (Hruby *et al.*, 1995), but in our assays its selectivity proved to be only 2 fold over the MC_3 receptor (Schiöth *et al.*, 1997a).

In the present study, we discovered that increasing the ring size of [Cys^4,D-Nal^7,Cys^{10}]α-MSH(4–10) by adding one amino acid (Gly^{10}) greatly favoured selectivity for the MC_4 receptor. Gly^{10} is not expected to play an important role in the binding of MSH peptides, at least not for the MC_1 and MC_3 receptors (Sahm *et al.*, 1994a,b). The 26 membered cyclic [Cys^4,D-Nal^7,Cys^{11}]α-MSH(4–11) analogue (HS964) had more than 12 fold MC_4/MC_3 selectivity, and more than 60 fold MC_4/MC_1 selectivity. Our results show that both the ring size and the D-Nal^7 is crucial to obtain these selective properties. Replacement of the bulky hydrophobic and aromatic D-Nal^7 with D-Phe^7 or with the bulky hydrophobic D-Cha^7 or with the bulky aromatic and hydrophobic D-Bpa^7 abolished the selective binding properties and lowered the affinities, in particular for the MC_4 receptor.

Glu^5 as well as Gly^{10} is believed not to be of major importance for the binding of MSH peptides to the MC receptors (Sahm *et al.*, 1994a,b). These residues might serve as coupling sites for tails which may increase the selectivity or the affinity of synthetic peptides. Our results indicate that the acidic and polar Asp^{10} does not favour the binding of the 26 membered cyclic peptide for all the receptors, without affecting the selectivity between the subtypes. On the other hand, the basic hydrophilic Arg^5 did not greatly affect the affinities except that it favoured the binding to the MC_3 receptor. This observation could be useful in the design of truly MC_3 receptor-selective substances.

His^6 together with Phe^7-Arg^8-Trp^9 make up the central core binding sequence of the MSH peptides. However, His^6 is probably less important than Phe^7, Arg^8 and Trp^9 for the binding of α-MSH. Interestingly, we have here observed that a replacement of His^6 by Ala^6 in a 26-membered ring resulted in higher affinity for the MC_3 and MC_4 receptors, but not for the MC_1 and MC_5 receptors. It is also interesting to note that we have previously shown that His^6/Ala^6 exchange in the linear ACTH(4–10) peptide resulted in great loss of affinity for the MC_1 receptor, but not the other subtypes (Schiöth *et al.*, 1997b), possibly indicating that the present cyclic peptide binds differently to the MC_1 receptor compared to the linear peptides. One of the major characteristics of the cyclic [Cys^4,D-Nal^7,Cys^{11}]α-MSH(4–11) peptides is the relatively low affinity for the MC_1 receptor compared to the natural linear MSH peptides. Taken together, this might indicate that the binding of His^6 to the MC_1 receptor is already disrupted in the 26 membered cyclic peptides.

Interestingly, a further increase of the cycle to the 29 member ring in HS010 resulted in MC_3 receptor-selective properties. It is tempting to speculate that the 23 member ring is optimal for the MC_1 receptor, where the MC_4 and the MC_3 receptors may favour 26 and 29 membered rings, respectively.

An N-terminal addition of Nle^3 (HS012) did not influence the binding affinities much, which may indicate that the Nle^3 position of the present cyclic series cannot mimic the binding interaction of the relatively important Met^4(Nle^4) in the linear α-MSH. However, C-terminal addition of the β-MSH sequence Pro^{12}-Pro^{13}-Lys^{14}-Asp^{15} did increase the affinity for all the receptors. The affinity of HS014 for the MC_4 receptor is close to that of NDP-MSH and 300 fold higher than that of α-MSH.

The most selective and potent compound for the MC_4 receptor found in the present study, HS014, was also shown to antagonize α-MSH stimulation in MC_3 and MC_4 receptor-transfected cells. It is conceivable therefore that HS014 is an MC_3 and MC_4 receptor antagonist. However, for the MC_1 and MC_5 receptors, HS014 was found to increase intracellular levels of cyclic AMP, but without reaching maximum levels.

Thus, these results seem to indicate that HS014 is a partial agonist at the MC_1 and MC_5 receptors.

The MC_3 and the MC_4 receptors are probably those MC receptors which are most abundantly expressed in the brain (Roselli-Rehfuss *et al.*, 1993; Chhajlani, 1996; Alvaro *et al.*, 1996). We and others have previously shown that γ-MSH has a much higher affinity for the MC_3 receptor than for the MC_4 receptor. The MC_4 receptor has in general low affinity for the natural MSH peptides and none of the peptides have a higher affinity for the MC_4 receptor than for the MC_3 receptor. Our new peptides add a new dimension to the possibilities to discriminate between the MC_3 and MC_4 receptors; the most useful substance probably being HS014 as it was the most selective and potent compound for the MC_4 receptor. The MC_5 receptor mRNA has been identified in a number of peripheral tissues and more recently also in adipocytes (Boston & Cone, 1996). The MC_1 receptor is also considered to have primarily peripheral distribution, despite evidence of some minor expression in both human and rodent brain (Xia *et al.*, 1995; Rajora *et al.*, 1997). The natural MSH peptides have the same potency order for the MC_5 receptor as for the MC_1 receptor, but in general much lower affinities (about or more than 100 times lower) for the MC_5 receptor. HS964 and HS007 are the first substances which have a higher affinity for the MC_5 receptor than for the MC_1 receptor. The unique MC_5/MC_1 selectivity of HS964 makes it an interesting candidate for discrimination of the peripheral effects mediated by the MC_5 and MC_1 receptors.

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Selective properties of C- and N-terminals and core residues of the melanocyte-stimulating hormone on binding to the human melanocortin receptor subtypes

Helgi B. Schiöth [a,*], Felikss Mutulis [a,b], Ruta Muceniece [a,c], Peteris Prusis [a], Jarl E.S. Wikberg [a,1]

[a] Department of Pharmaceutical Pharmacology, Uppsala University, Uppsala, Sweden
[b] Department of Medicinal Chemistry, Institute of Organic Synthesis, Riga, Latvia
[c] Laboratory of Pharmacology, Institute of Organic Synthesis, Riga, Latvia

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Abstract

We synthesised nine analogues of [Nle^4,D-Phe^7]α-MSH (melanocyte-stimulating hormone) (NDP) where (1) the N- or C-terminals were deleted or exchanged by those of β- or γ-MSH and (2) the core residues His^6, Phe^7, Arg^8 and Trp^9 were individually substituted by Glu^6, β-(2-naphthyl)-D-alanine (D-Nal^7), Lys^8 and His^9, respectively. We tested these analogues in ligand binding assays with cells transiently expressing the human melanocortin MC_1, MC_3, MC_4 and MC_5 receptors. The results show that the N-terminal segment (Ser^1-Tyr^2-Ser^3) of NDP was not important for binding to melanocortin MC_1 and MC_4 receptors whereas it affects binding to melanocortin MC_3 and MC_5 receptors. The C-terminal segment (Gly^{10}-Lys^{11}-Pro^{12}-Val^{13}) of NDP was clearly important for binding to all the four melanocortin receptor subtypes. The data indicate that the low affinity of γ-MSH for the melanocortin MC_4 receptor is due to its C-terminal (Asp^{10}-Arg^{11}-Phe^{12}). Substitution of D-Phe^7 by D-Nal^7 increased the affinity for the melanocortin MC_4 receptor but not for the other melanocortin receptor subtypes. The other core residue substitutions lowered the affinity in a differentiated manner for each of the melanocortin receptors. These results are valuable for the molecular modelling and design of selective drugs for the melanocortin receptors. © 1998 Elsevier Science B.V.

Keywords: Melanocortin receptor subtypes; MSH (melanocyte-stimulating hormone); Ligand binding; C-terminal; N-terminal; Core residues

1. Introduction

Molecular cloning has led to the identification of a family of five receptors for the melanocortic peptides (Chhajlani et al., 1993; Chhajlani and Wikberg, 1992; Gantz et al., 1993a,b; Mountjoy et al., 1992). The first member of this family was the well-characterised melanocortin MC_1 receptor which is expressed in melanocytes and melanoma cells and binds α-MSH (melanocyte-stimulating hormone) with high affinity. The melanocortin MC_1 receptor plays an important role in skin and fur pigmentation in a variety of vertebrates (Cone et al., 1996). The melanocortin MC_2 receptor (i.e., the ACTH (adrenocorticotropin) receptor) has a well-defined function in the regulation of steroid production in the adrenal gland. Much less is known about the physiological roles of the three newly discovered melanocortin (MC) receptors (melanocortin MC_3, MC_4, and MC_5. The melanocortin MC_3 receptor is found in the brain and in the placenta, gut tissues and the heart (Gantz et al., 1993a; Chhajlani, 1996). The melanocortin MC_4 receptor is found only in the brain, where it is distributed in several areas (Mountjoy et al., 1994). The melanocortin MC_4 receptor has recently been found to affect feeding in rodents and it might be important for weight homeostasis (Huszar et al., 1997; Fan et al., 1997). The melanocortin MC_5 receptor, which is less well characterised, is primarily located in various peripheral tissues but has also been found in the brain (Labbé et al., 1994; Fathi et al., 1995).

The melanocortin MC_2 receptor binds ACTH with high affinity but not the MSH peptides (Schiöth et al., 1996b).

* Corresponding author. Department of Pharmaceutical Pharmacology, Biomedical Center, Box 591, 751 24 Uppsala, Sweden. Tel: +46-471-4160; fax: +46-18-559718; e-mail: helgis@bmc.uu.se.

[1] Also corresponding author. E-mail: jarl.wikberg@farmbio.uu.se.

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The natural melanocortins (α-, β-, γ-MSH, ACTH) are bound to the other melanocortin receptors with a specific and individual affinity profile each, but still they all are bound with highest affinity by the melanocortin MC_1 receptor, with intermediate affinity by the melanocortin MC_3 receptor and lower affinity by the melanocortin MC_4 and MC_5 receptors. The natural MSH-peptides are thus selective only for the melanocortin MC_1 receptor, whereas ACTH is selective for the melanocortin MC_2 receptor. The newly described MSH analogue SHU9119 (cyclic[Nle^4,Asp^5,β-(2-naphthyl)-D-alanine (D-Nal^7),Lys^{10}]α-MSH-(4–10)) (Hruby et al., 1995) has approximately 2-fold higher selectivity for the melanocortin MC_4 receptor than for other melanocortin receptors (Schiöth et al., 1997b), and some ACTH-(4–10) analogues have been found to display certain selectivity for the melanocortin MC_4 receptor (Adan et al., 1994b).

There is little information available about how the MSH peptides bind to the different melanocortin MC receptor subtypes. This knowledge is essential for the design and synthesis of highly selective and potent ligands for the melanocortin MC_3, MC_4, and MC_5 receptors. The aim of the present study was to synthesise MSH peptides with different residues in the core and with different C- and N-terminal segments, using the high affinities ligand [Nle^4,D-Phe^7]α-MSH (NDP) as a model, and to investigate the subtype specific binding of these substances to the human melanocortin MC_1, MC_3, MC_4, and MC_5 receptors in order to provide information for molecular modelling and drug design.

2. Materials and methods

2.1. Chemicals

NDP (Sawyer et al., 1980) was purchased from Bachem, Switzerland. NDP was radio-iodinated by the chloramine T method and purified by HPLC (high performance liquid chromatography). D-Nal was purchased from Bachem, Switzerland. All other amino acid derivatives were purchased from PerSeptive, USA.

2.2. Peptide synthesis

The peptides tested in this study (except NDP) were synthesised in our laboratory by using the solid phase approach and then purified by HPLC. The correct molecular weights of the peptides were confirmed by mass spectrometry. The peptide sequences were assembled by using the Pioneer (PerSeptive) peptide synthesis system. Fmoc(9-fluorenylmethoxycarbonyl)–amino acid derivatives were used in coupling steps. When OPfp (pentafluorophenyl) esters were used, the synthesis cycle was as follows: (a) the Fmoc group was removed by 20% piperidine in DMF (*N,N*-dimethylformamide) (5 min); (b) to form a new peptide bound side chain-protected Fmoc–amino acid OPfp ester (4 eq.) and HOAt (1-hydroxy-7-azabenzotriazole) (4 eq.) were dissolved in DMF and circulated through the column for 30–60 min; (c) to cap residual amino groups the support was treated with 0.3 M Ac_2O (acetic anhydride) in DMF for 5 min. If free acids were used, then in step (b) side chain-protected Fmoc–amino acid (4 eq.), HATU (*O*-[7-azabenzotriazol-1-yl]1,1,3,3-tetramethyluronium hexafluorophosphate) (4 eq.) and DIEA (*N,N*-diisopropylethylamine) (4 eq.) were applied. For deprotection a reagent mixture (trifluoroacetic acid)–phenol–anisole–1,2-ethanedithiol–water, 82:2:2:2:2, 2.5 h) was used. The raw peptides formed were purified by HPLC (10 mm × 250 mm column, Vydac RP C18, 90A, 201HS1010, eluent—20–35% MeCN (acetonitrile) in water + 0.1% trifluoroacetic acid, detection at 240 nm).

2.3. Expression of receptor clones

The human melanocortin MC_1 and human melanocortin MC_5 receptors (Chhajlani and Wikberg, 1992; Chhajlani et al., 1993) were cloned into the expression vector pRc/CMV (In Vitrogen). The human melanocortin MC_3 and human melanocortin MC_4 receptors, cloned into the expression vector pCMV/neo, were a gift from Dr. Ira Gantz (Gantz et al., 1993ab). For receptor expression COS-1 (*CV-1 Origin, SV40*) cells were grown in Dulbecco's modified Eagle's medium with 10% foetal calf serum. Eighty-percent confluent cultures were transfected with the DNA mixed with liposomes in serum-free medium (for details see Schiöth et al., 1996b). After transfection, the serum-free medium was replaced by serum-containing medium and the cells were cultivated for about 48 h. Cells were then scraped off, centrifuged, and used for radioligand binding.

2.4. Binding studies

The transfected cells were washed with binding buffer (see Schiöth et al., 1995) and distributed into 96-well non-culture-coated plates, which were centrifuged and the binding buffer was removed. The cells were then immediately incubated in the well plates for 2 h at 37°C with 0.05 ml binding buffer in each well containing a constant concentration of [^{125}I]NDP and appropriate concentrations of the competing unlabelled ligand. After incubation the cells were washed with 0.2 ml of ice-cold binding buffer and detached from the plates with 0.2 ml of 0.1 N NaOH. Radioactivity was counted (Wallac, Wizard automatic gamma counter) and data were analysed with a software package suitable for radioligand binding data analysis (Wan System, Umeå, Sweden). Data were analysed by fitting to formulas derived from the law of mass action by the method generally referred to as computer modelling. The K_d values for [^{125}I]NDP for the melanocortin receptors were taken from Schiöth et al. (1995, 1996a). The binding

Peptide \ position nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
γ-MSH		Tyr	Val	Met	Gly	His	Phe	Arg	Trp	Asp	Arg	Phe			
β-MSH	Pro	Tyr	Arg	Met	Glu	His	Phe	Arg	Trp	Gly	Ser	Pro	Pro	Lys	Asp
α-MSH	Ser	Tyr	Ser	Met	Glu	His	Phe	Arg	Trp	Gly	Lys	Pro	Val		
NDP	Ser	Tyr	Ser	Nle	Glu	His	D-Phe	Arg	Trp	Gly	Lys	Pro	Val		
NDP(1-9)	Ser	Tyr	Ser	Nle	Glu	His	D-Phe	Arg	Trp						
NDP(4-13)				Nle	Glu	His	D-Phe	Arg	Trp	Gly	Lys	Pro	Val		
C-β-NDP	Ser	Tyr	Ser	Nle	Glu	His	D-Phe	Arg	Trp	Gly	Ser	Pro	Pro	Lys	Asp
C-γ-NDP	Ser	Tyr	Ser	Nle	Glu	His	D-Phe	Arg	Trp	Asp	Arg	Phe			
N-γ-NDP		Tyr	Val	Met	Gly	His	D-Phe	Arg	Trp	Gly	Lys	Pro	Val		
[Glu⁶]-NDP	Ser	Tyr	Ser	Nle	Glu	Glu	D-Phe	Arg	Trp	Gly	Lys	Pro	Val		
[D-Nal⁷]-NDP	Ser	Tyr	Ser	Nle	Glu	His	D-Nal	Arg	Trp	Gly	Lys	Pro	Val		
[Lys⁸]-NDP	Ser	Tyr	Ser	Nle	Glu	His	D-Phe	Lys	Trp	Gly	Lys	Pro	Val		
[His⁹]-NDP	Ser	Tyr	Ser	Nle	Glu	His	D-Phe	Arg	His	Gly	Lys	Pro	Val		

Fig. 1. Alignment of peptides used in the present study with natural MSH peptide. All peptides used in the present study had an acetyl-group at the N-terminal, except N-γ-NDP (which is similar to that of natural γ-MSH). All peptides used in the present study also had an amide group on the C-terminal, except for C-β-NDP which was free acid. The N-terminal H-Ala-Glu-Lys-Lys-Asp-Glu-Gly– sequence of β-MSH has been omitted from the figure.

assays were performed in duplicate wells and repeated three times. Untransfected COS-1 cells did not show any specific binding for [^{125}I]NDP.

3. Results

We tested NDP and the nine new NDP analogues on intact COS-1 cells that express the human melanocortin MC_1, MC_3, MC_4, and MC_5 receptors by competitive receptor binding assays using [^{125}I]NDP as radioligand. The structure of the substances are aligned with the structures of α-MSH, β-MSH, γ-MSH and NDP in Fig. 1. The resulting K_i values calculated from competition tests of these compounds are summarised in Table 1. The competition curves for NDP, NDP(1–9), NDP(4–13), [Glu⁶]NDP, [Lys⁸]NDP, [His⁹]NDP at the different melanocortin receptor subtypes are shown in Fig. 2. Schematic presentation of the pK_i values for each of the receptors are presented in Fig. 3.

As can be seen from Table 1, the deletion of the N-terminal of NDP (NDP(4–13)) or replacement of it by

Table 1
K_i values (mean ± S.E.M), obtained from competition curves, for MSH analogues at human melanocortin MC_1, MC_3, MC_4, and MC_5 receptors transfected in COS-1 cells together with relative affinity ratios

Ligand	Receptor						
	MC_1	MC_3	MC_4	MC_5	MC_3/MC_1	MC_4/MC_1	MC_5/MC_1
	K_i (nmol/l)	K_i (nmol/l)	K_i (nmol/l)	K_i (nmol/l)			
NDP	0.078 ± 0.020	0.653 ± 0.082	4.03 ± 0.22	3.43 ± 0.57	8.4	52	44
NDP(1–9)	0.345 ± 0.123	62.8 ± 33.4	51.2 ± 21.5	40.0 ± 6.7	180	150	120
NDP(4–13)	0.099 ± 0.037	4.85 ± 1.19	2.30 ± 0.44	15.3 ± 2.1	49	23	150
C-β-NDP	0.448 ± 0.092	3.05 ± 1.23	2.37 ± 0.95	8.81 ± 4.52	6.8	5.3	20
C-γ-NDP	0.850 ± 0.172	89.7 ± 6.8	513 ± 279	109 ± 23	110	600	130
N-γ-NDP	0.052 ± 0.013	0.722 ± 0.320	3.17 ± 1.28	3.31 ± 1.06	14	6163	
[Glu⁶]NDP	7.66 ± 2.64	122 ± 17	111 ± 49	134 ± 115	16	16	17
[D-Nal⁷]NDP	0.110 ± 0.080	0.740 ± 0.036	0.877 ± 0.278	2.51 ± 0.82	6.7	9.1	23
[Lys⁸]NDP	0.786 ± 0.136	24.5 ± 10.3	70.3 ± 5.2	57.7. ± 12.3	31	89	73
[His⁹]NDP	6.66 ± 2.64	2830 ± 830	4820 ± 1850	38000 ± 12000	64	720	5700
NDP(1–9)/NDP	4.4	96	13	12			
NDP(4–13)/NDP	1.3	7.4	0.57	4.5			
C-β-NDP/NDP	5.7	4.7	0.59	2.6			
C-γ-NDP/NDP	11	137	127	32			
N-γ-NDP/NDP	0.67	1.1	0.79	0.97			
[Glu⁶]NDP/NDP	98	187	28	39			
[D-Nal⁷]NDP/NDP	1.4	1.1	0.22	0.73			
[Lys⁸]NDP/NDP	10	38	17	17			
[His⁹]NDP/NDP	85	4300	1200	11000			

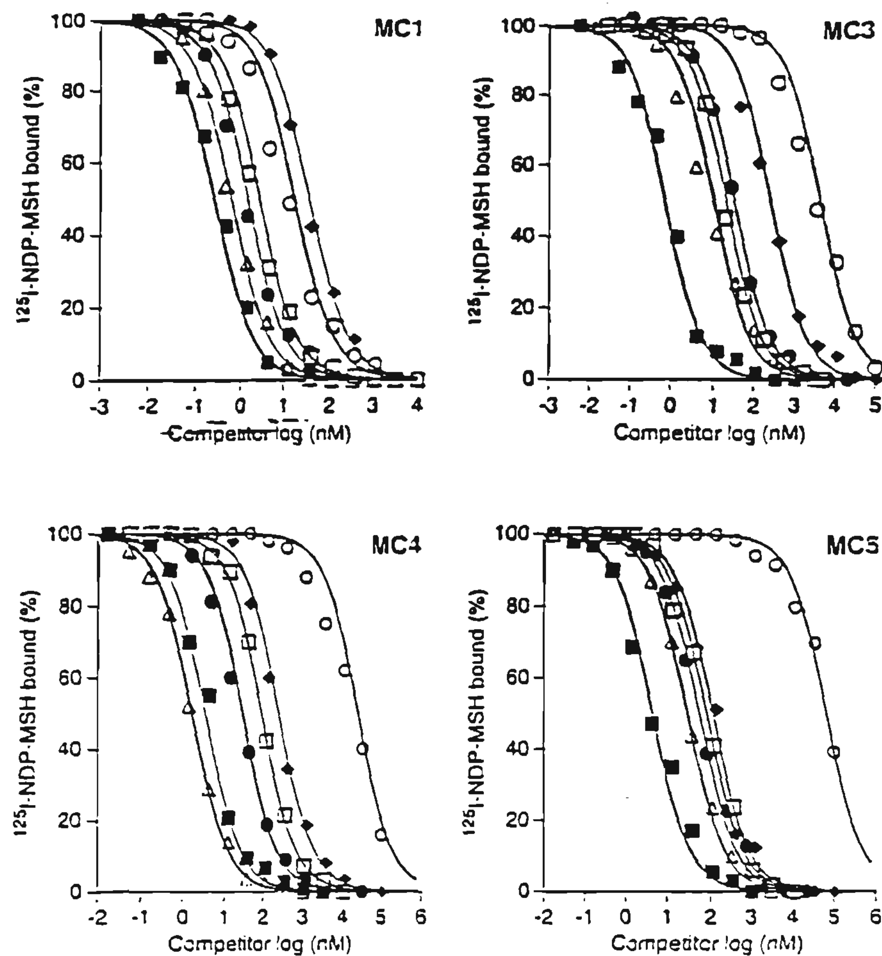

Fig. 2. Competition curves for NDP (■), NDP(1–9) (●), NDP(4–13) (△), [Glu^6]NDP (◆), [Lys^5]NDP (□), [His^8]NDP (○) binding to COS-1 cells transfected with the different melanocortin receptor subtypes. The curves were obtained by using a fixed concentration of [^{125}I]NDP and varying concentrations of the non-labelled competing peptide.

that of γ-MSH (N-γ-NDP) did not appreciably affect binding to the melanocortin MC_1 and MC_4 receptors. For the melanocortin MC_3 receptor, however, the deletion of the N-terminal of NDP clearly lowered affinity, whereas replacement of the NDP N-terminal with the N-terminal of γ-MSH did not affect binding. Deletion of the N-terminal of NDP clearly decreased the affinity for the melanocortin MC_5 receptor, but the NDP affinity was only slightly reduced when the N-terminal was replaced by that of γ-MSH.

Deletion of the C-terminal of NDP (NDP(1–9))and replacement of it by the C-terminal of β- or γ-MSH (C-β-NDP and C-γ-NDP, respectively) lowered the affinity for the melanocortin MC_1 receptor. For the melanocortin MC_3 receptor, deletion of the C-terminal and replacement of it with that of γ-MSH greatly lowered the affinity (around or more than 100-fold), whereas C-β-NDP displayed only a slightly lower affinity than NDP. The low affinity of C-γ-NDP was surprising to us, as γ-MSH has a slightly higher or similar affinity for the melanocortin MC_3 receptor as that of α-MSH (Schiöth et al., 1995). We repeated the synthesis of C-γ-NDP but the results were the same for all of the receptors. Replacement of the C-terminal of NDP by that of β-MSH slightly increased the affinity for the melanocortin MC_4 receptor, whereas deletion of the C-terminal resulted in 13-fold lower affinity. C-γ-NDP had 130-fold lower affinity for the melanocortin MC_4 receptor than did NDP. C-β-NDP showed only slightly lower affinity for the melanocortin MC_5 receptor than NDP whereas NDP(1–9) and C-γ-NDP displayed 12- and 32-fold lower affinity than NDP, respectively.

Replacement of the basic hydrophilic His^6 by the acidic hydrophilic Glu^6 reduced the affinity of NDP for all the melanocortin receptors, in particular for the melanocortin MC_3 (190-fold) and for the melanocortin MC_1 (98-fold) receptors, whereas the reductions for the melanocortin MC_4 (28-fold) and melanocortin MC_5 (39-fold) receptors was less. Replacement of the hydrophobic D-Phe^7 in NDP

by the hydrophobic but more bulky D-Nal[7] resulted in similar affinity for the melanocortin MC_1, MC_3, and MC_5 receptors but an about 4-fold increased affinity for melanocortin MC_4 receptor. Replacement of the basic hydrophilic Arg[8] with the structurally similar Lys[8] lowered the affinity for the melanocortin MC_1 receptor 10-fold, for the melanocortin MC_3 receptor 38-fold and for the melanocortin MC_4 and MC_5 receptors 17-fold. Replacement of the non polar hydrophobic Trp[9] by the basic hydrophilic His[9] lowered the affinity for the melanocortin MC_1 receptor 85-fold and for the melanocortin MC_3, MC_4, and MC_5 receptors more than 1000-fold.

4. Discussion

The melanocortin receptors are a family of 7TM (transmembrane) spanning receptors that bind melanocortic peptides. The melanocortin receptors display a number of common structural features and show considerable amino acid homology, especially within TM1, TM2, TM3 and TM7, which may contain key elements for the binding of MSH peptides. They are also characterised by short N- and C-terminal regions as well as a very small second extracellular loop. The core of the MSH peptide (i.e., His[6]-Phe[7]-Arg[8]-Trp[9]) is known to be crucial for MSH binding to melanocortin receptors (Eberle, 1988; Schiöth et al., 1995, 1996a, 1997a; Haskell-Luevano et al., 1996, 1997). All compounds that bind to the melanocortin receptors have important elements from this core (except the agouti peptide which has no homology to α-MSH). It is also evident that the C- and/or the N-terminal side chains of MSH peptides may play an important role in determining the subtype- specific binding, which is in particular displayed by γ-MSH, which has a comparatively much higher selectivity for the melanocortin MC_3 receptor than for the melanocortin MC_4 receptor (Adan et al., 1994a; Miwa et al., 1995; Schiöth et al., 1995, 1996a). NDP is an α-MSH analogue which has L-Phe[7] substituted by D-Phe[7], which led to a higher potency and prolonged biological activity, and Met[4] substituted by Nle[4], which further enhanced the affinity and stability of this peptide. NDP has earlier been shown by us and others to have a high affinity for all of the melanocortin receptors and is therefore suitable as a

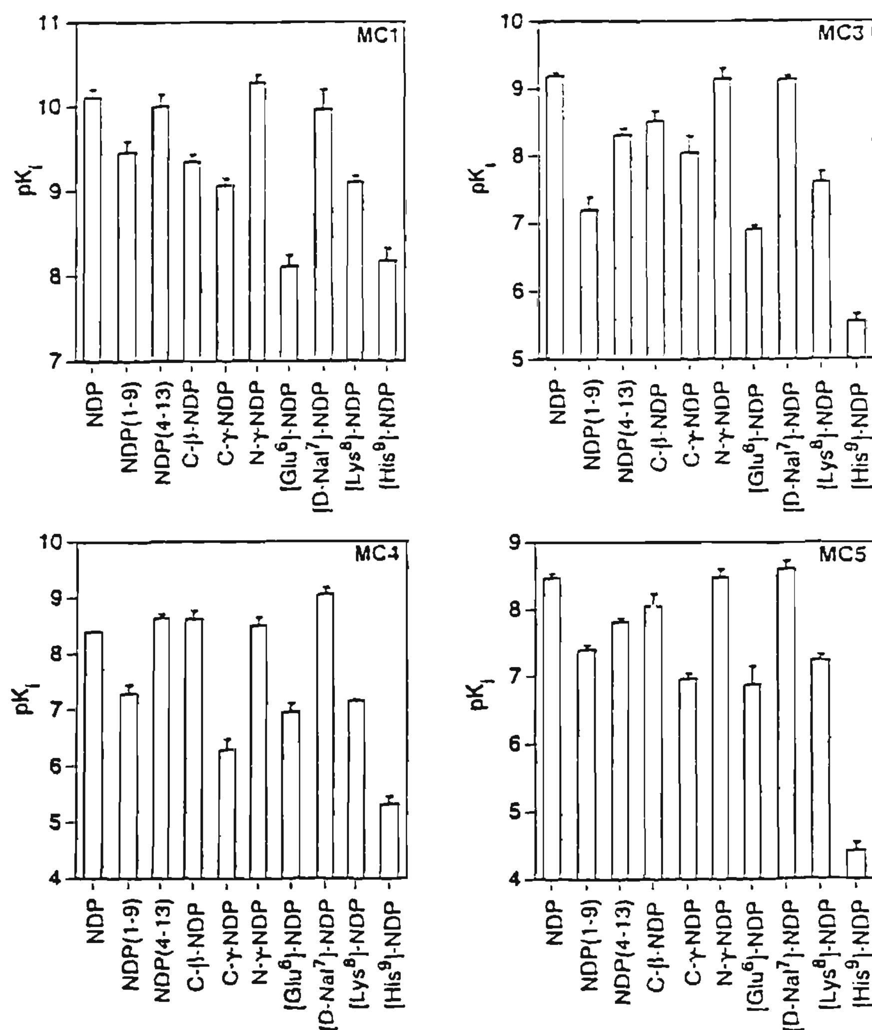

Fig. 3. Graphical presentation of the pK_i values for the different peptides for the melanocortin receptor subtypes.

reference substance for characterisation of the importance of the C- and N-terminals and core residues of MSH peptides for the binding to the human melanocortin receptors. Mutagenesis data indicate that there might be an alternative point of attachment for NDP and α-MSH at the melanocortin MC_1 receptor (Frändberg et al., 1994). However, more thorough testing with several linear and cyclic Phe^7-substituted MSH analogues (Schiöth et al., 1997c) has shown that this hypothesis may not be correct.

Our results show that the N-terminal segment (Ser^1-Tyr^2-Ser^3) in the NDP peptide is not important for binding to the melanocortin MC_1 receptor whereas the C-terminal segment (Gly^{10}-Lys^{11}-Pro^{12}-Val^{13}) is clearly important because deletion or changes in the C-terminal caused an about 5-fold decrease in affinity for the melanocortin MC_1 receptor. This is very much in line with early data on the melanotropic activity of MSH analogues on several melanophores (for review see Eberle, 1988), and with more recent data showing that individual replacement of N-terminal residues by Ala in α-MSH resulted in negligible influence on receptor binding or tyrosinase activity in murine melanoma cells, whereas Ala substitutions in the C-terminal, in particular the replacement of Pro^{12}, resulted in loss in affinity (Sahm et al., 1994b). The lack of importance of the N-terminal segment and the significance of the C-terminal for the melanocortin MC_1 receptor has also been shown by use of cyclic [Cys^4-Cys^{10}]α-MSH analogues in frog and lizard skin bioassays (Cody et al., 1984). The residues Lys^{11} and Pro^{12} were in particular shown to be important for the activity of cyclic analogues. Our present and earlier (Schiöth et al., 1995) data for the melanocortin MC_1 receptor seems to indicate that the differential binding of α-, β- and γ-MSH is related to the C-terminal sequence, where the absence of Lys^{11} in β-MSH and γ-MSH and Pro^{12} in γ-MSH causes the lower affinity of β-MSH and γ-MSH compared to α-MSH.

Much less is known about the influence of the N- and C-terminal segments of MSH peptides for binding to the other melanocortin receptors. Our present results show that both the N- and the C-terminal segments are important for binding to the melanocortin MC_3 receptor. This is in line with alanine scanning data for the rat melanocortin MC_3 receptor (Sahm et al., 1994b) which indicated the importance of both Tyr^2 in the N-terminal and Lys^{11} in the C-terminal, whereas other Ala substitutions in both terminals did not significantly affect ligand binding. Also, truncation of both the N- and C-terminals of MSH peptides influence the cAMP response of the rat (Adan et al., 1994b) and human melanocortin MC_3 receptors (Miwa et al., 1995).

The melanocortin MC_4 receptor can be distinguished from the melanocortin MC_3 receptor because the former binds γ-MSH with much lower affinity than α-MSH, whereas the latter shows a similar affinities for both of these peptides. Recently, it was shown that both linear and cyclic core MSH analogues are bound by melanocortin MC_3 and MC_4 receptors with equal affinity (Schiöth et al., 1997a), which indicates that it is indeed the N- or/and C-terminals that determine the differences in affinity of these receptors for α- and γ-MSH. Our present data show that the C-terminal of NDP but not the N-terminal is important for binding to the melanocortin MC_4 receptor. Our data indicate that it may be the C-terminal end of γ-MSH (Asp^{10}-Arg^{11}-Phe^{12}) that perturbs binding to the melanocortin MC_4 receptors. However, it should be considered that chimeric or engineered peptides may not bind in the same manner as natural peptide hormones simply due to incompatibility of the different structural elements which are artificially brought together. The effects of deletions or replacements of terminal residues may be caused by a direct contribution of the amino acid residues involved or the changes may cause indirect effects due to conformational changes of core residues.

The melanocortin MC_5 receptor binds the different MSH peptides with the same order of preference as the melanocortin MC_1 receptor but in general with much lower all over affinity. Our present data show that both the N- and C-terminals are important for the binding of MSH peptides to the melanocortin MC_5 receptor. Our data indicate that it is in particular the C-terminal which determines the differential binding of the MSH peptides to this receptor.

Replacement of Phe^7 by D-Phe^7 is the only substitution that is known to enhance the affinity of the core of α-MSH. Now we demonstrate that replacement of D-Phe^7 by D-Nal^7 further enhances the affinity for the melanocortin MC_4 receptor but not for the other melanocortin receptor subtypes. Replacement of D-Phe^7 by D-Nal^7 in the cyclic lactam analogue MTII resulted in SHU9119, which has been shown to be a melanocortin MC_4 receptor antagonist (Hruby et al., 1995). However, SHU9119 showed higher affinity for the melanocortin MC_3 and MC_5 receptors compared to MTII (Schiöth et al., 1997b), which reveals that the D-Nal^7/D-Phe^7 substitution has a different effect in the lactam analogue than the linear NDP used in the present study. The other substitutions in the MSH core resulted in more or less pronounced loss in affinity for the different melanocortin receptors, as one might have predicted. The above-mentioned alanine scanning experiments by Sahm et al. (1994b), which were performed with murine melanoma cells and rat melanocortin MC_3 receptors, as well as earlier structure–activity studies (Eberle, 1988), have indicated that Arg^8 and Trp^9 are the most important residues in the MSH core for receptor binding. It is interesting to note that the relatively subtle change in the Arg side chain, converting it to a Lys, resulted in a more than 10-fold loss in affinity for all the melanocortin receptors. It is not unexpected that the substitution of His^6 by Glu^6 or of Trp^9 by His^9, which changes the polarity of the residue in respective position, causes a much more dramatic decrease of the affinities. Interestingly, these changes depend to a large extent on the receptor subtype. These

differences might be useful to verify or reject hypotheses formulated on the basis of results from molecular modelling studies of the melanocortin receptors or from direct mutagenesis studies which pinpoint the specific interaction between residues in the ligand and the receptors.

It is also important for the design of compounds which bind selectively to a specific receptor subtype to determine residues or regions whose modification can increase the selectivity. Taken together, our present data show that both the melanocortin MC_1 and MC_4 receptors are not sensitive to changes in the N-terminal of the MSH peptide, whereas the melanocortin MC_3 and MC_5 receptors recognise important binding elements in this part of the MSH peptide. Moreover, binding to the melanocortin MC_4 and MC_3 receptors seems in particular to be sensitive to changes in the C-terminal of the MSH peptide, whereas the melanocortin MC_1 and MC_5 receptors are affected in a more subtle way by changes in this part of an MSH peptide. Furthermore, His^6 replacement by Glu^6 reduced binding to the melanocortin MC_3 and MC_1 receptors to a much greater extent than it did binding to the melanocortin MC_4 and MC_5 receptors. The Trp^9 replacement by His^9 reduced binding to the melanocortin MC_4 and MC_3 receptors to a much greater extent than to the melanocortin MC_1 receptor. The D-Phe^7/D-Nal^7 or the Arg^3/Lys^3 substitutions did not lead to any marked changes in the selectivity of the MSH ligand for the melanocortin MC receptor subtypes. The substitution of NDP by D-Nal^7 (resulting in [D-Nal^7]NDP) gave the ligand with the highest affinity for the melanocortin MC_4 receptor, a property which might find potential use in studies of the pharmacology of the melanocortin MC_4 receptor.

5. Unlinked References

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Acknowledgements

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BRIEF COMMUNICATION

Selectivity of [Phe-I[7]], [Ala[6]], and [D-Ala[4],Gln[5],Tyr[6]] Substituted ACTH(4–10) Analogues for the Melanocortin Receptors

HELGI B. SCHIÖTH,*[1] RUTA MUCENIECE AND JARL E. S. WIKBERG

Department of Pharmaceutical Pharmacology, Uppsala University, Uppsala, Sweden

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SCHIÖTH, H. B., R. MUCENIECE AND J. E. S. WIKBERG. *Selectivity of [Phe-I[7]], [Ala[6]], and [D-Ala[4],Gln[5],Tyr[6]] substituted ACTH(4–10) analogues for the melanocortin receptors.* PEPTIDES 18(5) 761–763, 1997.—We tested [Ala[6]]ACTH(4–10) and [Phe-I[7]]ACTH(4–10) (putative MC receptor antagonists), [D-Ala[4],Gln[5],Tyr[6]]ACTH(4–10) (BIM 22015), and ACTH(4–10) with radioligand binding using transiently expressed human MC_1, MC_3, MC_4, and MC_5 receptors. [Phe-I[7]]ACTH(4–10) had higher affinity for the MC_3, MC_4, and MC_5 receptors but lower for the MC_1 compared to ACTH(4–10). [Ala[6]]ACTH(4–10) did not bind the MC_1 receptor but had highest affinity for the MC_4 receptor. The data indicate that the His[6] has a specially important role in binding to the MC_1 receptor. The BIM 22015 did not bind to these MC receptor subtypes, which indicates that the neurotrophic and myotrophic properties that are attributed to this peptide are mediated by some other receptor. © 1997 Elsevier Science Inc.

Melanocortin receptor subtypes MSH ACTH Ligand binding BIM 22015

PRO-OPIOMELANOCORTIN is a large polypeptide that is posttranslationally cleaved into peptides with melanotrophic, adrenocortic, or opiate activities. The melanocortins, which include the ACTH and MSH peptides, are primarily known for their role in regulation of steroid production in the adrenal gland and skin pigmentation, respectively. In addition to these well-known effects, administration of melanocortin peptides has been reported to stimulate nerve regeneration, increase retention of learned behavior, induce grooming, decrease fever, increase heart rate and blood pressure, and to have anti-inflammatory and analgesic effects (4,10).

By molecular cloning, five melanocortin receptor subtypes were recently identified (2,3,5,6,9). The MC_1 has high affinity for α-MSH (14,16) and is expressed in melanoma cells and in rat and human brain (3,19). The MC_2 is the ACTH receptor expressed in the adrenal gland (9). The MC_2 receptor has high affinity for ACTH but does not bind to the MSH peptides (13). The three recently discovered receptor subtypes, MC_3, MC_4, and MC_5, bind the natural MSH peptides with relatively lower affinity and are all expressed in the brain (8,15–17). The MC_5 also has a prominent peripheral distribution (8,17).

A recent report (1) describes that the ACTH (4–10) analogues, [Ala[6]]ACTH(4–10) and [Phe-I[7]]ACTH(4–10), may be specific antagonists for MC receptor subtypes. [Ala[6]]ACTH(4–10) inhibited an α-MSH-induced cAMP response of rat MC_3 and ovine MC_5 receptors, but only weakly antagonized the activation of human MC_4 receptor. [Phe-I[7]]ACTH(4–10) antagonized these receptors equally well. [Phe-I[7]]ACTH(4–10), but not [Ala[6]]ACTH(4–10), inhibited α-MSH-induced grooming in rats. Another ACTH(4–10) analogue, BIM 22015 ([D-Ala[4],Gln[5],Tyr[6]]ACTH(4–10)), increased rate of development of tetanic tension and contraction in a rat muscle (18). BIM 22015 also showed neurotrophic stimulation of nerve branching and prevented denervation muscle atrophy.

The aim of this study was to determine the binding properties of these ACTH(4–10) analogues by radioligand binding on the human MC_1, MC_3, MC_4, and MC_5 receptors expressed in a single eukaryotic cell line.

METHOD

Peptides

[Nle[4], D-Phe[7]]α-MSH was purchased from Saxon Biochemicals GmbH, Germany. [Nle[4], D-Phe[7]]α-MSH was radioiodinated by the chloramine-T method and purified by HPLC. The specific

[1] Requests for reprints should be addressed to Helgi B. Schiöth, Department of Pharmaceutical Pharmacology, Biomedical Center, Box 591, 751 24 Uppsala, Sweden.

activity of [^{125}I][Nle^4, D-Phe^7]α-MSH was approximately 2000 μCi/nmol. BIM 22015 {[D-Ala^4,Gln^5,Tyr^9]ACTH(4–10)} [H-(D-Ala)-Gln-Tyr-Phe-Arg-Trp-Gly-NH_2] and [Ala^6]ACTH-(4–10) (H-Met-Glu-Ala-Phe-Arg-Trp-Gly-OH) were synthesized by Medprobe AS, Norway. ACTH(4–10) (H-Met-Glu-His-Phe-Arg-Trp-Gly-OH) and [Phe-I^7]ACTH(4–10) [H-Met-Glu-His-(para-iodo-Phe)-Arg-Trp-Gly-OH] were purchased from Bachem, Switzerland.

Expression of Receptor Clones

The human MC_1 and human MC_5 receptors (2,3) were cloned into the expression vector pRc/CMV (InVitrogen). The human MC_3 and human MC_4 receptor DNAs, cloned into the expression vector pCMV/neo, were a gift from Dr. Ira Gantz (5,6). For receptor expression, COS-1 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Eighty percent confluent cultures were transfected with the DNA mixed with liposomes in serum-free medium [for details see (15)]. After transfection, the serum-free medium was replaced with the serum containing medium and the cells were cultivated for about 48 h. Cells were then scraped off, centrifuged, and used for radioligand binding.

Binding Studies

The transfected cells were washed with binding buffer (16) and distributed into 96-well plates (approximately 40,000 cells/well). The cells were then incubated for 2 h at 37°C with 0.05 ml binding buffer in each well, containing a constant concentration of [^{125}I][Nle^4, D-Phe^7]α-MSH and appropriate concentrations of an unlabeled ligand. After incubation, the cells were washed with 0.2 ml of ice-cold binding buffer and then detached from the plates with 0.2 ml of 0.1 N NaOH. Radioactivity was counted (Wallac, Wizard automatic gamma counter) and data analyzed with a software package for radioligand binding analyses (Wan System AB, Umeå, Sweden). Data were either analyzed by fitting to formulas derived from the law of mass action by the method generally referred to as computer modeling, or by fitting to the four-parameter logistic function. K_i values were calculated by using the Cheng and Prusoff equation. The K_d value for [^{125}I][Nle^4, D-Phe^7]α-MSH for the MC_1, MC_3, and MC_5 receptors was taken from (16) and for the MC_4 from (15). The binding assays were performed in duplicate wells and repeated three times. Untransfected COS-1 cells did not show any specific binding to [^{125}I][Nle^4, D-Phe^7]α-MSH.

RESULTS

The human DNAs encoding the MC_1, MC_3, MC_4, and MC_5 were transiently expressed in COS-1 cells for ligand binding. The K_i values for the ACTH(4–10) analogues were evaluated in competition with [^{125}I][Nle^4, D-Phe^7]α-MSH on intact cells. The data obtained are given in Table 1 together with the relative affinity ratios of [Phe-I^7]ACTH(4–10)/ACTH(4–10) and [Ala^6]ACTH(4–10)/ACTH(4–10). The assays were performed using the same approach as we used earlier for the evaluation of several natural melanocortic peptides (15,16). Competition curves for each of the receptors for the peptides are shown in Fig. 1.

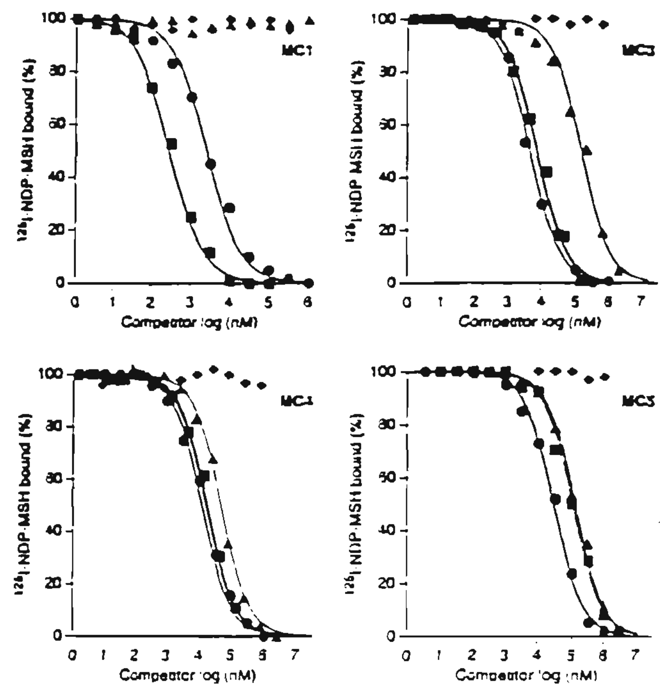

FIG. 1. Competition curves of ACTH(4–10) (■), [Phe-I^7]ACTH(4–10) (●), [Ala^6]ACTH(4–10) (▲), and BIM 22015 (◆) on COS-1 cells transfected with the MC_1, MC_3, MC_4, or MC_5 obtained by using a fixed concentration of ~2 nM [^{125}I][Nle^4, D-Phe^7]α-MSH and varying concentrations of the nonlabeled competing peptide.

The data show that the ACTH(4–10) has highest affinity for the MC_1 receptor and lower for the MC_3, MC_4, and MC_5 receptors. The ACTH(4–10) shares the same preference order for the MC receptor subtypes as α-MSH, ACTH, and other natural peptides: MC_1 > MC_3 > MC_4 > MC_5 (15,16). The [Phe-I^7]ACTH(4–10) had ninefold lower affinity for the MC_1 receptor, slightly higher affinity for the MC_3 and MC_4 receptors, and threefold higher affinity for the MC_5 receptor compared to the

TABLE 1

K_i VALUES (MEAN ± SEM) OBTAINED FROM COMPETITION CURVES, FOR MSH ANALOGUES ON MC_1, MC_3, MC_4, AND MC_5 TRANSFECTED COS-1 CELLS

Ligand	Receptor			
	MC_1 K_i (μmol/l)	MC_3 K_i (μmol/l)	MC_4 K_i (μmol/l)	MC_5 K_i (μmol/l)
ACTH (4–10)	0.294 ± 0.021	7.65 ± 1.39	18.0 ± 4.1	103 ± 19
[Phe-I^7]ACTH (4–10)	2.65 ± 0.30	4.80 ± 1.10	12.8 ± 2.1	29.2 ± 7.8
[Ala^6]ACTH (4–10)	>3000	104 ± 33	67.0 ± 16.0	125 ± 48
BIM 22015	>1000	>1000	>1000	>1000
[Phe-I^7]ACTH(4–10)/ACTH(4–10)	9.0	0.63	0.71	0.28
[Ala^6]ACTH(4–10)/ACTH(4–10)	>10,000	14	3.7	1.2

ACTH(4–10). The subtype preference order is the same as for ACTH(4–10).

[Ala[5]]ACTH(4–10) did not bind to the MC_1 receptor even at 3 mM concentration. [Ala[6]]ACTH(4–10) had highest affinity for the MC_4 receptor and slightly lower for the MC_3 and the MC_5 receptors. [Ala[5]]ACTH(4–10) had lower affinity for all the subtypes than the ACTH(4–10). BIM 22015 did not displace [^{125}I][Nle[4], D-Phe[7]]α-MSH bound to the MC_1, MC_3, MC_4, or MC_5 receptors at concentration up to 1 mM.

DISCUSSION

Stimulation of the cloned MC receptors by natural MSH/ACTH peptides results in an increase of intracellular cAMP (8,17). Each of the MC receptors (except MC_2) has a unique affinity profile for the natural MSH peptides, although these peptides show highest affinity for the MC_1 receptor and lower affinities for the MC_3, MC_4, and MC_5 receptor subtypes (15,16). The physiological role of these receptors remains unknown and thus it is urgent to identify compounds that are selective for these subtypes. Hruby et al. (7) synthesized the cyclic lactam analogue SHU9119, which was claimed to be an antagonist for the human MC_4 receptor but an agonist for the human and mouse MC_1 receptor. Adan et al. (1) has identified ACTH(4–10) analogues that antagonized α-MSH-induced cAMP response of rat MC_3 and ovine MC_5 receptors and grooming in rats. These ACTH(4–10) analogues and BIM 22015 have not been tested by radioligand binding on all the human MC receptors.

Our results show that the replacement of Phe[7] in the ACTH(4–10) by para-iodo-Phe[7] results in loss in affinity for the MC_1 receptor but a slight increase in affinity for the other subtypes. The possible steric hindrance of the iodine seems to favor the binding to the low affinity subtypes (i.e., the MC_3, MC_4, and MC_5 receptors) compared with the high-affinity MC_1 receptor.

His[6] has been demonstrated by intensive structure–activity analyses using different melanophores and melanoma cells to be one of the most important binding residues in the MSH peptide (4). These assay systems refer to the MC_1 receptor. Furthermore, Ala[6] substitution of His[6] in α-MSH resulted in about 100-fold and 6-fold reduction in affinity for murine MC_1 (11) and the rat MC_3 (12) receptors, respectively. Here we found that replacement of His[5] in the ACTH(4–10) resulted in a more than 10,000-fold loss in affinity for the MC_1 receptor, and about 14-fold loss in affinity for the MC_3 receptor but only in a slight loss for the MC_4 and the MC_5 receptors. The data indicate the His[5] has an especially important role in the binding to the MC_1 receptor, which is not shared by the MC_4 and MC_5 receptors.

The BIM 22015, which does not only have the His[5] replaced but also lacks the very important Met[4] (4,11,12), did not bind to the MC receptor subtypes. The data indicate that the neurotrophic and myotrophic properties that are attributed to this peptide are mediated by some other receptor. This may not be so surprising as we and others (8,15,16) have previously shown that another ACTH analogue, ORG 2766 [(O_2)-Met-Glu-His-(D-Lys)-Phe-OH], which may also influence peripheral nerve regeneration, does not bind to the cloned MC receptor subtypes.

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Selectivity of Cyclic [D-Nal[7]] and [D-Phe[7]] Substituted MSH Analogues for the Melanocortin Receptor Subtypes

HELGI B. SCHIÖTH,*[1] RUTA MUCENIECE,*‡ FELIKSS MUTULIS,*|| PETERIS PRUSIS,*
GUNNAR LINDEBERG,† SHUBH D. SHARMA,§ VICTOR J. HRUBY§ AND JARL E. S. WIKBERG*[1]

*Department of Pharmaceutical Pharmacology and †Department of Medicinal and Physiological Chemistry, Uppsala University, Uppsala, Sweden
‡Laboratory of Pharmacology, and ||Department of Medicinal Chemistry, Institute of Organic Synthesis, Riga, Latvia
§Department of Chemistry, University of Arizona, Tucson, Arizona

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SCHIÖTH, H. B., R. MUCENIECE, F. MUTULIS, P. PRUSIS, G. LINDEBERG, S. D. SHARMA, V. J. HRUBY AND J. E. S. WIKBERG. *Selectivity of cyclic [D-Nal[7]] and [D-Phe[7]] substituted MSH analogues for the melanocortin receptor subtypes.* PEPTIDES **18**(7), 1009–1013, 1997.—The binding of the 2 cyclic lactam MSH (4–10) analogues (MTII, SHU9119), and 5 cyclic [Cys[4], Cys[10]]α-MSH analogues were tested on cells transiently expressing the human MC1, MC3, MC4 and MC5 receptors. The results indicate a differential importance of the C-terminal (Lys-Pro-Val) and N-terminal (Ser-Tyr-Ser) of cyclic [Cys[4], Cys[10]]α-MSH analogues in binding to the MC receptor subtypes. Substitution of D-Phe[7] by D-Nal(2')[7] in both the cyclic lactam MSH (4–10) and the cyclic disulphide MSH (4–10) analogues resulted in a shift in favour of selectivity for the MC4 receptor, the disulphide analogue, [Cys[4], D-Nal(2')[7] Cys[10]]α-MSH (4–10) (HS9510), showing the highest selectivity for the MC4 receptor among all the substances tested. However, the cyclic lactams displayed an over all higher affinity for the MC receptors, than any of the cyclic disulphide MSH (4–10) analogues. © 1997 Elsevier Science Inc.

Melanocortin receptor subtypes MSH MTII SHU9119 HS9510 Ligand binding

THE melanocortic peptides are primarily known for their role in regulation of adrenal steroid production (ACTH) (26) and pigmentation (α-MSH) (7). The MSH and ACTH peptides have also a variety of both central and peripheral effects. These peptides are reported to affect learning, memory, behaviour, inflammation, pyretic control, analgesia, blood pressure, neurotropy, as well as influence events surrounding parturition (7,16).

Molecular cloning of the five melanocortin (MC) receptor subtypes (MC1-5) by us and others (3,4,8,9,15) has provided tools for systematic studies of the molecular mechanisms underlying the above mentioned effects. The natural hormones are not subtype selective except that α-MSH is selective for the MC1 receptor, ACTH is selective for the MC2 receptor and γ-MSH has preference for the MC3 receptor. Each of the newly discovered MC3, MC4 and MC5 receptors show unique affinity profiles, although in general they display lower affinity for MSH peptides than the MC1 receptor (14,23–25). The MC2 receptor is distinctly different from the other MC receptor subtypes by not binding the MSH peptides (22). The physiological roles of the MC3, MC4 and MC5 receptors remain largely unknown. These receptors are all expressed in the brain where it is likely that they participate in mediation of the various central effects of the melanocortins (14).

Cyclic disulphide [Cys[4], Cys[10]]MSH analogues were synthesized in the early 80's and shown to be potent melanotropes in lizard and frog melanocyte bioassays (20). Recently, a new class of cyclic lactam MSH analogues were synthesized which also are highly potent melanotropins (1). The most important of these lactams are SHU9119 and melanotan II (MTII). MTII causes penile erections in humans (6) and SHU9119 is a potential MC4 receptor selective antagonist (12).

In this study, we synthesized new cyclic substances in order to investigate the selective binding of [D-Phe[7]]- and [D-Nal(2')[7]]-substituted cyclic disulphide MSH analogues in comparison with cyclic lactam α-MSH analogues (SHU9119 and MTII) to the human MC1, MC3, MC4 and MC5 receptors, aiming for back-

[1] Requests for reprints should be addressed to Helgi B. Schiöth and Jarl E. S. Wikberg, Department of Pharmaceutical Pharmacology, Biomedical Center, Box 591, 751 24 Uppsala, Sweden; E-mail: helgis@bmc.uu.se; jwikberg@bmc.uu.se

Peptide \ position nr.	1	2	3	4	5	6	7	8	9	10	11	12	13
α-MSH	Ser	Tyr	Ser	Met	Glu	His	Phe	Arg	Trp	Gly	Lys	Pro	Val
NDP-MSH	Ser	Tyr	Ser	Nle	Glu	His	D-Phe	Arg	Trp	Gly	Lys	Pro	Val
(1-13)D	Ser	Tyr	Ser	*Cys*	Glu	His	D-Phe	Arg	Trp	*Cys*	Lys	Pro	Val
(1-13)L	Ser	Tyr	Ser	*Cys*	Glu	His	Phe	Arg	Trp	*Cys*	Lys	Pro	Val
(4-13)D				*Cys*	Glu	His	D-Phe	Arg	Trp	*Cys*	Lys	Pro	Val
(4-10)D				*Cys*	Glu	His	D-Phe	Arg	Trp	*Cys*			
HS9510				*Cys*	Glu	His	D-Nal	Arg	Trp	*Cys*			
Melanotan II				Nle	*Asp*	His	D-Phe	Arg	Trp	*Lys*			
SHU9119				Nle	*Asp*	His	D-Nal	Arg	Trp	*Lys*			

FIG. 1. Alignment of α-MSH and NDP-MSH to the MSH analogues evaluated in this study. All peptides have an acetyl-group on the *N*-terminus and an amide group on the *C*-terminus. The amino acid residues which make up the ring closure in the cyclic compounds are shown underlined in italics.

ground information for 3D modelling of the different MC receptor subtypes.

METHOD

Peptides

α-MSH, [Nle^4, D-Phe^7]α-MSH (NDP-MSH) (21) and [Cys^4, D-Phe^7, Cys^{10}]α-MSH (4–13) ((4–13)D) (10,21) was purchased from Saxon Biochemicals GmbH, Germany. [Cys^4, D-Phe^7, Cys^{10}]α-MSH (1–13) ((1–13)D) and [Cys^4, L-Phe^7, Cys^{10}]α-MSH (1–13) ((1–13)L) were synthesized by Scandinavian Peptide Synthesis. [Cys^4, D-Phe^7, Cys^{10}]α-MSH (4–10) ((4–10)D), and HS9510 were synthesised in our laboratories using the solid phase approach and purified by HPLC. The correct molecular weights of the peptides were confirmed by mass spectrometry. The HS9510 peptide sequence was assembled using Pioneer, PerSeptive Biosystems peptide synthesis system. Fmoc-PAL-PEG-PS support and Fmoc-Cys(Trt)-OPfp, Fmoc-Trp(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-D-Nal-OH, Fmoc-His(Trt)-OH and Fmoc-Glu(OBu^t)-OPfp were used in coupling steps. When OPfp esters were used the synthesis cycle was as follows: (a) the Fmoc group was removed by 20% piperidine in DMF (5 min); (b) to form a new peptide bound side chain protected Fmoc-aminoacid OPfp ester (4 eq.) and HOAt (4 eq.) were dissolved in DMF and circulated through the column for 30–60 min; (c) to cap residual amino groups the support was treated with 0.3M Ac_2O in DMF for 5 min. If free acids were used the step (b) was: side chain protected Fmoc-aminoacid (4 eq.), HATU (4 eq.) and DIEA (4 eq.) were applied. After addition of the second Cys residue the Fmoc group was removed and Ac_2O applied as mentioned before. For the deprotection a reagent mixture (TFA–phenol–anisole–1,2-ethanedithiol–water, 82:2:2:2:2, 2.5 h) was used. To form the S-S bond the product was dissolved in a minimal amount of DMSO and heated at 65°C under argon for 36 h. The raw HS9510 formed, was purified by HPLC (10 × 250 mm column, Vydac RP C18, 90A, 201HS1010, eluent–24% MeCN in water + 0.1% TFA, detection at 240 nm). Yield 24%. R_f0.69 (1-butanol–pyridine–AcOH–water, 4:1:1:2). k′ = 3.1 (eluent mentioned above). MS data: 1071.4 (M + H). SHU9119 and melanotan II (MTII) were synthesized as described previously (1,12). NDP-MSH was radioiodinated by the Chloramine T method and purified by HPLC. The specific activity of [^{125}I][Nle^4, D-Phe^7]α-MSH was approximately 2000 μCi/nmol.

Expression of Receptor Clones

The human MC1 and human MC5 receptor (3,4) were cloned into the expression vector pRc/CMV (In Vitrogen). The human MC3 and human MC4 receptor DNAs, cloned into the expression vector pCMV/neo, were a gift from Dr. Ira Gantz (8,9). For receptor expression, COS-1 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Eighty percent confluent cultures were transfected with the DNA mixed with liposomes in serum free medium (for details see (24)). After transfection, the serum-free medium was replaced with growth medium and the cells were cultivated for about 48 h. Cells were then scraped off, centrifuged, and used for radioligand binding.

Binding Studies

The transfected cells were washed with binding buffer (25) and distributed into 96 well plates (approximately 40,000 cells/well). The cells were then incubated for 2 h at 37°C with 0.05 ml binding buffer in each well, containing a constant concentration of [^{125}I]NDP-MSH and appropriate concentrations of an unlabelled ligand. After incubation, the cells were washed with 0.2 ml of ice-cold binding buffer and detached from the plates with 0.2 ml of 0.1 N NaOH. Radioactivity was counted (Wallac, Wizard automatic gamma counter) and data analysed with a software package for radioligand binding analyses (Wan System, Umeå, Sweden). Data were either analysed by fitting it to formulas derived from the law of mass-action by the method generally referred to as computer modelling, or by fitting to the four parameter logistic function. K_i-values were calculated by using the Cheng and Prusoff equation (2). The K_d value for [^{125}I][Nle^4, D-Phe^7]α-MSH for the MC1, MC3 and MC5 receptor was taken from (25) and for the MC4 from (24). The binding assays were performed in duplicate wells and repeated three times. Untransfected COS-1 cells did not show any specific binding to [^{125}I]NDP-MSH.

RESULTS

We tested the 2 cyclic lactam MSH analogues MTII and SHU9119, 5 cyclic [Cys^4, Cys^{10}]α-MSH analogues, and nonlabelled NDP-MSH on intact COS-1 cells expressing the human MC1, MC3, MC4 and MC5 receptors with competitive receptor binding assays using [^{125}I]NDP-MSH as radioligand. The structure of the substances we tested are aligned with the structures of α-MSH and NDP-MSH in Fig. 1. For MT II (1) and SHU9119 (12) the ring is closed via the side chains of Lys and Asp. As seen from the Fig. 1, MT II has a D-Phe in position 7, whereas SHU9119 has a D-Nal(2′)^7 (D-2′-naphthylalanine). For all the other cyclic compounds the ring is closed via a disulphide bridge between Cys^4 and Cys^{10} and the substances in this series have L-Phe, D-Phe or D-Nal(2′) in position 7. The K_i values resulting from calculations

TABLE 1

K_i VALUES (MEAN ± SEM) OBTAINED FROM COMPETITION CURVES FOR MSH ANALOGUES ON MC1, MC3, MC4 AND MC5 TRANSFECTED COS-1 CELLS

Receptor Ligand	MC1 K_i (nM)	MC3 K_i (nM)	MC4 K_i (nM)	MC5 K_i (nM)
α-MSH	0.230 ± 0.089	31.5 ± 3.9	900 ± 97	7160 ± 860
NDP-MSH	0.109 ± 0.010	0.469 ± 0.038	2.93 ± 0.34	5.50 ± 0.11
(1–13)D	0.105 ± 0.033	1.78 ± 0.28	0.87 ± 0.50	470 ± 118
(1–13)L	2.06 ± 0.15	91.6 ± 14.0	37.7 ± 15.0	9730 ± 1050
(4–13)D	0.140 ± 0.046	2.00 ± 0.30	1.11 ± 0.26	3790 ± 520
(4–10)D	175 ± 36	475 ± 88	72.5 ± 10.5	15000 ± 4500
HS9510	148 ± 48	216 ± 47	37.0 ± 5.2	5150 ± 530
Melanotan II	0.686 ± 0.109	34.1 ± 4.4	6.60 ± 0.82	46.1 ± 7.9
SHU9119	0.714 ± 0.161	1.20 ± 0.30	0.360 ± 0.059	1.12 ± 0.31

of the radioligand binding competition test of these compounds are summarized in Table 1. The competition curves for the peptides on the different MC receptor subtypes are shown in Fig. 2.

As seen from Table 1 the affinities of the two cyclic lactams were similary high for the MC1 receptor, although being lower compared to the affinity of NDP-MSH, (4–13)D and (1–13)D for the MC1 receptor. However, for the MC4 and MC5 receptors SHU9119 had 8-, and 5-fold higher affinity than NDP-MSH, respectively. It also showed 28-, 18-, and 41-fold higher affinity, respectively, for the MC3, MC4 and MC5 receptors compared to MT II. In fact SHU9119 had the highest affinity among any substance that we have hitherto tested on the MC4 and MC5 receptors. SHU9119 showed quite similar affinities for all the evaluated MC receptor subtypes, with a minor preference for the MC4 receptors (see Table 1 and Fig. 2).

Among the cyclic disulphide compounds both (1–13)D and (4–13)D showed almost identical high affinity for the MC1R, as NDP-MSH. The (1–13)L, had as expected, much lower affinities for all the subtypes; the affinities being, respectively, 1600-, 51-, 43-, and 21-fold lower for the MC1, MC3, MC4 and the MC5 compared with the (1–13)D. The (1–13)L, (1–13)D and (4–13)D showed similar or slightly higher affinity for the MC4 receptor compared to the MC3 receptor. This makes a clear distinction between the [Cys^4, Cys^{10}]α-MSH (1/4–13) peptides from the linear natural MSH peptides that we have tested earlier, since the later all share the same MC1 > MC3 > MC4 > MC5 preference order (10,12). (4–10)D had a unique preference order for the different subtypes: MC4 > MC1 > MC3 > MC5. Replacement of the D-Phe^7 in (4–10)D by D-Nal(2′)^7 (HS9510) resulted in higher affinity for the MC3, MC4 and MC5 receptors, whereas the affinity of MC1 receptor was not affected. HS9510 showed 4-, 6-, and 140-fold higher affinity for the MC4 receptor than for the MC1, MC3 and MC5 receptors, respectively.

DISCUSSION

A large number of MSH analogues had been synthesized and tested on melanophores and melanoma cells prior to the cloning of the different MC receptor subtypes (for review see (7,13). One of the most important findings that emerged from these studies was the was the discovery of NDP-MSH (21). The D-Phe^7 substitution of L-Phe^7 in melanocortic peptides led both to a higher potency and prolonged biological activity (13). Moreover, it was found that substitution of Met^4 by Nle^4 further enhanced the affinity and stability of the peptides. The NDP-MSH had earlier been shown by us and others (14,24,25) to have very high affinity for all of the MC receptors (excluding the MC2 receptor). Cyclic [Cys^4, Cys^{10}]α-MSH analogues are known to be very potent and long acting compounds in frog skin bioassays, but these compounds have hitherto not been tested on the newly discovered MC receptor subtypes. Since such compounds are expected to show less flexibility, information of their binding properties for the different MC receptors are of value for ligand docking and molecular modelling of the MC receptors 3D structure (11,17).

Our present results indicate that the N-terminal (Ser^1-Tyr^2-Ser^3) is not very important for the binding to MC receptors, as (1–13)D had similar affinity as (4–13)D, except for the MC5 receptor where (1–13)D had 8-fold higher affinity compared to (4–13)D. The C-terminal seems to have a much greater importance as the (4–10)D had much lower affinities for all the subtypes, compared with (4–13)D. This may not be surprising, at least not for the MC1 receptor, as the C-terminal tripeptide (Lys^{11}-Pro^{12}-Val^{13}) was reported to be very important for the activity of [Cys^4, Cys^{10}]α-MSH analogues in frog and lizard skin bioassays (5). Interestingly, the loss of binding caused by lack of a C-terminal affects binding to the MC1 receptor to a much greater extent than for the MC4 receptor. Moreover, the cyclic [Cys^4, Cys^{10}]α-MSH analogues have a relatively higher affinity for the MC4 receptor compared with the natural linear MSH peptides (24,25) as all the cyclic [Cys^4, Cys^{10}]α-MSH analogues showed a similar or higher affinity for the MC4 receptor than for the MC3 receptor. The data indicate that both the disulphide bridge and the loss of the C-terminal favours MC4 receptor binding, especially when compared with the MC1 receptor. This relative increase in affinity for the MC4 receptor was further enhanced by replacement of the D-Phe^7 by the bulky aromatic amino acid D-Nal(2′)^7 in HS9510. HS9510 had higher affinity for the MC4 receptor than for the all the other receptor subtypes; the selectivities of the compound being a 4-fold MC4/MC1 preference, a 6-fold MC4/MC3 preference and a 140-fold MC4/MC5 preference.

The two cyclic lactam MSH (4–10) analogues had higher affinities than the corresponding cyclic disulphide bridged (4–10)D and HS9510 compounds. This might, at least partially, be explained by the fact that the lactam analogues contain Nle in position 4, where the disulphide analogues have Cys^4. The Met^4 in the natural MSH peptides is very important for binding as has been shown in earlier studies on melanophores (7). Ala^4 replacement of the Met^4 in α-MSH also resulted in a 140-fold loss in affinity for a mouse melanoma cell receptor (18), and a 14-fold loss of affinity for a rat MC3 receptor (19). The replacement of the Met^4 by Nle^4 in α-MSH has also been shown to increase the affinity to all the

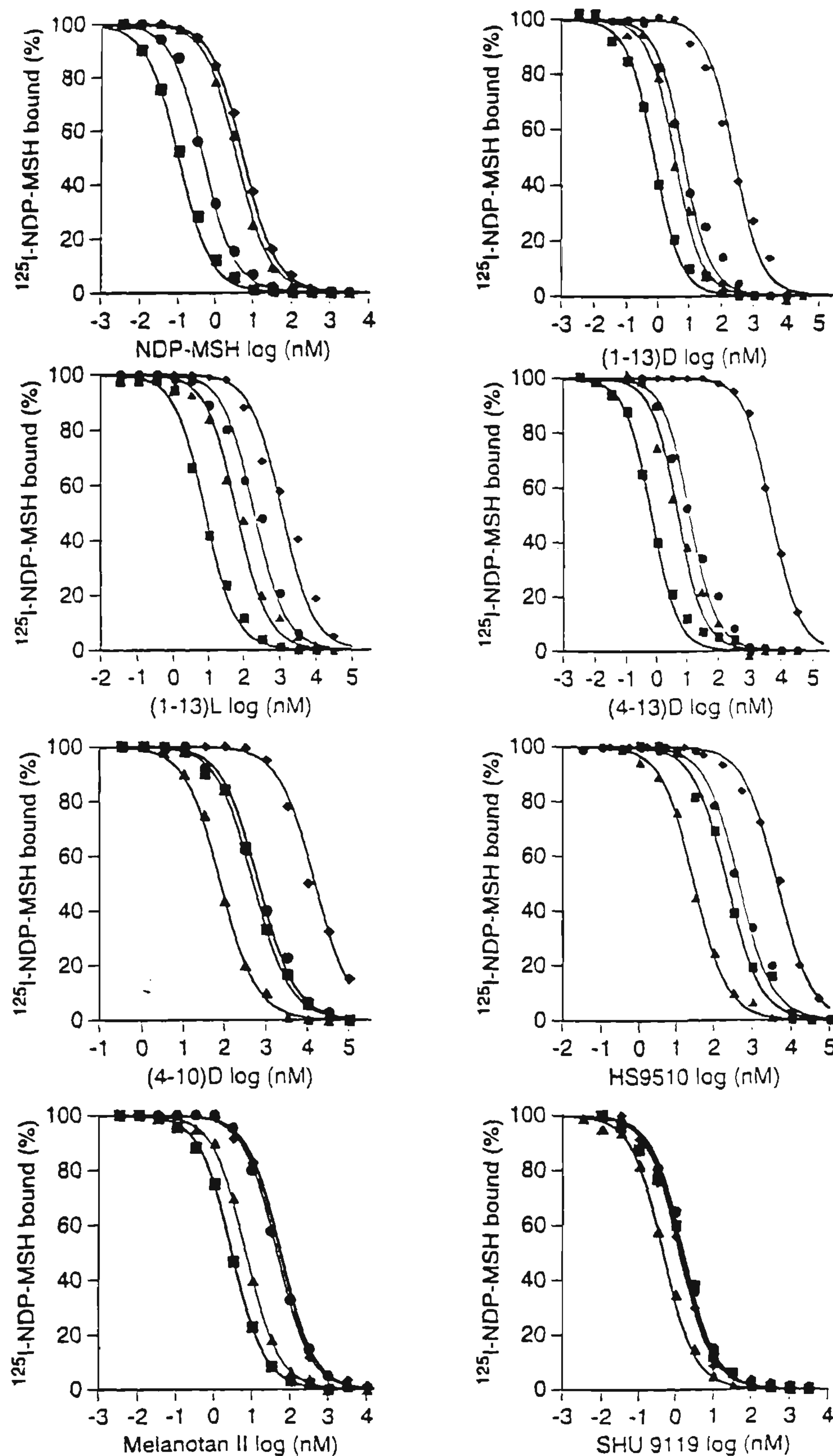

FIG. 2. Competition curves of different MSH analogues for COS-1 cells transfected with the MC1 (■), MC3 (●), MC4 (▲) or MC5 (◆) receptor clones, obtained by using a fixed concentration of ~2 nM [^{125}I]NDP-MSH and varying concentrations of the non-labelled competing peptide. Competing peptides used are indicated on the abscissa for each panel.

different subtypes (24). SHU9119 showed especially high affinities for the MC4 and MC5 receptors, the affinities being higher than for any other tested substance. As already pointed out above, the affinities of SHU9119 were very similar for all the different subtypes thus indicating that it is essentially non-selective for the MC receptor subtypes, which is in agreement with previous data (12). However, when measured as antagonists in a functional assays, SHU9119 was found to be about 10-fold selective for the MC4 vs. the MC3 receptor (12). Nevertheless, interpretation of these functional data should be made with caution as the SHU9119 was a partial agonist for the MC3 and an antagonist for the MC4 receptor.

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Characterization of the binding of MSH-B, HP-228, GHRP-6 and 153N-6 to the human melanocortin receptor subtypes

H. B. Schiöth*, R. Muceniece†, J. E. S. Wikberg*

*Department of Pharmaceutical Pharmacology, Uppsala University, Uppsala, Sweden
†Laboratory of Pharmacology, Institute of Organic Synthesis, Riga, Latvia

Summary We determined the binding affinities of the MSH analogues MSH-B, HP-228 and 153N-6 and of the enkephalin analogue GHRP-6 on a single eukaryotic cell line transiently expressing the human MC1, MC3, MC4 and MC5 receptors. Moreover, we tested the binding and cAMP response of MSH-B in comparison with α-MSH on murine B16 melanoma cells. Our results indicate that MSH-B has a potency similar to that of α-MSH and that these two peptides induce similar cAMP responses in murine B16 melanoma cells. HP-228 has its highest affinity for the MC1 receptor. For the other receptors, it has slightly higher affinity for the MC5 receptor than for the MC3 and MC4 receptors. 153N-6 was found to be selective for the MC1 receptor. GHRP-6 was found to bind to the MC1 and the MC5 receptors despite its low structural homology with α-MSH. [D-Lys³]GHRP-6 bound to all the four MC receptors with similar affinities. The structurally related Met-enkephalin and the functionally related GHRH, as well as LHRH and somatostatin-14 did not bind to these MC receptors. The low affinity of the GH-releasing/enkephalin peptides may indicate that they do not interact with the MC receptors at pharmacologically relevant concentrations.

INTRODUCTION

The melanocortin peptides are known primarily for their role in skin pigmentation and regulation of steroid production in the adrenal gland. In addition, the melanocortins, which include the natural α-, β- and γ-MSH and ACTH, have a broad array of other physiological functions which are much less understood. These include effects on behaviour, memory, thermoregulation, pain perception, nerve regeneration, inflammation, blood pressure and parturition.[1,2]

Cloning of five melanocortin (MC1–5) receptor subtypes has opened new possibilities to elucidate the physiological actions of the melanocortins and their receptors.[3–7] The MC1 receptor has high affinity for α-MSH,[8] plays an important role for pigmentation and is expressed in melanoma cells.[3,4] The MC2 receptor is the ACTH receptor; it binds ACTH with high affinity but not the MSH peptides[9] and is expressed in the adrenal gland.[4] The physiological roles of the other three MC receptors, whose existence was not known prior to their cloning, have been much less characterized. The MC3 receptor is expressed mainly in the brain but has also been detected in peripheral tissues like the gut, placenta and heart.[5,10,11] The MC4 receptor is found only in the brain and has recently been related to weight homeostasis.[12,13] The MC5 receptor is found in the brain and has also a wide peripheral distribution but still has a much less characterized physiological role.[14,15]

α-MSH is selective for the MC1 receptor and ACTH is selective for the MC2 receptor, but none of the natural MSH peptides or other hormones are know to be selective for the newly discovered MC3, MC4 and MC5 receptors.[8,9,16,17] There are only a few reports on specific synthetic analogues for these subtypes.[18,19] More basic knowledge is needed about the binding of the MC receptor subtypes to MSH peptides to elucidate the subtype-specific properties which may allow construction of selective compounds.

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Correspondence to: Helgi B. Schiöth and Jarl E. S. Wikberg, Department of Pharmaceutical Pharmacology, Biomedical Center, Box 591, 751 24 Uppsala, Sweden. Fax: + 46 18 559718; E-mail: helgis@bmc.uu.se, jarl.wikberg@farmbio.uu.se

Table 1 Alignment of peptides evaluated in this study

Peptide position no.	1	2	3	4	5	6	7	8	9	10	11	12	13
α-MSH	Ser	Tyr	Ser	Met	Glu	His	Phe	Arg	Trp	Gly	Lys	Pro	Val
MSH-B	[1]Gly	Tyr	Arg	Met	Gln	His	Phe	Arg	Trp	Gly	Gln	Pro	Leu[2]
MNHdFRWG				Met	Asn	His	D-Phe	Arg	Trp	Gly			
HP-228				Nle	Gln	His	D-Phe	Arg	D-Trp	Gly			
153N-6					Met	Pro	D-Phe	Arg	D-Trp	Phe	Lys	Pro	Val
GHRP-6						His	D-Phe	Ala	Trp	D-Phe	Lys		
[D-Lys[3]]GHRP-6						His	D-Phe	D-Lys	Trp	D-Phe	Lys		

All the peptides have an amide group in the C-terminus. For MSH-B is (1) H-Val-Gln-Glu-Ser-Ala-Asp- in the N-terminus and (2) -Pro-NH_2 in the

Recently, new MSH analogues were isolated from the pituitary gland of a primitive vertebrate, the sea lamprey.[20] One of the peptides, termed MSH-B, was 10 times more potent than α-MSH in a frog skin assay. Another new synthetic MSH analogue, HP-228 was shown to inhibit induction of nitric oxide synthase in vivo.[21] A new MSH antagonist, 153N-6, was identified by screening of a library of MSH(5–13) analogues measuring phototransmission through *Xenopus laevis* dermal melanophores.[22] Structural modifications of Met-enkephalin analogues led to the discovery of potent GH-releasing peptide GHRP-6 (also termed SK&F 110679).[23] A peptide identical to GHRP-6 was identified in a search for a competitive MSH antagonist using frog skin bioassay.[24]

The aim of the present study was to determine the affinities of the above-mentioned and related peptides to the cloned human MC1, MC3, MC4 and MC5 receptors expressed in a single eukaryotic cell line in order to (1) determine if these peptides may serve as leads for synthesis of new subtype selective ligands and (2) clarify their potential usefulness to elucidate the physiological function of the different MC receptor subtypes.

MATERIALS AND METHODS

Peptides

GHRH(1–29) (Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-NH_2), LHRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), somatostatin-14 (Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys, cyclic via Cys-Cys disulphide bridge) and Met-enkephalin (Tyr-Gly-Gly-Phe-Met) were purchased from Neosystem SA, France. 153N-6 was synthesized by Medprobe AS, Norway and also puchased from Bachem, Switzerland. [Nle[4],D-Phe[7]]α-MSH, α-MSH, MSH-B, GHRP-6, [Lys[3]]GHRP-6, HP-228 and 153N-6 were purchased from Bachem, Switzerland. [Nle[4],D-Phe[7]]α-MSH was radio-iodinated by the chloramine T method and purified by HPLC. In the text, we use the position numbering of α-MSH as shown in Table 1.

Expression of receptor clones

The human MC1[3] and human MC5[7] receptor genes were cloned into the expression vector pRc/CMV (InVitrogen). The human MC3[5] and human MC4[6] receptor DNAs, cloned into the expression vector pCMV/neo, were gifts from Dr Ira Gantz. For receptor expression, COS-1 (CV-1 Origin, SV40) cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Eighty per cent confluent cultures were transfected on 100-mm cell culture dishes with the DNA (approximately 1 µg DNA for every 1 × 10^6 cells) mixed with liposomes in serum-free medium. After transfection, the serum-free medium was replaced with growth medium and the cells were cultivated for about 48 h. Cells were then scraped off, centrifuged, and used for radioligand binding.

cAMP assay

Murine B16-F1 melanoma cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Cells were harvested and incubated for 30 min at 37°C with 0.05 ml serum-free Dulbecco's modified Eagles medium in each tube, containing 30 µM IBMX and appropriate concentrations of α-MSH or MSH-B. After incubation with the indicated drugs, cAMP was extracted with perchloric acid at final concentration 0.4 M. After centrifugation, the protein-free supernatants were neutralized with 5 M KOH/1 M TRIS. 0.05 ml of the neutralized cAMP extract or a cAMP standard (dissolved in distilled water) was added to a 96-well microtitre plate. The content of cAMP was then estimated essentially according to Nordstedt & Fredholm[25] by adding to each well [^{3}H]cAMP (0.14 pmol, approximately 11 000 cpm, specific activity 54 Ci/mmol, Amersham) and bovine adrenal binding protein and incubating at 4°C for 150 min. Standards containing non-labelled cAMP were also assayed concomitantly with the samples. The incubates were thereafter harvested by filtration on Whatman GF/B filters using a semiautomatic Brandel cell harvester. Each filter was rinsed with 3 ml 50 mM Tris/HCl pH 7.4. The filters were

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Table 2 K_i values (mean ± SEM) obtained from competition curves for the peptides tested in this study on MC1, MC3, MC4 and MC5 receptor transfected COS-1 cells

Receptor ligand	MC1 K_i (nmol/L)	MC3 K_i (nmol/L)	MC4 K_i (nmol/L)	MC5 K_i (nmol/L)
α-MSH	0.240 ± 0.030	60.0 ± 7.5	645 ± 67	9220 ± 980
MSH-B	0.763 ± 0.141	39.3 ± 4.0	1480 ± 340	1400 ± 760
*MNHdFRWG	2.79 ± 0.08	350 ± 120	285 ± 34	962 ± 250
HP-228	1.62 ± 0.20	73.9 ± 17.0	74.2 ± 18.6	53.4 ± 18.8
153N-6	40.0 ± 2.9	470 ± 24	1340 ± 144	2400 ± 280
GHRP-6	82 000 ± 68.000	>1 000 000	>1 000 000	153 000 ± 43 000
[Lys³]GHRP-6	51 200 ± 30.400	44 200 ± 10 600	120 000 ± 29 000	26 500 ± 9800
GHRH(1–29)	>300 000	>1 000 000	>1 000 000	>1 000 000

*Values for MNHdFRWG are taken from Schiöth et al.[25]

punched out and put into scintillation vials with scintillation fluid and counted. The cAMP assays were performed in duplicate wells and repeated three times.

Binding studies

The transfected cells were washed with binding buffer[3] and distributed into 96-well plates (approximately 40 000 cells/well). The cells were then incubated for 2 h at 37°C with 0.05 ml binding buffer in each well, containing a constant concentration of [^{125}I][Nle^4,D-Phe^7]α-MSH and appropriate concentrations of an unlabelled ligand. After incubation, the cells were washed with 0.2 ml of ice-cold binding buffer and detached from the plates with 0.2 ml of 0.1 N NaOH. Radioactivity was counted (Wallac, Wizard automatic gamma counter) and data analysed with a software package for radioligand binding analyses (Wan System, Umeå, Sweden) by fitting it to formulas derived from the law of mass-action by the method generally referred to as computer modelling. The K_d values for [^{125}I][Nle^4,D- Phe^7]α-MSH for the MC1, MC3, MC4 and MC5 receptors were taken from Schiöth et al.[3,21] The binding assays were performed in duplicate wells and repeated three times. Untransfected COS-1 cells did not show any specific binding to [^{125}I][Nle^4,D-Phe^7]α-MSH.

RESULTS

The human DNAs for the MC1, MC3, MC4 and MC5 receptors were transiently and independently expressed in COS-1 cells for competitive receptor binding assays using [^{125}I][Nle^4,D-Phe^7]α-MSH as radioligand. The expression levels of the different receptor subtypes were similar (data not shown). The K_i values resulting from calculations of the competition curves for α-MSH, MSH-B, HP-288, 153N-6, GHRP-6 and [D-Lys^3]GHRP-6, are summarized in Table 2, together with our previous results of MNHdFRWG (Met-Asn-His-(D-Phe)-Arg-Trp-Gly-NH_2).[26] Competition curves for α-MSH, MSH-B, HP-288, 153N-6, GHRP-6 and [D-Lys^3]GHRP-6 using cells expressing the MC1, MC3, MC4 and MC5 receptor clones are shown in Figure 1.

In addition to testing the binding of α-MSH and MSH-B to the human MC1 receptor expressed in COS cells, we also tested the binding to murine B16 melanoma cells. The K_i values for α-MSH and MSH-B were 0.900 ± 0.045 and 0.910 ± 0.063 nM, respectively. The data show that the affinity of the sea lamprey MSH-B was similar to that of α-MSH in the melanoma cells, but for the recombinant human MC1 receptor expressed in COS cells the affinity of MSH-B was about 3-fold lower than that of α-MSH. For the other receptor subtypes, the MSH-B had affinity similar to that of α-MSH for the MC3 receptor, 2 times lower affinity for the MC4 receptor and about 7 times higher affinity for the MC5 receptor. The preference order of MSH-B for the different receptors is the same as for α-MSH except that MSH-B has similar affinity for the MC4 and MC5 receptors, where, in contrast, α-MSH had more than 10 times higher affinity for the MC4 receptor compared with the MC5 receptor. Both α-MSH and MSH-B are selective for the MC1 receptor.

Murine B16 melanoma cells were stimulated by α-MSH and MSH-B and the cAMP responses were measured. The cAMP responses are shown in Figure 2. The EC_{50} values calculated from these experiments were 0.460 ± 0.202 nM for MSH-B and 2.92 ± 2.41 nM for α-MSH.

HP-228 shares structural similarity with [Nle^4,D-Phe^7]α-MSH(4–10) (Table 1) as well as with the analogue MNHdFRWG, which we have evaluated previously. We found that HP-228 has similar affinity for the MC1 receptor as MNHdFRWG, but about 5-fold higher affinity for the MC3 and MC4 receptors, and 18-fold higher affinity for the MC5 receptor than MNHdFRWG (Table 2).

153N-6 showed its highest affinity for the MC1 receptor and, similarly to α-MSH, lower affinity for the MC3, MC4 and MC5 receptors. 153N-6 was found to be selective for the MC1 receptor, which may not be surprising as 153N-6 was discovered by using tests on dermal melanophores.[22] However, our results are quite discrepant with those of Chhajlani,[27] the most important difference being Chhajlani's K_i value of 955 ± 35.7 nM of 153N-6 for the MC1 receptor compared with our K_i value of 40.0 ± 2.9 nM. Our value is closer to the 11 nM IC_{50} of

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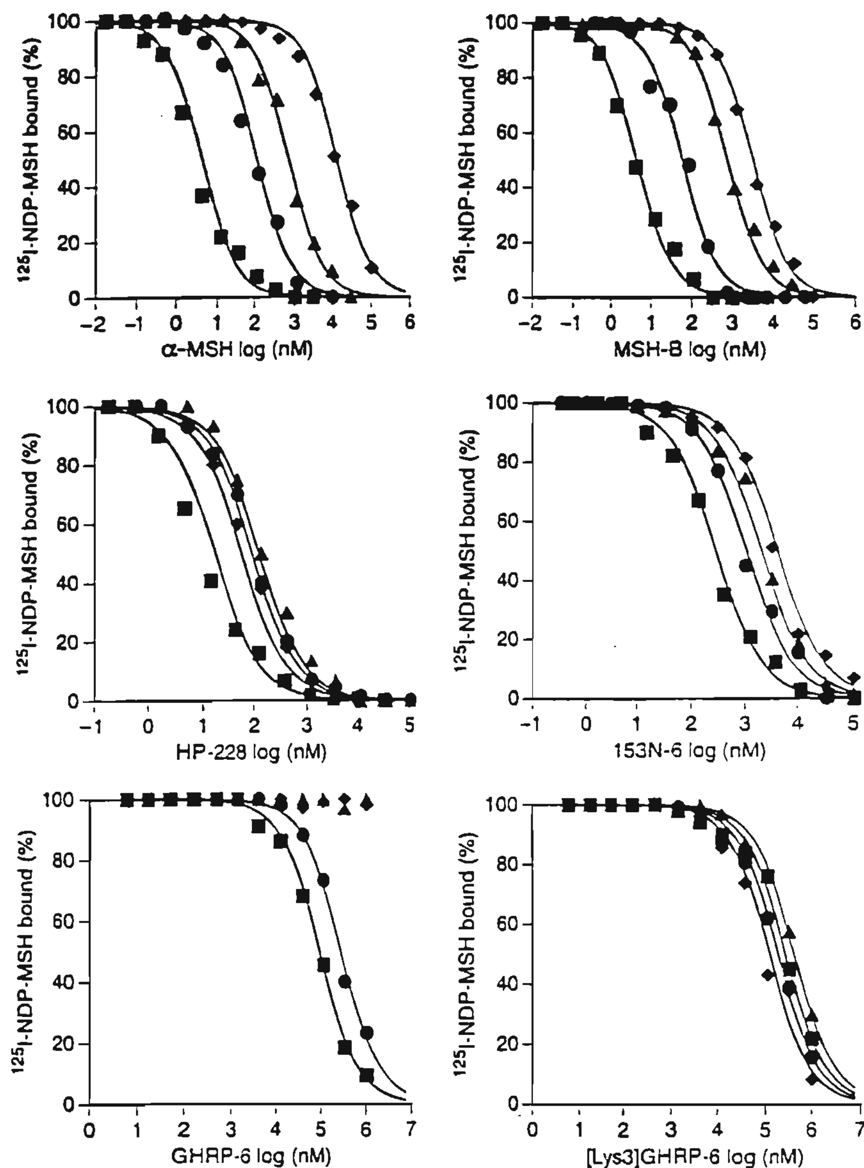

Fig. 1 Competition curves of α-MSH, MSH-B, HP-288, 153N-6, GHRP-6 and [D-Lys[3]]GHRP-6, obtained on COS-1 cells transfected with the MC1 (■), MC3 (●), MC4 (▲) or MC5 (◆) receptor clones, obtained by using a fixed concentration of [^{125}I][Nle[4],D-Phe[7]]α-MSH and varying concentrations of the non-labelled competing peptide. Competing peptides used are indicated on abscissa for each panel.

153N-6 for melanin dispersion in melanophores reported by Jayawickreme et al.[22] Because of these discrepancies, we repeated our testing 6 times for the MC1 receptor. Still, all these tests gave consistently the same results. Moreover, we also tested the affinity of commercially available 153N-6 (from Bachem) for the MC1 receptor. The K_i value for this peptide on the MC1 receptor were also similar to our earlier K_i values received by using custom-synthesized peptide from Medprobe (which are reported in Table 2). Chhajlani's 153N-6 peptide is unfortunately no longer available for retesting.

GHRP-6 bound to the MC1 and MC5 receptors, but with low affinities (Table 2). Up to 1 mM concentrations of GHRP-6 did not displace [^{125}I][Nle[4],D-Phe[7]]α-MSH bound to the MC3 or the MC4 receptors. [D-Lys[3]]GHRP-6, however, bound to all of the MC receptors. [D-Lys[3]]GHRP-6 bound to the MC1 receptor only with slightly higher affinity than GHRP-6. GHRH(1–29) caused partial displacement of [^{125}I][Nle[4],D-Phe[7]]α-MSH bound to the MC1 receptor at a concentration of 300 μM, but not from the other receptor subtypes. 1 mM of either LHRH, somatostatin-14 and Met-enkephalin did

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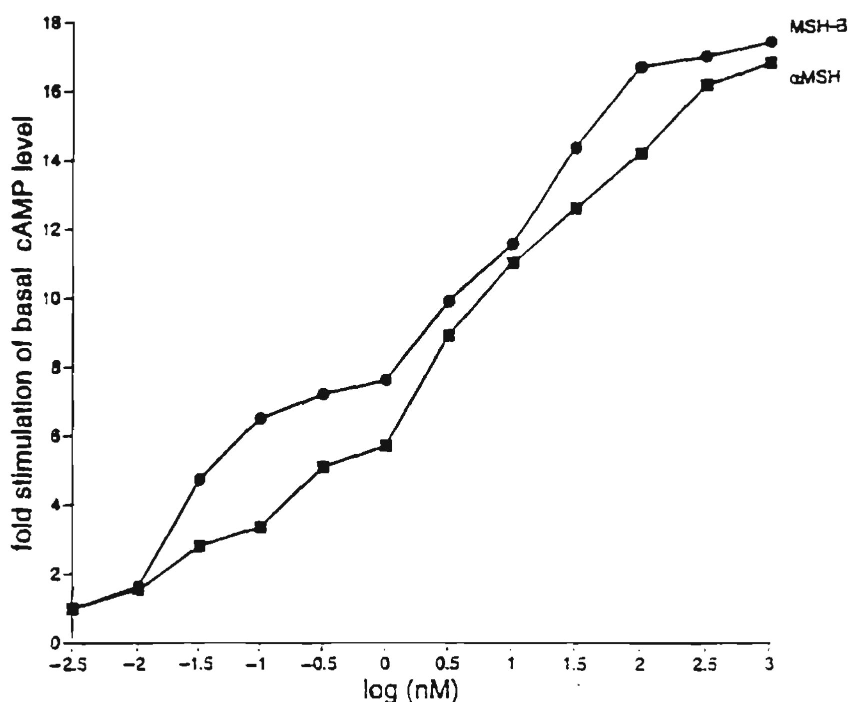

Fig. 2 Fold increase in cAMP response of α-MSH and MSH-B on B16 murine melanoma cells.

not displace [^{125}I][Nle^4,D-Phe^7]α-MSH bound to any of the melanocortin receptors.

DISCUSSION

The melanocortic peptides share a common core of four amino acids: His^6-Phe^7-Arg^8-Trp^9. All compounds known to bind the MC receptors (except agouti, which is a large peptide that does not show any homology with α-MSH) have important elements from this core. As the natural MSH hormones are not selective for the MC3, MC4 and MC5 receptors, and no highly selective synthetic MSH analogues are available, it is tempting to search for other peptides that might bind to the MC receptors, since structural elements in such binders might guide the design and development of selective compounds for each of the MC receptor subtypes. MSH-B, HP-228 and GHRP-6 have not been tested by radioligand binding prior to this study. Moreover, these peptides have not hitherto been tested on the different human MC receptor subtypes.

MSH-A and MSH-B are two peptides that were recently isolated from the pituitary gland of the sea lamprey.[20] MSH-B was reported to be 10 times more potent than α-MSH in a frog skin assay.[20] MSH-B has a free amino group at the N-terminus where α-MSH is acetylated. The N-terminal acetyl group in α-MSH is known to have importance for binding.[8] MSH-B has identical sequence with α-MSH in positions 4–10, which includes the main MSH core, and the important Met.[4] Our results indicate that MSH-B has a potency similar to that of α-MSH for the mammalian MC1 receptor, both in binding to the recombinant human MC1 receptor and to the murine B16 melanoma cell MC1 receptor, as well as in eliciting a cAMP response in the latter cells. We have shown previously that desacetyl α-MSH has higher affinity for the MC3 and MC5 receptors than α-MSH.[3] Interestingly, the MSH-B also has higher affinity for the MC3 and MC5 receptors than α-MSH. Perhaps the N-terminal acetylation is the reason for the differences in affinities of α-MSH and MSH-B, despite the fact that the N-terminus is much longer for MSH-B than for α-MSH.

It has been known for some time that α-MSH acts centrally to inhibit fever, and that it modulates inflammation in the periphery.[28] HP-228 is an α-MSH analogue that has been shown to protect against hypotensive and toxic actions of lipopolysaccharides;[29] an action which may involve induction of nitric oxide production. Moreover, HP-228 was shown to inhibit induction of nitric oxide synthase in vivo.[21] HP-228 does not have any major structural differences compared with [Nle^4,D-Phe^7]α-MSH(4–10). The only difference in the amino acid sequence is that the acidic hydrophilic Glu^5 is replaced by the more neutral Gln^5, and Trp^9 is replaced by D-Trp^9 in HP-228. Glu^5 is not believed to have major importance for the receptor binding, at least not for the murine MC1 and the rat MC3 receptors.[10,31] When comparing HP-228 with MNHdFRWG, it should be considered that HP-228 is N-terminally acetylated whereas MNHdFRWG is not. Our results show that HP-228 shares the α-MSH and

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[Nle^4,D-Phe^7]α-MSH binding properties as it has highest affinity for the MC1 receptor. It also shares the properties of MNHdFRWG and other core MSH peptides by not discriminating between the MC3 and the MC4 receptors.[26] Interestingly, HP-228 has slightly higher affinity for the MC5 receptor than the MC3 and MC4 receptors. HP-228 has 18- and 170-fold higher affinity for the MC5 receptor than MNHdFRWG and α-MSH, respectively. The MC1 receptor is primarily found peripherally in melanocytes and in testis[35] but has also recently been found in murine macrophages where it may participate in autocrine modulation of nitric oxide synthase.[36] The MC5 receptor has been identified in a number of peripheral tissues including tissues associated with the immune system such as lymph nodes, leukocytes, spleen, bone marrow and thymus.[14,15,32–34] The MC3 and MC4 receptors are, on the other hand, found primarily in the brain. HP-228 has its highest affinity for the MC1 receptor and it seems to be the most likely candidate to mediate the immunomodulatory effects of HP-228. The relatively high affinity of HP-228 for the MC5 receptor may perhaps indicate that the MC5 receptor may also participate in eliciting the immunomodulatory effects of HP-228.

GHRP-6 is a Met-enkephalin analogue[23] and a potent GH-releasing peptide, which has also been shown to be active in frog skin bioassay.[24] GHRP-6 does not show much structural similarity with α-MSH (Table 1). It shares only His^6, Trp^9 and Lys^{11} with α-MSH. Phe^7 is replaced by another non-polar hydrophobic residue, D-Trp^7 but, most importantly, Arg^8 in α-MSH is missing in GHRP-6. [D-Lys^3]GHRP-6, which is a putative GHRP-6 antagonist, has closer structural similarity to the MSH peptides. It has the structurally related D-Lys^3 instead of Arg^8. [D-Lys^3]GHRP-6 has, as could be expected, higher affinity for all the MC receptors (especially for the MC3 and MC4 receptors) when compared with GHRP-6. Interestingly, [D-Lys^3] GHRP-6 has only slightly higher affinity for the MC1 receptor compared with GHRP-6 which may indicate that the basic hydrophilic residue in position 8 is not as important for the MC1 receptor as it is for the other MC receptor subtypes. The structurally related Met-enkephalin and the functionally related GHRH did not bind to the MC receptors. The low affinity of the GH-releasing/enkephalin peptides may indicate that they do not interact with the MC receptors at pharmacologically relevant concentrations.

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Expression of Functional Melanocortin 1 Receptors in Insect Cells

Helgi B. Schiöth,* Arja Kuusinen,† Ruta Muceniece,‡ Michael Szardenings,* Kari Keinänen,† and Jarl E. S. Wikberg*,§

*Department of Pharmaceutical Pharmacology, Uppsala University, Uppsala, Sweden; †VTT Biotechnology and Food Research, Espoo, Finland; ‡Laboratory of Pharmacology, Institute of Organic Synthesis, Riga, Latvia; and §Wa Pharm, Umeå, Sweden

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We expressed epitope-tagged human melanocortin 1 receptor (MC1R) in insect cells using two different recombinant baculovirus constructs; one of which encoded MC1R with an N-terminal Flag epitope and a C-terminal polyHis tag, while the other encoded the MC1R with a C-terminal Myc tag. The constructs were used to infect Sf9 insect cells. For both constructs, immunoblotting with tag-specific antibodies demonstrated the presence of the receptor in the infected cells. The infected Sf9 cells were characterized by radioligand binding using [^{125}I][Nle^4, D-Phe^7]α-MSH. Both saturation and competition analysis, using α-, β-, and γ_1-MSH on the tagged MC1R expressed in the insect cells, gave binding constants and potency orders that were undistinguishable from those obtained on MC1R expressed in COS cells. The expression level obtained (in the order of pmoles of binding sites per mg of protein) will now facilitate attempts to purify the receptor. This is the first report that demonstrates a functional expression of recombinant melanocortin receptor in nonmammalian cells. © 1996 Academic Press, Inc.

The melanocortic peptide α-MSH was one of the earliest peptide hormones to be isolated (1). Although the α-MSH and the related hormones β-MSH and γ_1-MSH have a broad range of biological functions (2), the precise physiological roles of the MSH peptides in humans remains largely unknown.

Molecular cloning has identified a family of G-protein coupled receptors that bind and are stimulated by melanocortic peptides (3–7). This family includes five subtypes which were named by their order of cloning. The melanocortin 1 receptor (MC1R) is expressed in human melanocytes where it has a regulatory role in pigmentation as it induces melanoneogenesis after binding to α-MSH. Recent observations also show that MC1R is present in the periaqueductal gray area of human and rat brains (8). The MC2R, which is expressed in the adrenal gland, has high affinity to ACTH which binding induces corticosteroidogenesis (7). The MC3R and MC4R are primarily expressed in the brain where they are proposed to contribute to the behavioral, neurochemical and neurotrophic effects of melanocortic peptides (5,6,9–11). The MC5R has wide peripheral distribution in addition to its presence in the brain (3,12–16).

The molecular cloning of the MCR genes has opened new avenues to study the mechanisms for ligand interactions and the structure of the melanocortic receptors. The MCR subtypes have been individually expressed in mammalian cell lines and their binding to MSH peptides have been characterized (17). Site-directed mutagenesis and molecular modelling of the MC1R has revealed a putative interaction of individual amino acids in the receptor with the MSH ligands (18,19).

The baculovirus expression system is established as one of the most efficient methods for high-level production of functional recombinant proteins (for a review, see Ref. 20). Several G-protein-coupled receptors have been successfully expressed in baculovirus-infected insect cells, and levels comparable to or higher than those obtained in other expression hosts were achieved

Abbreviations: MSH, melanocyte stimulating hormone; MCR, melanocortin receptor; Sf9, *Spodoptera frugiperda*; PCR, polymer chain reaction; COS, CV-1 Origin, SV40.

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(21–23). In the present study we demonstrate high level expression in insect cells of affinity-tagged human MC1Rs, which are pharmacologically undistinguishable from native MC1Rs.

MATERIALS AND METHODS

Chemicals. The [Nle^4, D-Phe^7]α-MSH, α-MSH, β-MSH and γ_1-MSH (H_2N-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-NH_2) were purchased from Saxon Biochemicals GmbH, Germany. [Nle4, D-Phe7]α-MSH was radioiodinated by the Chloramin T method and purified by HPLC.

Construction of plasmids. The DNA sequence encoding the human MC1 receptor (EMBL/GenBank acc.no.X67594) was amplified by PCR using the following primers (written in 5'–3' direction): GTTGGTTCCATGGCTGTGCAGGGATCT-CAGAGAAGACTTCTGGGC (5' sense primer: the underlined sequence encodes the amino acids 1–12 of MC1R; NcoI site in boldtype) and GTTGGTTCTAGACCACGAGCATGTCAGCAC (3' antisense primer: the underlined sequence encodes the amino acids 312–317 of the MC1 receptor; XbaI site shown in bold type). The human MC1 gene (4) in the vector pRc/CMV served as a template. The PCR fragment was excised from an agarose gel, digested with NcoI and XbaI and cloned into two different derivatives of pFASTBAC1 (Gibco-BRL) harboring affinity tags ((24) and K.K., unpublished). Fig. 1 illustrates the structural details of the resulting expression constructs, designated as pK506-1 and pK508-7, encoding MC1R with an N-terminal signal peptide (derived from ecdysteroid UDP-glucosyltransferase of *Autographa californica* nuclear polyhedrosis virus (25)), a Flag epitope (26), and a C-terminal polyHis tag (referred to as Flag-MC1R-His), and MC1R with a C-terminal Myc tag (referred to as MC1R-Myc), respectively. The PCR generated MC1R DNA inserts in pK506-1 and pK508-7 were sequenced to verify their correctness. Recombinant baculoviruses were then generated by using the Bac-to-Bac system (Gibco-BRL) based on site-specific transposition of the expression cassette carried by the recombinant plasmids into a baculovirus shuttle vector (bacmid) maintained in *E. coli* (27). The recombinant bacmids were introduced into *Spodoptera frugiperda* (Sf9) cells by lipofection (Cellfectin, Gibco-BRL) followed by harvest of the recombinant virus from the culture supernatant three days after the transfection.

Expression of MC1R in COS cells. The human MC1R was cloned into the expression vector pRC/CMV (In Vitrogen-Corp., USA) (4). For receptor expression, COS cells were grown in Dulbecco's modified Eagle's medium with 10% foetal calf serum. Eighty percent confluent cultures were transfected with the DNA mixed with liposomes in serum free medium. The liposomes were the commercially available Lipofectin (BRL, USA) or produced according to Cambell (28). After transfection the serum-free medium was replaced with the serum containing medium and the cells were cultivated for ca 48 h. Cells were then scraped off, centrifuged and used for radioligand binding.

Expression of MC1R in Sf9 cells. Standard protocols were used for the maintenance and infection of Sf9 insect cells (29). Cells growing as 50–100 ml suspension cultures (2–3 × 106 cells/ml) in spinner flasks at +27°C were harvested 72 hours after the infection by centrifugation, and analyzed for radioligand binding and by immunoblotting as described below. Sf-900 II (Gibco-BRL) was used as the growth medium for insect cells.

Immunoblotting. Cells were dissolved in SDS and resolved by electrophoresis in 15% SDS-polyacrylamide gels, and transferred to nitrocellulose. The blots were probed with anti-Flag M2 antibody (IBI Kodak), or with anti-Myc 9E10 antibody (30), followed by anti-mouse IgG-alkaline phosphatase conjugate (Bio-Rad). Alternatively, the blots were detected by using a polyclonal antiserum raised against a synthetic peptide corresponding to the N-terminal sequence of MC1R (8), followed by anti-rabbit IgG-alkaline phosphatase conjugate (Bio-Rad). Finally, the blots were developed by using 5-bromo-4-chloro-3-indoylphosphate and nitroblue tetrazolium substrates (Promega).

Binding studies. The Sf9 and COS cells were used directly after the infection/transfection procedure or were taken from frozen stocks. The cells were washed with binding buffer (Minimum Essential Medium with Earle's salts, 25 mM HEPES, pH 7.0, 0.2% bovine serum albumin, 1 mM 1,10-phenanthroline, 0.5 mg per liter leupeptin and 200 mg per liter bacitracin), and then distributed into multi well plates for binding. The cells were then incubated for 2 h at 37°C with binding buffer in each well, containing an appropriate concentrations of [^{125}I][Nle^4, D-Phe^7]α-MSH and unlabelled ligand. After the incubation plates were put on ice, cells washed with ice-cold binding buffer and detached from the plates with 0.1 N NaOH. Radioactivity was counted (Wallac, Wizard automatic gamma counter) and data analyzed with the BindAid software (Wan System AB, Umeå, Sweden). By using BindAid B_{max} and K_d values were thus obtained by fitting saturation curves to formulas derived from the law of mass-action by using the method generally referred to as multicurve modelling. K_i-values were calculated from IC_{50} values of competition curves by using the Cheng and Prusoff (31) equation; the IC_{50} values being obtained by fitting the curves to the four parameter logistic function. For saturation analysis 12 concentrations of [^{125}I][Nle^4, D-Phe^7]α-MSH, ranging 0.02 up to 8 nM, were used. Non-specific binding was determined in the presence of 3 μM [Nle^4, D-Phe^7]α-MSH. All binding assays were performed in duplicate wells in three repeated experiments.

RESULTS

We expressed epitope-tagged human MC1R in insect cells using two different recombinant constructs (see Fig. 1 for details). The recombinant baculoviruses v506-1, which encodes MC1R with an insect virus-derived signal peptide, N-terminal Flag epitope and a C-terminal polyHis tag

FIG. 1. Outline of the Flag-MC1R-His and MC1R-Myc expression constructs.

(Flag-MC1R-His), and v508-7 which encodes the receptor with a C-terminal Myc tag (MC1R-Myc), were used to infect Sf9 insect cells. In western blotting (Fig.2) anti-Flag M2 antibody detected a major 30–32 kDa species, and a minor 64 kDa species in the cells infected with v506-1. No M2 immunoreactivity was observed in cells infected with v508-7, or in uninfected cells (not shown). On the other hand, anti-Myc tag antibody 9E10, recognized a 30–32 kDa major band and

FIG. 2. Immunoblot showing the expression of MC1R in insect cells. Blots were probed with anti-Flag M2 mAb, 9E10 mAb, or with a polyclonal anti-MC1R antiserum as indicated. Lanes: 1, v506-1-infected cells; 2, v508-7-infected cells; 3, control cells infected for expression of a glutamate receptor. The positions of the molecular mass markers are shown at the left (trypsin inhibitor, 20 kDa; triosephosphate isomerase, 26 kDa; maltose binding protein, 43 kDa; bovine serum albumin, 66 kDa).

a 64 kDa minor band in cells expressing MC1R-Myc (infected with v508-7) but not in cells expressing Flag-MC1R-His (v506-1). The labeling illustrates the high specificity of the tag-specific antibodies and demonstrates expression of both MC1R versions in Sf9 cells. Furthermore, a polyclonal anti-MC1R antiserum (8) recognized a broad 30–32 kDa band in cells infected with either v506-1 or v508-7, but not in Sf9 cells expressing a nonrelevant control protein (glutamate receptor GluR-6). From the calculated molecular sizes of Flag-MC1R-His (37.2 Da) and MC1R-Myc (36.4 kDa), it is likely that the smaller 30–32 kDa band represents the monomeric receptor while the 66 kDa band possibly is a receptor dimer.

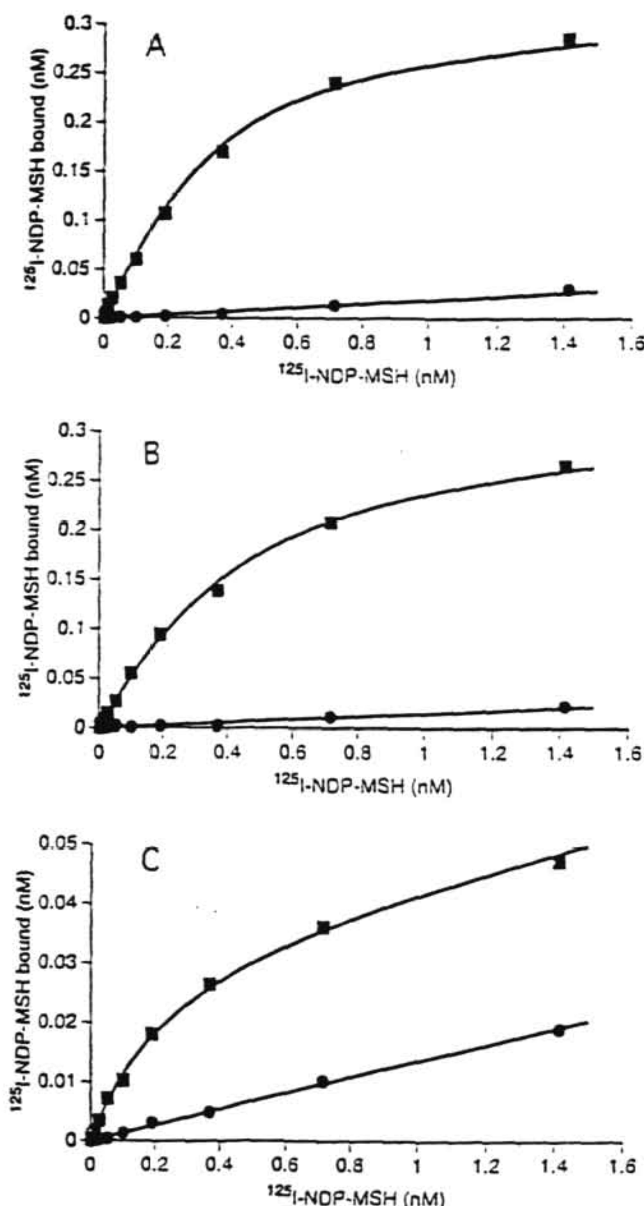

FIG. 3. Saturation curves of [^{125}I][Nle^4, D-Phe^7]α-MSH obtained from MC1-COS cell (A), MC1R-Myc/Sf9 (B), and Flag-MC1R-His/Sf9 (C). The figures show total binding (■) and binding in the presence of 3 μM cold [Nle^4, D-Phe^7]α-MSH (●) for each receptor. Lines represent the computer modeled best fit of the data assuming that ligands bound to one site.

The ligand binding properties of the MC1R expressed in Sf9 cells were characterized in a radioligand binding assay using intact cells. As can be seen in Fig 3, the binding of [^{125}I][Nle^4, D-Phe^7]α-MSH to Sf9 cells infected with v506-1 (Flag-MC1R-His/Sf9), v508-7 (MC1R-Myc/Sf9), as well as to COS cells transiently transfected for expression of the MC1R (MC1R/COS) occured to saturable high affinity sites. Analysis of the binding data by nonlinear curve-fitting revealed that for all three cases binding was to a single class of binding sites with the K_ds being 110 ± 11, 169 ± 31 and 120 ± 14 pmol/l (mean ± S.E.M) for the MC1R/COS cells, Flag-MC1R-His/Sf9 and MC1R-Myc/Sf9 cells, respectively. The B_{max} values obtained from these tests were

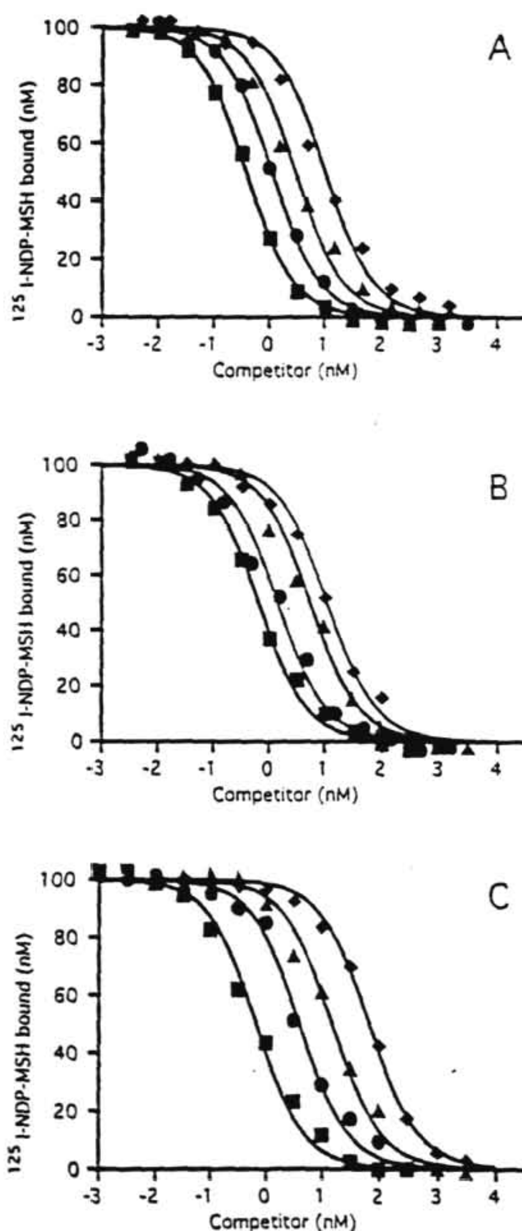

FIG. 4. Competition curves of [Nle^4, D-Phe^7]α-MSH (■), α-MSH (●), β-MSH (▲), and γ_1-MSH (♦) obtained from MC1-COS cell (A), MC1R-Myc/Sf9 (B), and Flag-MC1R-His/Sf9 (C) using a fixed concentration of [^{125}I][Nle^4, D-Phe^7]α-MSH.

1.35 ± 5, 1.56 ± 7 and 0.175 ± 0.005 pmol/mg of total protein for the MC1R/COS cells, Flag-MC1R-His/Sf9 and MC1R-Myc/Sf9 cells, respectively. We further investigated the pharmacological properties of the three expressed MC1Rs by analyzing the ability of some MSH peptides to compete for the [^{125}I][Nle^4, D-Phe^7]α-MSH binding. Fig. 4, show representative competition curves for [Nle^4, D-Phe^7]α-MSH, α-MSH, β-MSH and γ_1-MSH, and Table 1 lists the cognate K_i values. All the tested peptides showed the same order of potency ([Nle^4, D-Phe^7]α-MSH > α-MSH > β-MSH > γ_1-MSH), as well as closely similar IC_{50} values in all the three cells types. Thus, the ligand binding characteristics of the insect cells expressing affinity-tagged MC1Rs are in good agreement with the pharmacological properties of the MC1R expressed in COS cells ((17) and this study), as well as with those naturally present in melanoma cells (32).

DISCUSSION

Previously, MC1Rs have been expressed in mammalian cell systems, like COS, 293 human embryonic kidney cells, Hepa cells, L-cells (murine fibroblast), CHO and Y1 cells (4,5,10,12,13,34). All these cells, whether expressing MC1R in a transient or stable manner, bind MSH peptides in an identical fashion, and thus seem suitable for the pharmacological analysis of the receptor. However, these cell lines may not be ideal for large-scale preparation of MCRs. This is because these cells show low and variable expression of the receptor. Furthermore, scaling up mammalian cell expression is expensive and impractical.

Recombinant baculovirus-based insect cell expression system generally yields high expression levels of functional proteins also in suspension culture. In the present study, insect cells were engineered for expression of two different epitope-tagged versions of the human MC1R. By immunoblotting using tag-specific monoclonal antibodies a dominant 30–32 kDa was detected in the infected insect cells. The molecular sizes predicted by the amino acid sequences of Flag-MC1R-His and MC1R-Myc are slighly higher (36–37 kDa). Partial proteolysis of the receptor is not the likely source for the size difference, because the 30–32 kDa band carried the C-terminal Myc tag in MC1R-Myc and the N-terminal Flag epitope in Flag-MC1R-His. A more plausible explanation for the faster than expected electrophoretic migration of the tagged receptors may be an incomplete denaturation of the samples. We did not boil our samples in SDS prior to electrophoresis because heating invariably led to the conversion of the immunoreactive material into aggregates (also observed for photolabelled MC1R frm B16 melanoma cells (35)), which barely penetrated into the separating gel in SDS-PAGE (data not shown). The extreme hydrophobicity of MC1R, with its high content (50%) of leucine, isoleucine, valine, phenylalanine and alanine, may contribute to this behaviour.

The infected Sf9 cells expressed functional MC1Rs in high numbers. Both tagged receptors expressed in the present study appear to be functionally indistinguishable from the MC1R ex-

TABLE 1

K_d and K_i values (mean ± S.E.M), obtained from saturation and competition curves for MSH peptides on MC1R/COS, Flag-MC1R-His/Sf9 and MC1R-Myc/Sf9 cells

Ligand	MC1R/COS (nmol/liter)	Flag-MC1R-His/Sf9 (nmol/liter)	MC1R-Myc/Sf9 (nmol/liter)
[^{125}I][Nle^4, D-Phe^7]α-MSH^a	0.110 ± 0.011	0.169 ± 0.021	0.120 ± 0.014
[Nle^4, D-Phe^7]α-MSH^b	0.0993 ± 0.004	0.117 ± 0.016	0.0913 ± 0.017
α-MSH^b	0.294 ± 0.061	0.511 ± 0.090	0.182 ± 0.033
β-MSH^b	0.950 ± 0.061	1.23 ± 0.24	1.05 ± 0.04
γ_1-MSH^b	7.69 ± 1.21	12.6 ± 3.7	3.12 ± 0.45

^{a} K_d values from saturation analyses.
^{b} K_i values from competition analyses.

pressed in COS cells as judged from the binding data, both when compared to the mammalian cell expressed MC1Rs characterised in the present report, as well when compared to earlier published data (17,32).

In conclusion, tagged MC1Rs expressed in Sf9 insect cells displayed ligand-binding pharmacology identical to that observed in MC1R expressed in mammalian cells. The expression level obtained (in the range of pmoles of binding sites per mg of protein) will now facilitate the purification of the receptor by using immobilized metal chelation affinity chromatography (His tag) or immunoaffinity chromatography (Flag and Myc epitopes). Such purified receptors will be useful for further biochemical and pharmacological characterization of the MC1R.

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Phage Display Selection on Whole Cells Yields a Peptide Specific for Melanocortin Receptor 1*

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Michael Szardenings‡§, Susanna Törnroth‡, Felikss Mutulis‡¶, Ruta Muceniece‡**, Kari Keinänen‡‡, Arja Kuusinen‡‡§§, and Jarl E. S. Wikberg‡

*From the ‡Department of Pharmaceutical Pharmacology, Uppsala University, S-75 124 Uppsala, Sweden, the ¶Department of Medicinal Chemistry, Latvian Institute of Organic Synthesis, LV-1006 Riga, Latvia, the **Laboratory of Pharmacology, Latvian Institute of Organic Synthesis, LV-1006 Riga, Latvia, and the ‡‡VTT Biotechnology and Food Research, FIN-02044 Espoo, Finland*

A phage display system for the selection of peptides binding to heterologously expressed human melanocortin receptor 1 on the surface of insect cells has been established. It could be shown that phage particles displaying the natural ligand α-melanocyte-stimulating hormone bind selectively to cells expressing this receptor and that these phages exhibit biological activity on mouse B16F1 melanoma cells. Insect cells were superior to other cell lines tested and have been used to select binders from a small library, in which critical determinants (Phe^7-Arg^8-Trp^9) were kept, whereas the flanking regions where allowed to variate freely. One peptide displaying little similarity with native hormone was found that binds to the receptor also in its free form with an affinity of 7 nM. It showed a remarkable selectivity for this receptor, because it binds to the other melanocortin receptor subtypes with a maximum affinity of 21 μM. This is the first time phage display has been used successfully with G-protein-coupled receptors lacking an extracellular binding domain.

Phage display techniques, *i.e.* the display of libraries of peptides, enzymes, antibodies, and other proteins on the surface of bacteriophages and selection of functional sequences thereof, have undergone a rapid development (1–3). Originally used as selection systems to identify peptide epitopes (4, 5), phage display is today in use for almost any kind of problem that involves the interaction of peptides and proteins with other materials. Most of the published literature deals with the development of antibodies (6–8). With the use of this technique one comes close to the power of the human immune system. Other milestones were the display of enzymes (9), enzyme inhibitors (10), hormones (11), and cloning of active protein domains (12), to list only a few applications. Among the numerous publications about the display of hormones there are only a few dealing with the bioactivity of the phage (for examples see Refs. 13 and 14), because in almost all cases purified domains of cell surface receptors are used for the selection of binders from libraries (for examples see Refs. 11 and 15). Only a very few successful experiments are published with receptors displayed on living cells and in all such cases the receptors bind the ligands with extracellular domains (16–18).

The selection of random libraries of small peptides is naturally rendered more difficult when working with impure target proteins. Libraries of larger protein ligands usually contain common structural features directing and restricting binding of phages to the intended target. Especially G-protein-coupled receptors for small ligands that bind the ligands within their transmembrane helices have been the exclusive target for chemical peptide and other compound libraries (19–23). These can be analyzed and deconvoluted by other means than just the affinity of the compounds, as has been shown for example for the MC1 receptor (24), but they lack the complexity of bacteriophage displayed libraries. Because these receptors have not yet been purified in quantities and in a quality sufficient for the standard panning of phages, the selection on isolated membranes or whole cells remains the only possibility.

Good candidates for studying whether phage panning on such receptors is possible in principle against these odds are the recently cloned melanocortin receptors. Some of their ligands, the family of melanocortin peptides, have been known already for a long time for their effects on pigmentation in melanocytes (α-MSH)[1] and for regulation of steroid production in the adrenal gland (adrenocorticotropic hormone). Already in early studies it was recognized that they display numerous other effects (25, 26), which is now confirmed and explained by molecular cloning of different receptor subtypes.

The first receptor to be cloned was the melanocortin receptor (MC1) of the melanocytes, soon followed by the identification of the adrenocorticotropic hormone receptor of the adrenal gland (MC2) and three other receptor subtypes (MC3, MC4, and MC5) with initially unknown functions (27–31). The distribution of these receptors in different tissues is known (29, 31–33), but the cellular function of these three subtypes is still not identified. Only the MC4 receptor, which is found in many tissues of the brain (30, 34), has recently been identified to be involved in weight homeostasis (35, 36).

Aside from the MC2 receptor, all of them bind the 13-amino acid-long α-MSH or analogues with nanomolar and subnanomolar affinities (37–40), but ligands highly specific for a single receptor subtype have not been found up to now. Intense studies have been carried out on the characterization and identification of residues involved in binding of the receptors, and the synthesis of numerous natural and unnatural peptides have been tried to achieve peptides with selectivity for a single

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§ To whom correspondence should be addressed: Dept. of Pharmaceutical Pharmacology, Biomedical Center, Box 591, S-75 124 Uppsala, Sweden. Fax: 46-18-559718; E-mail: msz@bmc.uu.se.

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[1] The abbreviations used are: α-MSH, α-melanocyte-stimulating hormone; gpIII, product of bacteriophage *fd* geneIII; MC, melanocortin; PCR, polymerase chain reaction.

receptor (24, 41, 42). A common and essential motif of natural MSH peptides is a stretch of four amino acids (His[6]-Phe[7]-Arg[8]-Trp[9]) believed to be essential for binding, an usually short N terminus preceding this sequence, and a less size-restricted C-terminal sequence (25). These are features that should allow the design of phage libraries, where the C terminus is linked to the phage protein. It should also be possible to improve the selection procedure by using only partially randomized sequences with the motif His[6]-Phe[7]-Arg[8]-Trp[9] or parts thereof. This should restrict specific phage binding to the receptor protein.

This report shows that phages displaying an α-MSH-*gp*III fusion protein bind specifically to cells expressing the MC1 receptor and even retain a biological activity. A selection system based on heterologously expressed receptors on the cell surface was established and was exploited to select novel peptides specific for the MC1 receptor from a phage display library.

MATERIALS AND METHODS

Cloning Procedures—Plasmid pComb3d derived from pComb3 (43) with the light chain cloning sites removed as a *Nhe*I fragment (44) and pG3H6 (45), a derivative of pHEN (7), were used in this study. The plasmids were cut with *Eco*RI/*Sma*I and *Pst*I/*Xho*I, respectively. PCR reactions were carried out in 100-μl volumes with 5 μM dNTPs and 2 units of Vent or exo⁻-Vent-polymerase (Biolabs) in the buffer supplied with the enzymes. In a typical PCR reaction about 500 ng of template DNA and 20 pmol of each primer were used. The PCR reactions were carried out in a Biometra Trio PCR cycler using the following cycling: three cycles of 94 °C for 30 s, 48 °C for 30 s, and 72 °C for 5 min; seventeen cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 5 min); and then one cycle of 72 °C for 10 min. The primers were kinased with T4 polynucleotide kinase (Boehringer Mannheim) prior to PCR. The oligonucleotides used in this study were MS-4pcr (5′-AACAGGTTTACCCCAACGGAAGTGCTCCATAGAGTAAGAGGCCA-TGGCTGGTTGGGCAGC-3′) and MS-5pcr (5′-VNNVNNVNN-VNNCCAACGGAAVNNVNNVNNVNNVNNAGAGGCCATGGCTGG-TTGGGCAGCGAGTA-3′) (N = A, C, G, or T; V = A, C, or G) with a 3′ sequence complementary to the *pel*B leader sequence gene in both vectors and cp3–1 (5′-CCCGGGGCCGCATAGACTGTT-3′) and cp3–198 (5′-CCCGGGGGCGGTGGCTCTCCA-3′) partially complementary to the 5′ region of *gene*III in the vectors pG3H6 and pComb3d, respectively.

PCR products were purified by agarose gel electrophoresis (46). Ligation reactions were carried out with T4 ligase at a DNA concentration of 4–6 μg/ml in 500-μl volumes at 15 °C overnight. For standard transformation a simple one-step procedure was used (47). For library construction the ligation products (3 μg) were purified on a silica support (JetPure, Genomed) and used for 12 individual electroporations of XL1-Blue using Electroporator II (InVitrogen) according to the manufacturer's descriptions.

Preparation of Phage—All media used were ISO approved and obtained from Difco or Oxoid. To avoid counterselection due to overexpression of the *gp*III fusion protein only freshly transformed bacteria were used. Precultures of XL1-Blue harboring the phagemid were grown in Antibiotic-Medium-3 with 30 μg/ml tetracycline and 250 μg/ml ampicillin at 37 °C. dYT medium (10 g/liter yeast extract, 16 g/liter BactoTryptone, 5 g/liter NaCl) with ampicillin was inoculated 1:100 with this culture, and library transformed cells were directly added to about 200 ml in a 1-liter erlenmeyer flask. At an A_{555} of 0.5–0.7 the culture was superinfected at a multiplicity of infection of 5 with low titer M13KO7 (48) prepared as in Ref. 49. After 1 h, kanamycin (30 μg/ml) and optionally isopropyl β-D-thiogalactoside (0.5 mM) were added. The culture was shaken an additional 8–12 h, the bacteria were removed carefully in 1–2 centrifugation steps, and phages were precipitated in the cold by addition of ¼ volume filtered 20% PEG6000, 2.5 M NaCl and centrifugation at 20,000 × *g*. The resulting phage pellet was resuspended in ¹⁄₁₀ of the original volume 100 mM HEPES (pH 7.2), centrifuged to remove insoluble debris, precipitated a second time, and finally resuspended in ¹⁄₁₀₀ of the original volume HEPES buffer. These phage preparations did not disturb the growth of HEK293 or COS-1 cells if diluted 1:500 in cell culture medium. Aliquots of purified libraries were stored at −70 °C with 50% glycerol.

Expression of Melanocortin Receptors—The melanocortin receptors were cloned into pZeoSV (InVitrogen). The resulting vectors were used for transformation of the mammalian cell lines HEK293 (50) and COS-1 (51) using liposomes (52) as described previously (53). Cells were cultivated in Dulbecco's modified Eagle's medium with 10% fetal calf serum at 7% CO_2.

Transfected cell lines were kept under selection of 180 μg/ml, respectively, 250 μg/ml zeocin for at least 2–3 days. COS-1 cells could even be distributed two or three times under selection pressure without loss of receptors. Sf9 (54) and High Five (InVitrogen; Ref. 55) insect cell lines were grown in synthetic medium (SF900II, Life Technologies, Inc.) and infected with baculovirus v508–5 carrying a gene for the hMC1 receptor as described (40) in T80 flasks. 48 h after infection the cells were dissociated by pounding the flasks several times on the table and collecting cells by centrifugation.

Peptide Synthesis—Peptides were synthesized on a Pioneer Peptide Synthesis System (PerSeptive Biosystems) and purified by high pressure liquid chromatography. The molecular weight of the peptides was confirmed by mass spectrometry.

cAMP Stimulation—B16F1 cells expressing the mMC1 receptor (56) were grown as above. Cells were detached from almost confluent adherent cultures and incubated for 30–60 min at 37 °C in ordinary growth medium containing 0.5 mM of the phosphodiesterase inhibitor 3-iso-butyl-1-methyl-xanthine. 50-μl aliquots of the hormone dilutions in growth medium were prepared in 1.5-ml tubes and placed in a water bath at 37 °C. For the stimulation about 3 × 10^5 cells in 450 μl were quickly added to each tube to obtain immediate mixing. After 20 min, 50 μl of 4.4 M perchloric acid were added, mixed, neutralized after a few minutes by the addition of 50 μl of base (5 M KOH, 1 M Tris), and centrifuged. The determination of cAMP in the resulting supernatant was carried out as described (57).

Binding, Competition, and Selection Experiments—All binding and selection experiments with phages as well as radio ligand competition experiments were carried out in buffers and under conditions as described previously for COS-1 (37) and Sf9 (40) cells; in addition, 1 mM 2-mercaptoethanol was added to protect the free sulfhydryl groups of some peptides. Phage binding experiments were usually carried out with total phagemid titers of 1 × 10^9 colony-forming units/ml. For competition experiments with pGEM7 (Promega) phagemid solution usually contained about 2% pMS4-23 or pMS4-26. 2.5 × 10^6 insect cells or 1 × 10^6 COS-1 were incubated at 37 °C gently shaken in a water bath in 4 ml of phage suspension in binding buffer (Ref. 37, minimal essential medium with proteinase inhibitors) for 30 min. The cells were washed four times with 4 ml of binding buffer after centrifugation at a maximum of 1000 × *g* for 10 min. Finally only 1 ml of binding buffer was added, and the incubation was extended to 30 min. Cells were harvested by centrifugation at 5000 × *g*, and aliquots of the cell pellet and this final supernatant were used to infect logarithmic cultures of XL1-blue at different dilutions. After 30 min of incubation at 37 °C, the bacteria were plated on agar plates containing 250 μg/ml ampicillin, 0.5 mM isopropyl β-D-thiogalactoside, and 40 μg/ml X-Gal. Blue/white colonies were counted after 20 h of incubation in the dark at 37 °C. In library panning the entire cell pellet was used to infect 100 ml of XL1-blue at A_{555} = 0.4–0.6 and grown as described above. For library analysis an aliquot was plated on agar plates containing 250 μg/ml ampicillin 1–2 h after the infection.

RESULTS

Investigations on the Selection System—To display α-MSH in an active form on bacteriophages constructs were made by long inverse PCR amplification of the entire plasmids. Although we are still not satisfied with the quality of the DNA obtained, other groups recently applied a similar approach for codon mutagenesis (58) and deletions (59), indicating that this still could be a useful approach for the generation of peptide libraries in smaller vectors. Two constructs were made originally, based on the vectors pG3H6 (45) and a pComb3 derivative (43, 44) fusing the sequence of human α-MSH to the N terminus (pMS4-23 from pG3H6) and amino acid 198 (pMS4-26 from pComb3d) of bacteriophage *fd gene*III as outlined in Fig. 1. Cyclizing the PCR product with DNA ligase resulted in vectors containing no additional sequences above those needed for the phage display. pMS4-23 behaved similarly to pMS4-26, but we did not study this construct in great detail, because it turned out to be difficult to obtain clean phage preparations due to lysis of the bacterial host. Both constructs required induction by isopropyl β-D-thiogalactoside, otherwise no enrichment on

```
                                          /-------------------
                                          MetLysTyrLeuLeuProThr
5' [illegible] 3'
1
-----------pelB leader--------------------\
AlaAlaAlaGlyLeuLeuLeuLeuAlaAlaGlnProAlaMetAlaGlnValLysLeuLeu
5' [illegible] 3'
61           3' [illegible]
             MSK 5-per                    )
    5' [illegible]
              W  R  F                 S

                /EcoRI                     /SmaI
GluValAspGlyIleAspLysLeuAspIleGluPheLeuGlnProGlyGlySerThrSer
5' [illegible] 3'
121
             /------- geneIII (Pro198-Ser406) ------------
GlyGlyGlyGlySerProPheValCysGluTyrGlnGlyGlnSerSerAspLeuProGln
5' [illegible] 3'
5' [illegible] 3'
ProGlyGlyGlyGlySerPro
   cp3-198
```

Fig. 1. Location of primer hybridization sites for library generation with long inverse PCR. Nucleotides 1–180 of pComb3d are displayed. Primer sequences and resulting amino acid sequences are in *bold*, denoted in one-letter codes for MS5-per to indicate the inverted direction. MSH5-per results in a direct fusion of the degenerated sequence to the *pelB* leader sequence. cp3–198 with a Pro-Gly-Gly-Gly linker hybridizes to Ser[198] of bacteriophage *fd geneIII*. Ligation of the PCR product "removes" approximately 70 nucleotides from the polylinker site.

Table I
Enrichment of pMS4–26 phagemids over pGEM7 phagemids

Experiments presented here were carried out using COS-1, baculovirus-infected SF9 and High Five cells expressing the MC1 receptor, infected SF9 cells expressing a subunit of the glutamate receptor (65), and ordinary High Five cells as a control. The table shows the enrichment of pMS4–26 over pGEM7 relative to the original relation 1:50 in the final washing solution and the cell pellet (see "Materials and Methods") and the number of experiments carried out. Each individual experiment had been carried out with different cells and/or phage preparations, showing a clear enrichment for the α-MSH displaying phages at least in the insect cell pellets. No. exp., number of experiments.

	COS mc1		SF9 glu		SF9 mc1		Hi-5		Hi-5 mc1	
	Wash	Pellet	Wash	Pellet	Wash	Pellet	Wash	Pellet	Wash	Pellet
Average enrichment	×1.72 ± 0.21	×1.75 ± 0.75	×1.5 ± 1.65	×1.71 ± 1.07	×2.5 ± 0.55	×7.05 ± 3.22	×1.69 ± 0.41	×2.76 ± 1.3	×2.32 ± 0.94	×15.9 ± 1.34
No. exp.	$n = 5$	$n = 5$	$n = 4$	$n = 4$	$n = 6$	$n = 6$	$n = 2$	$n = 2$	$n = 4$	$n = 4$

cells expressing the MC1 receptor was found (data not shown).

The phagemids obtained were tested for specific binding to the hMC1 receptor heterologously expressed in the mammalian cell lines HEK293 and COS-1, as well as baculovirus-infected insect cells. We tried to improve the amounts of receptor generally obtained in our laboratory in mammalian cell culture using the vector pZEO-SV (InVitrogen). COS-1 cells expressing the large SV40 antigen maintained this plasmid due to its SV40 origin and could be replated at least three times under selection without loss of receptor expression, reducing the experimental effort involved in the transfection of cells, but these procedures did not improve the number of binding sites significantly above levels published earlier (40). Insect cells infected with baculovirus v508-6 (40) turned out to express at least the same number of receptors per cell, although they are substantially smaller in size.[2]

Because panning with adherently growing cells failed, gentle centrifugation of detached cells had to be used instead. HEK293 cells lysed during these procedures. COS-1 cells were more stable and used for more intensive studies together with different insect cell lines. The binding experiments were carried out with pMS4-26 in direct competition with a 50-fold excess of phagemids derived from pGEM7, which allowed easy evaluation of the binding experiments after infecting *Escherichia coli* XL1-blue and counting blue (pGEM7) and white colonies on X-Gal plates (Table I). Because incubation in the final washing step for more than 1 h was not sufficient to remove the majority of bound phage, we used also the resuspended cell pellet to infect XL1-blue. The addition of the MC1 ligand [Nle[4], D-Phe[7]]α-MSH (2 μM) had no effect on phage elution in the final step, but the same concentration did abolish all enrichment when applied in the first incubation step (data not shown). This is not unusual for this receptor, because the complexes of the MC1 receptor with natural and synthetic ligands also exhibit high stability and low dissociation constants (60). In general only a very weak enrichment of pMS4-26 phage particles over pGEM7 on COS-1 expressing the receptor could be seen. This may be caused by a high unspecific binding of phage particles to the membranes under the conditions used for selection, and a similar tendency had been found earlier (17). Recently published data about peptides selected on insect cells revealed a strong preference for sequences containing tryptophan and arginine as present in all displayed peptides (18). There seemed to be a slight enrichment of pMS4-26 on insect cells without the MC1 receptor, but the statistics of these data were weak and seemed not to rectify alternating library selection on insect cells and mammalian cells as had been necessary to obtain ligands for the urokinase receptor (17). The difference of binding on Sf9 and High Five cells may simply be due to a slightly higher expressional level found with the latter. These expression levels vary even within our lab, and the conclusion that those cells are in general better should not be drawn from these data.

Aside from evaluating specific binding, the bioactivity of the α-MSH displaying phages was tested by investigating cAMP stimulation in B16F1 (56) cells (Fig. 2). There is about one active peptide displayed per two or three phages according to these results, if one assumes a stimulatory efficacy close to that of α-MSH. These results were further substantiated by Western blotting experiments with phagemid supernatant. Using a monoclonal antibody against *gp*III (61), the blots showed an estimated amount of about one fusion protein per ten full length *gp*III for pMS4-26 phagemids (Fig. 3). Western blots with polyclonal antibodies against α-MSH showed a strong band for pMS4-26 and only a very weak response for pMS4-23, above an overall high background (not shown).

Library Construction and Selection—A small library (about 10^5 individual clones) was constructed to test this phage dis-

[2] M. Szardenings, unpublished results.

play system using a partially randomized oligonucleotide (MS-5pcr) that kept the MSH-core sequence Phe[7]-Arg[8]-Trp[9], which is believed to be most essential for receptor binding, to direct phage binding toward the receptors. The Ser at the junction with the *pelB* leader sequence was also maintained to reduce

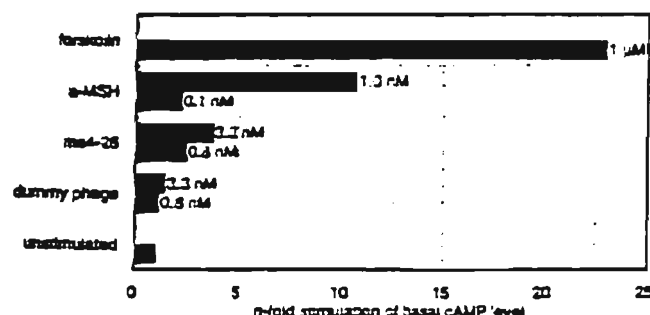

FIG. 2. Stimulation of murine B16F1 melanoma cells with pMS4-26 derived phage particles. The figure compares the relative stimulation by this phage at a 0.3 and 3.3 nM concentration to a phage prepared from unmodified pComb3d at the same concentrations, α-MSH (0.1 and 1.0 nM) and 1 μM forskolin.

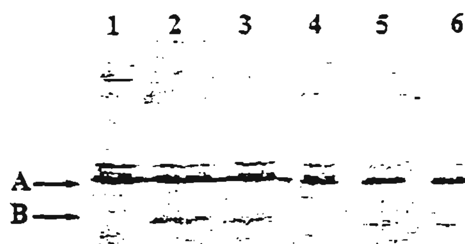

FIG. 3. Western blot of phage particles. ~3 × 10^{12} and ~1 × 10^{12} phage particles from pGEM7 (*lanes 1* and *3*) and two different preparations of pMS4-26 (*lanes 2* and *5* and *lanes 3* and *6*) were run on a 10% SDS-polyacrylamide gel electrophoresis, blotted on nitrocellulose membrane (Bio-Rad), and detected with a primary monoclonal antibody against *gp*III (61) and a secondary antibody coupled to alkaline phosphatase (Sigma). The *arrows* point toward the band corresponding to the native *gp*III (*A*) and the truncated fusion protein with the α-MSH peptide (*B*); the band above *A* is probably an artifact of the SDS-polyacrylamide gel electrophoresis, largely depending on the amount of protein in *A*. This picture was taken with a CCD72 video system (Dage-MTI Inc.) and analyzed with NIH image software (Wayne Rasband, NIH). Evaluation of counts over background for the different dilutions showed that the larger bands are about 3–15 times more intense than the smaller bands, depending evaluation conditions and the inspected lane.

TABLE II
Analysis of the library selection experiments

The library phages were mixed 1:50 with pGEM7-derived phagemids. The percentage of library phage present in each selection step was determined in the blue/white screening assay.

Round of selection	Wash	Pellet
	%	
1	1.9	0.7
2	32.0	34.0
3	19.0	33.0

the amount of sequences that may not be processed by the leader peptidase. We observed problems in the specificity of PCR reactions, low transformation efficiencies of the PCR derived vector DNA as well as truncated sequences in the resulting libraries. These were partially abolished by using exo⁻-Vent polymerase, indicating mispriming and partial digestion of the degenerated sequence by exonuclease activities during the PCR reaction. Three rounds of selection were run on infected High Five insect cells. Blue/white colony screening experiments were carried out in parallel by mixing library phages with pGEM7 phages to evaluate the binding properties of the entire library (Table II). After three rounds of selection, a significant enrichment of cell surface bound phages could be found.

To our surprise numerous mutated vectors were found to be enriched in the final library that carried deletions in the cloning region. 50 clones were analyzed, 43 had changes that could be easily detected by restriction analysis, and only 4 could be sequenced unambiguously (Table III). All peptides were synthesized as amides to avoid an extra negative C-terminal charge, and affinities to the hMC1 receptor were characterized in standard ligand binding assays with both the insect cell and the COS-1 expressed receptor (Table IV and Fig. 4). Values obtained for both cell types were identical as could be expected from earlier studies (40) and are therefore not listed separately. The only high affinity ligand MS-04 was further tested for its selectivity for the hMC1 receptor compared with the other MSH binding receptors (Table V). To rule out possible oxidation or dimerization caused by Cys[12], an analogue with Ala[12] (MS-4ala), was also synthesized and tested; it behaved similar but had an overall lower affinity.

The peptide MS-04 was also compared with α-MSH and [Nle[4], D-Phe[7]]α-MSH in a cAMP stimulation experiment on B16F1 cells. MS-04 exhibited very weak agonistic activity (Fig. 5).

DISCUSSION

The data presented here prove that panning of phage displayed peptide libraries on whole cells expressing the MC1 receptor using partially randomized libraries can yield specific high affinity ligands. This opens new ways to find leads for the design of potential ligands for receptors of this type. It may be useful soon to obtain the desired highly selective ligands for the other MC receptors, which are needed to enable studies on their biological function in more detail.

We have shown that α-MSH displaying phages not only bind specifically to cells expressing the MC1 receptor, but they also exhibit biological activity. Based on this knowledge we have been able to select binders from a phage display library. The library used in the selection was rather small with only 10^5 individual clones, but our intention was first to test whether this system yields ligands for the target receptor before putting effort into the preparation of large libraries. The degenerated oligonucleotide MS-5pcr renders random genes with no adenine in the third position of all randomized triplets to reduce the number unwanted of stop codons (TAA, TGA) in this

TABLE III
Comparison of phage display-derived synthesized peptides with α-MSH and the primer defined sequence

Underlined and double underlined sequences in the α-MSH sequence are taken from Ref. 63; substitution by alanine in these positions resulted in loss of affinity by a factor of more than 10 and 100 times, respectively.

MS4pcr	Ser*******************Phe-Arg-Trp***************ProGly...
MS-01	Ser-Ser-Leu-Glu-Cys-Ser-Phe-Arg-Trp-Gly-Pro-Glu-His-NH_2
MS-02	Ser-Val-Thr-Val-Val-Pro-Phe-Arg-Trp-Tyr-Ser-Cys-Ser-NH_2
MS-03	Ser-Leu-Asp-Phe-Asn-Ser-Phe-Arg-Trp-Cys-Ser-Ala-Leu-NH_2
MS-04	Ser-Ser-Ile-Ile-Ser-His-Phe-Arg-Trp-Gly-Leu-Cys-Asp-NH_2
MS-04ala	Ser-Ser-Ile-Ile-Ser-His-Phe-Arg-Trp-Gly-Leu-Ala-Asp-NH_2
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2

TABLE IV
K_i values for the different peptides on the MC1 receptor

All values are in nM rounded to the first three digits. std. dev., standard deviation; n.o.e., number of experiments.

Substance	MS-01	MS-02	MS-03	MS-04	MS-04ala
K_i mean	3700.0	43000.0	15900.0	7.6	29.5
K_i std. dev.	580.0	12600.0	4070.0	2.4	14.4
n.o.e.	4	4	4	10	12

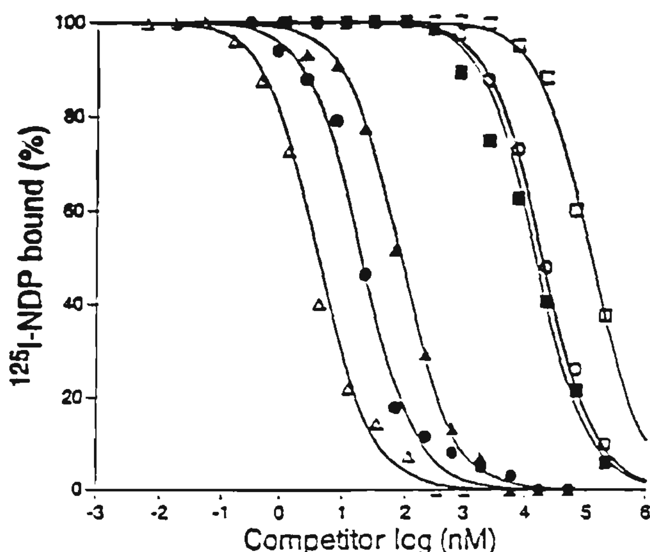

FIG. 4. **Radio ligand binding with the MC1 receptor.** Competition curves of α-MSH (△), MS-04 (●), MS-04ala (▲), MS-01(■), MS-02 (□), and MS-03 (○) obtained on SF9 cells infected with baculovirus v508-6 (40) were obtained at a fixed concentration of ~0.3 nM [^{125}I][Nle[4], D-Phe[7]]α-MSH (*^{125}I-NDP*).

TABLE V
K_i values for MS-04, MS-04ala, and the natural ligand α-MSH (37) on different melanocortin receptors

All values are in nM rounded to the first three digits.

Receptor	MC1	MC3	MC4	MC5
MS-04	7.6	21,000 ± 11,000	>>50,000[a]	>>50,000[a]
MS-04ala	29.5	120,000 ± 50,000	>>50,000[a]	>>50,000[a]
α-MSH	0.21	53.2	2,033	4,990

[a] Cells expressing MC4 and MC5 have under some circumstances given some inconsistent binding in the 10–100 μM range but with very bad statistics of the calculated curve. Regularly we have not observed any competition with 200 μM as the highest competitor concentrations running control experiments with [Nle[4], D-Phe[7]]α-MSH in parallel.

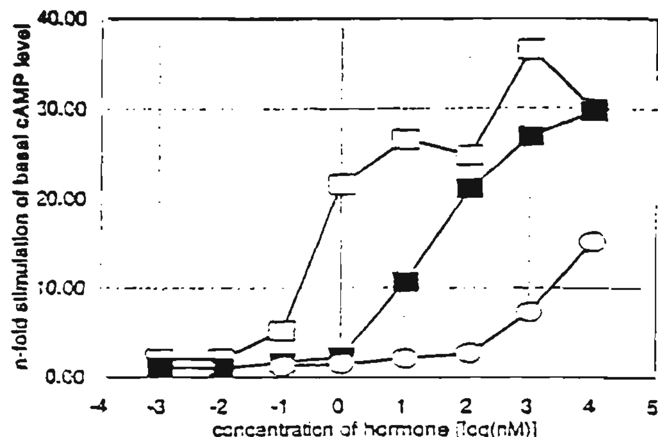

FIG. 5. **Stimulation of B16F1 cells with MS-04.** The cells were stimulated with the strongest stimulator known, NDP ([Nle[4], D-Phe[7]]α-MSH) (□), the natural ligand, α-MSH (■), and MS-04 (○) as described under "Materials and Methods."

library (62).

The deletion problems observed among the selected clones are of minor importance for the final results, because the size of the library would anyway not allow real statistical analysis of multiple clones. These truncated clones may have been selected by some affinity to other surface proteins of the insect cells. At a first glance the variable regions of the four sequences determined in the end do not very much resemble each other or α-MSH, aside from His[6] in one sequence and Gly[10] in two sequences and the amino acids defined in the primer sequence (Ser[1]-Phe[7]-Arg[8]-Trp[9]). Only one of these sequences, MS-04 with 7.6 nM, has a submicromolar affinity to the MC1 receptor (Table IV and Fig. 4). This is in accordance with the idea that the structure of peptides fused to *gp*III will often be different from the soluble peptide due to interactions within the fusion protein (1). It also supports the idea that His[6] and Gly[10] are of at least structural importance for the binding of the free peptide, and their substitution by other amino acids may be the reason for the low affinity of the other peptides. Table III summarizes the results obtained with Ala scanning of α-MSH (63). It can be seen that positions important for binding and biological activity in α-MSH are the worst conserved in the low affinity peptides obtained by phage selection.

Met[4] has been replaced by Nle in the best known binder [Nle[4], D-Phe[7]]α-MSH, and hydrophobic amino acids are found in this area in all four peptides. MS-04 has again the closely related Ile[4] in this position, whereas the low affinity peptides contain amino acids more different from Met in this position. Small amino acids, among them four cysteines, seem to be slightly over represented and none of the amino acids with large side chains (Arg, Trp, Lys, Met, and Gln) were found, aside from Glu, which might be favored as a charge compensation to Arg[8]. The cysteines made it necessary to run all assays in the presence of reducing agent, otherwise the measured binding constants were not reproducible.

The affinity to the heterologously expressed receptors was identical for insect cells and COS-1 cells, confirming the structural identity of both receptor proteins (40) and excluding any bias during the selection impeded by the local environment of the receptor. The most important observation is that the peptide MS-04 binds not tightly to any of the other receptors, as most artificial and natural ligands do, which excludes the possibility of a binding caused solely by interactions of the conserved region (His[6]-Phe[7]-Arg[8]-Trp[9]). MS-04 discriminates more than ten times better between the MC1 receptor and the closely related MC3 receptor than the natural ligand α-MSH. The importance of the flanking regions in the peptide, selected by phage display, is further confirmed by the fact that a single replacement of Cys[12] to Ala in MS-04/MS-04ala reduced the affinity to the MC1 receptor. This position is also of importance for the binding affinity of α-MSH (Table III). It indicates that the peptides presented here interact with the receptor differently. This could be confirmed for MS-04, which exhibits only week agonistic activities at rather high concentrations (Fig. 5). It can therefore be concluded that the conservation of the N- and C-terminal regions among all natural MSH peptides (26) is most important for agonistic behavior but not for the binding itself.

In summary we have shown that selection of bacteriophage presented peptides is possible even on peptide receptors like the melanocortin receptors by targeting the peptide toward the receptor with a conserved sequence motif. The peptides obtained in this work are already valuable for the design of new ligands for the MC1 receptor and may also turn out to be useful for further modelling studies (64). MS-04 is the most selective

ligand for the MC1 receptor described so far. Now more efforts are needed to create larger libraries and select them also on the other melanocortin receptor subtypes. The door is wide open for new specific peptides to examine the role of these receptors *in vivo*.

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