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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 *Xba*I/*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

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                  ** TM 1 ****---------- IL 1 ----------*** TM 2 *********
mc1-protein   60: AlaThrIleAlaLysAsnArgAsnLeuHisSerProMetTyrCysPheIleCysCysLeu
mc1-DNA      180: gccaCCaTcGcCAaGAACcGgAACCTGCACTCaCCcATGTACTgCTTcaTCTGCtGCCTG
                                  <--GGACGTGAGTGGGTACATGA-5' (P1)
mc3-DNA      180: ctggCCgTgGtCAgGAACgGcAACCTGCACTCcCCgATGTACTtCTTtcTCTGCaGCCTG
mc3-protein   60: LeuAlaValValArgAsnGlyAsnLeuHisSerProMetTyrPhePheLeuCysSerLeu

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                  --------- IL 2 ----------**********  TM 4 *****
mc1-protein  146: PheTyrAlaLeuArgTyrHisSerIleValThrLeuProArgAlaArgArgAlaValAla
mc1-DNA      438: TTcTACGCaCTgCGCTACCACAGCATCgTGACCcTGccGcgGGCgCggcgagccgTtGcG
                       (P2)5'-TACCACAGCATCgTGACC-->
mc3-DNA      438: TTtTACGCgCTcCGCTACCACAGCATCaTGACCgTGagGaaGGCcCtcaccttgaTcGtG
mc3-protein  146: PheTyrAlaLeuArgTyrHisSerIleMetThrValArgLysAlaLeuThrLeuIleVal

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                  ******** TM 6 ************************************-- EL 3 -
mc1-protein  246: LeuGlyIlePhePheLeuCysTrpGlyProPhePheLeuHisLeuThrLeuIleValLeu
mc1-DNA      731: CTGGGCaTtTTCtTCcTCTGCTGGGgCCCCTTCTTCCTgCAtCTcacaCTCATCgTCctC
                       (P3) 5'-TCTGCTGGGGCCCCTTCTT-->
mc3-DNA      734: CTGGGCgTgTTCaTCtTCTGCTGGGcCCCCTTCTTCCTcCAcCTggtcCTCATCaTCacC
mc3-protein  247: LeuGlyValPheIlePheCysTrpAlaProPhePheLeuHisLeuValLeuIleIleThr
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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.

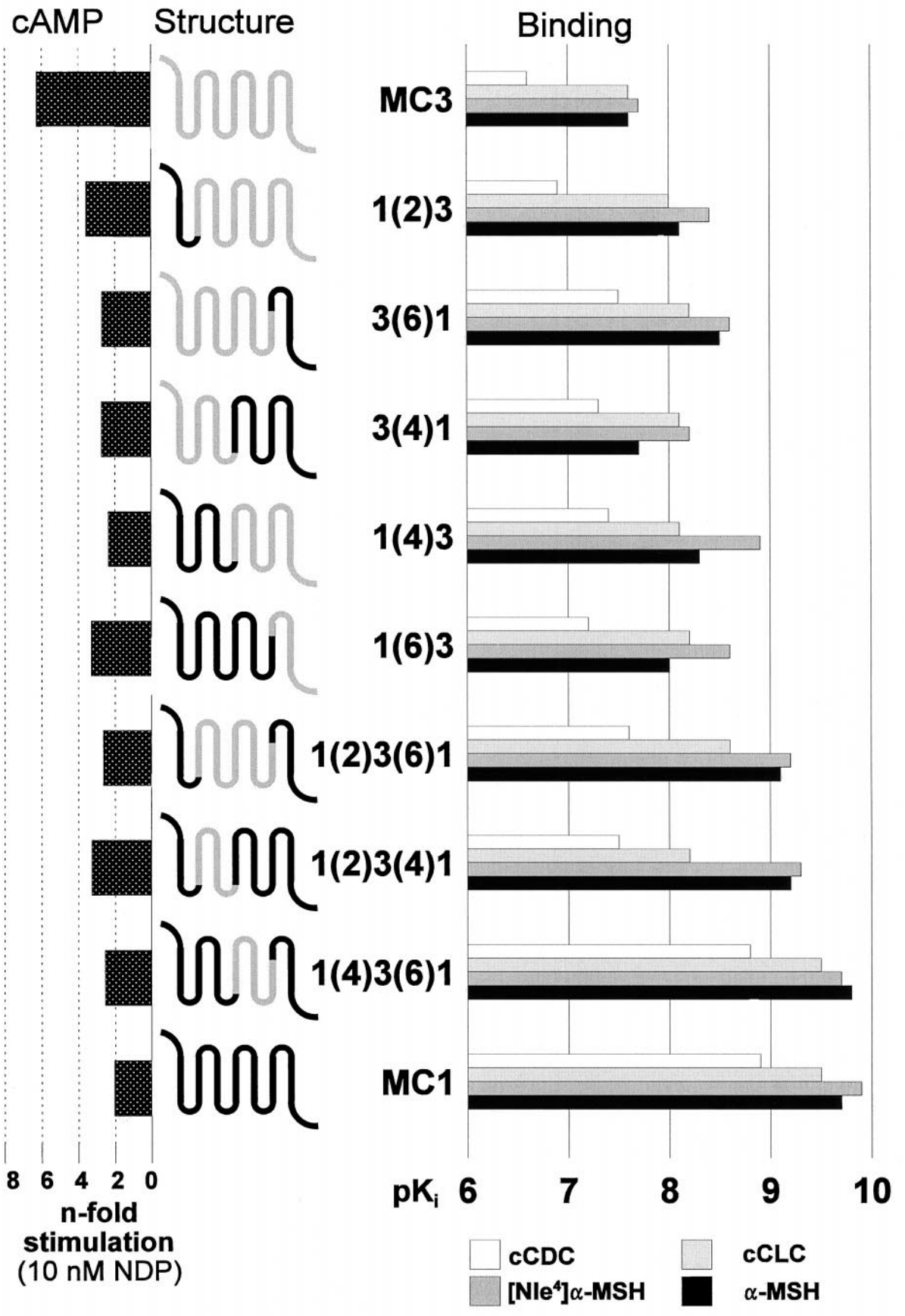

Fig. 2. Schematic representation of the structure of the chimeras aligned with graphical presentation of cAMP response and pK_i values for [Nle^4]α-MSH, α-MSH, cCDC, and cCLC.

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

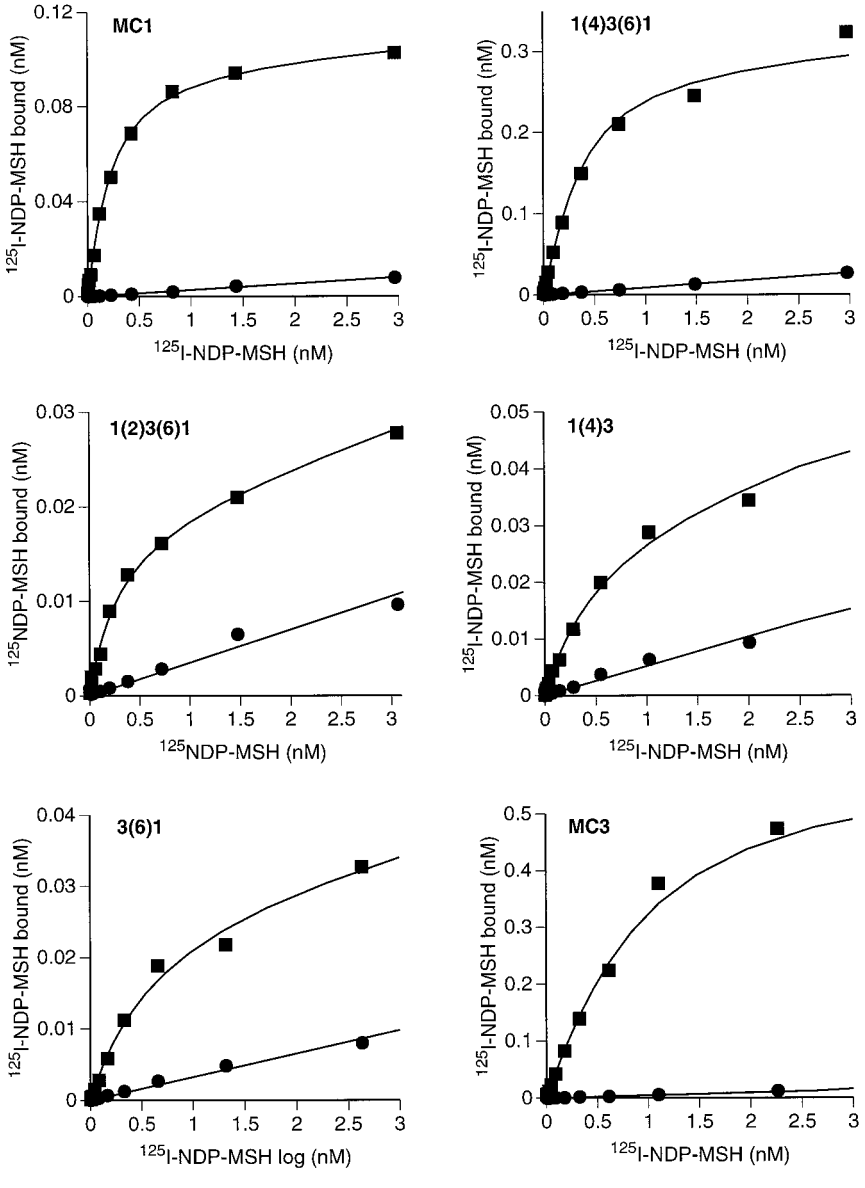

Fig. 3. Saturation curves of [^{125}I]NDP-MSH obtained from transfected COS cells. The figures show total binding (■) and binding in the presence of 3 μM cold NDP-MSH (●) for the MC1, 1(4)3(6)1, 1(2)3(6)1, 1(4)3, 3(6)1, and MC3 receptors. *Lines*, computer-modeled best fit of the data using a one-site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

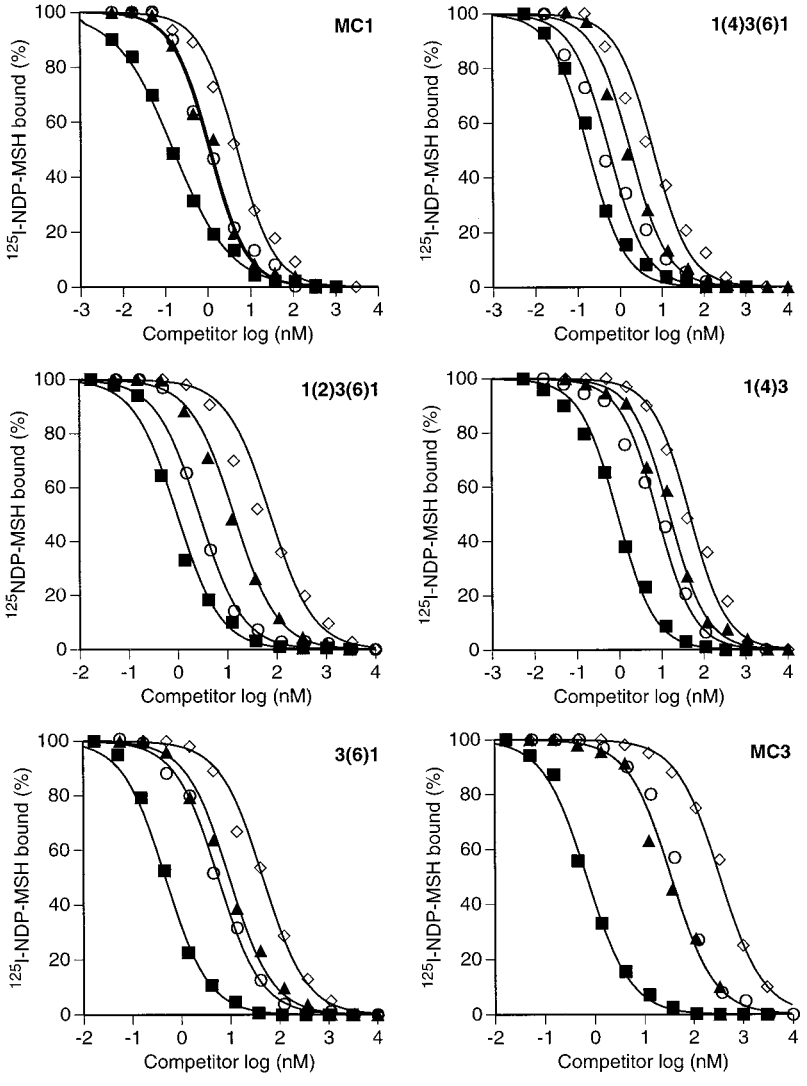

Fig. 4. Competition curves of NDP-MSH (■), α-MSH (○), cCDC (▲), and cCLC (◇) obtained on the MC1, 1(4)3(6)1, 1(2)3(6)1, 1(4)3, 3(6)1, and MC3 receptors by using a fixed concentration of [^{125}I]NDP-MSH.

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