Conformation of the Core Sequence in Melanocortin Peptides Directs Selectivity for the Melanocortin MC3 and MC4 Receptors*

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The melanocortins, adrenocorticotrophic hormone, α-, β-, and γ-melanocyte-stimulating hormone (MSH),1 are derived from the precursor protein pro-opiomelanocortin. Pro-opiomelanocortin is expressed in the pituitary gland, in two brain nuclei (1), and in several peripheral tissues (2). Effects of melanocortins have been described on behavior (3), metabolism (4), fever, inflammation (5), analgesia (6), addiction (7), nerve regeneration (8), and the cardiovascular system (9, 10). Since virtually all [125I]NDP-α-MSH-binding sites overlap with expression of either MC3R and/or MC4R mRNA, these are the main MC receptors in the brain affecting the variety of effects of melanocortin peptides (11–13). The melanocortin (MC) receptor subtypes, MC1R, MC2R, MC3R, MC4R, and MC5R (14–20) constitute a subfamily within G-protein-coupled receptor superfamily. MC receptors differ in ligand binding specificity as well as in tissue distribution. Insight into these two aspects is essential to understand the physiological functions of the pro-opiomelanocortin/MC receptor system. Therefore, it is essential to identify ligands that can discriminate between the MC3R and the MC4R.

The development of selective ligands for the MC receptors has been hampered by the absence of detailed knowledge about the structural requirements of peptide ligands for selective MC receptor binding and activation. Nevertheless, it has been demonstrated that HFRW (MSH (6–9)) forms the core sequence of melanocortins, which is necessary to bind to all MC receptors (21–23). Ligand selectivity may therefore be determined by residues outside the core region either through a selective interaction with different receptor subtypes, by altering folding of the core sequence, or by a combination of both.

Although the MC3R and MC4R both recognize α-MSH, the affinity of α-MSH is 50-fold higher for the MC3R. Of the three forms of γ-MSH, γ1- and γ2-MSH (11 and 12 amino acid residues, respectively) are most related, while γ3-MSH has an extended C terminus. In vivo amidation of the C-terminal Gly12 residue of γ2-MSH results in the formation of γ1-MSH with an C-terminal Phe11-amide. In mammals, the natural forms of γ-MSH contain an additional N-terminal Lys residue (3). α-MSH and Lysγ-MSH both contain the core sequence, HFRW, a Tyr residue at position 2 and a Met residue at position 4, while N- and C-terminal residues and the residue at position 5 differ (Fig. 1). Recently it was shown that Asp10 in Lysγ-MSH determined MC3R selective activation (24). However, it is not clear whether replacement of Asp10 in Lysγ-MSH increases binding affinity or only increases efficacy for the MC4R. Moreover, it remains to be determined whether there exists a direct selective interaction of Asp10 with the MC3R, or whether Asp10 induces a peptide structure that is favorable for the MC3R. To solve this problem a more detailed analysis is required regarding the contribution of each individual amino acid in the ligand to receptor binding and activation. Therefore, the aim of this study was to gain insight into the molecular mechanism of the selectivity of γ2-MSH for the MC3R versus the MC4R. Using a gain of function approach, we tested [Nle4]α-MSH and [Nle4]Lysγ2-MSH and derivatives with exchanged amino acid residues on in vitro binding and activation of wild type and chimeric MC3R/MC4R and mutant MC4Rs. We demonstrate here that the selectivity of γ2-MSH for the MC3R versus the MC4R is determined by a single amino acid residue in the ligand and, to a large extent, a single residue in the receptor. A new concept is proposed in which MC3R/MC4R selectivity is determined by how the melanocor-

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1 The abbreviations used are: α-MSH, γ1-MSH, γ2-MSH, and γ3-MSH, Lysγ2-MSH, and derivatives with exchanged amino acid residues on in vitro binding and activation of wild type and chimeric MC3R/MC4R and mutant MC4Rs. We demonstrate here that the selectivity of γ2-MSH for the MC3R versus the MC4R is determined by a single amino acid residue in the ligand and, to a large extent, a single residue in the receptor. A new concept is proposed in which MC3R/MC4R selectivity is determined by how the melanocor-

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Fig. 1. Comparison of the amino acid sequence of α-MSH and Lys-γ2-MSH.

MATERIALS AND METHODS

Peptides—NDP-α-MSH (Nle^4,D-Phe^2]-α-MSH), α-MSH, and γ2-MSH were purchased from Bachem Feinchemicalien, Bubendorf, Switzerland. Lys-γ2-MSH was synthesized by the National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, The Netherlands. γ1-MSH was a gift from Organon, Oss, The Netherlands. MT-II was a gift from V. Hruby (Tucson, AZ). [D-Tyr^7]MT-II, [Nle^4]MT-II, [Nle^4,D-Phe^7]2-MSH, and [Nle^4,Phe^12]2-MSH were synthesized by Salomon (29, 30). In short, after prelabeling with 500 μCi of [125I]Iodine (NEN Life Science Products Inc.) in a concentration of 2 μCi/ml, the 293 cells were incubated for 20 min at 37 °C in phosphate-buffered saline containing 0.1 mM isobutylmethylxanthine (IBMX), 1 μM forskolin, and agonist in a concentration ranging from 10^{-11} to 10^{-5} M. The cells were harvested and [125I]Iodination was determined. For each peptide 12 duplicate data points were measured. EC_{50} values were then calculated with a 95% confidence interval using GraphPad Prism software (sigmoidal dose-response curve fitting, variable slope).

Receptor Binding Assay—Transfected HEK 293 cells were grown in poly-L-lysine-coated 24-well Costar plates. Two days after transfection, the cells were incubated with 100,000 cpm of [125I]NDP-α-MSH and various concentrations of peptides diluted in binding buffer consisting of Ham’s F-10 medium (Life Technologies, Inc.) (pH 7.4) containing 2.5 mM calcium chloride, 0.25% bovine serum albumin, 10 μM Hepes, and 50 μg/ml (150 KIU/ml) aprotinin. After incubation for 30 min at room temperature, the cells were washed twice with ice-cold Tris-buffered saline containing 2.5 mM calcium chloride and lysed in 1 mM sodium hydroxide. Radioactivity of the lysates was counted in a Packard Cobra gamma-counter. Competition curves were fitted from 11 duplicate data points with GraphPad Prism software. Nonlinear regression, one site competition, K values were calculated using the Cheng and Prusoff equation with 95% confidence interval. Experiments were repeated at least twice with the same results.

RESULTS

Characterization of the Reference Peptides—In order to delineate α/γ-MSH selectivity we first excluded influence of peptide length, N- and C-terminal modifications, and oxidation of the Met residue, and therefore used synthetic [Nle^4]-α-MSH and [Nle^4]Ac-Lys-γ2-MSH-NH₂ as reference peptides (the latter will be referred to as [Nle^4]Lys-γ2-MSH).

Table I shows that, using displacement of [125I]NDP-α-MSH, the affinity for [Nle^4]-α-MSH, as compared with α-MSH, was increased about 2-fold for both the MC3R and the MC4R. Also [Nle^4]Lys-γ2-MSH showed increased affinity for both the MC3R and the MC4R as compared with γ1-MSH, γ2-MSH, Lys-γ2-MSH, and [Nle^4]γ2-MSH. On the MC4R, [Nle^4]Lys-γ2-MSH displayed a 2-fold lower affinity and 20-fold lower activity of cAMP-dependent β-galactosidase activity than [Nle^4]-α-MSH (Table II). On the MC3R, however, [Nle^4]Lys-γ2-MSH exhibited a 3.5-fold higher affinity and similar activity as compared with [Nle^4]-α-MSH. Even though [Nle^4]Lys-γ2-MSH had a 6-fold lower affinity for the MC4R than for the MC3R, [Nle^4]Lys-γ2-MSH maintained selectivity for the MC3R, since the affinity is almost 50 times higher for the MC3R than for the MC4R. Thus, [Nle^4]Lys-γ2-MSH and [Nle^4]Ac-Lys-γ2-MSH Residues in Binding and Activation of the MC3R and MC4R—First, each amino acid residue in [Nle^4]-α-MSH was substituted separately for the corresponding residues of [Nle^4]Lys-γ2-MSH. When Ser^1, Ser^3, Glu^5, Pro^12, or Val^13 were substituted for Lys^1, Val^3, Gly^5, Phe^12, and Gly^13 respectively, no significant effect was observed on affinity or activity for the MC3R or the MC4R as
compared with reference peptide [Nle4]-α-MSH (upper panel of Table I). The only exception was [Nle4,Phe12]-α-MSH, which exhibited a 2-fold decrease in activity for the MC4R and a 2-fold increase in affinity for the MC3R. However, when Gly10 or Lys11 of [Nle4]-α-MSH were substituted for Asp10 or Arg11 a clear loss of affinity and activity was observed for both the MC3R and MC4R. When the Gln2 to Gly2 substitution was combined with the Gly10 to Asp10 substitution in [Nle4]-α-MSH, the affinity and activity for the MC4R decreased more than 3-fold, whereas the affinity for the MC3R remained the same as for [Nle4]-α-MSH.

Next, each amino acid residue in [Nle4]Lys-γ2-MSH was substituted for corresponding residues of [Nle4]-α-MSH (lower panel of Table II). When Lys1 was substituted for Ser1, a slight decrease in affinity was observed for both MC3R and MC4R but the activity remained unaffected. Substitution of Arg11 for Lys11 slightly increased activity for both receptors. Substitution of Val12 for Ser12 or Gly12 for Val12 decreased affinity for only the MC4R, while the activity remained unaffected. With respect to binding affinity, [Val12]Lys-γ2-MSH displayed the largest difference between MC3 and MC4 as did [Ser12]Lys-γ2-MSH in activation. Interestingly, when Asp10 was substituted for Gly10, a 5-fold increase in both affinity and activity for the MC4R was observed, while the MC3R was unaffected.

[Nle4,Gly10]-Lys-γ2-MSH had an even higher affinity for the MC4R than [Nle4]-α-MSH. The Phe12 for Pro12 substitution also gave an increase in affinity and activity of more than 3-fold for the MC4R, but not for the MC3R. Strikingly, the Asp10 to Gly10 substitution combined with the Phe12 to Pro12 substitution further increased the affinity to almost 13-fold for the MC4R, but there was no additive effect of these two substitutions on MC4R activation.

Receptor Domains Involved in [Nle4]Lys-γ2-MSH Selective Binding—The MC3R and the MC4R share 58% overall amino acid identity and 76% similarity. The transmembrane regions (TM) show the highest degree of homology while the intra- and extracellular loops (IC and EC) have lower homology (Fig. 2). To identify regions of the MC3R responsible for [Nle4]Lys-γ2-MSH selectivity a series of MC3R and MC4R chimeric receptors were generated with boundaries in stretches of amino acid residues with complete homology in order to minimize effects on receptor folding. These chimeric receptors were designed to determine the contribution of each of the extracellular domains to ligand recognition. Thus, the first extracellular loop with or without the N-terminal domain (named 3B and 3AB, respectively), the second (3C) and third (3D) extracellular loop of the MC4R were swapped individually with the corresponding domain of the MC3R. These chimeric receptors were transfected into 293 HEK cells and the affinities of NDP-α-MSH, [Nle4]-α-MSH, [Nle4]Lys-γ2-MSH, and [Nle4,Gly5,Asp10]-α-MSH were determined (the latter two peptides displayed MC3R selectivity).

Fig. 3 summarizes ligand affinity for the MC3R, MC4R, and five chimeric receptors. All chimeras bound [125I]NDP-α-MSH, demonstrating that they were all expressed on the plasma membrane. In general, the affinities of MSH peptides for MC3R and chimera 3AB, 3B, and 4D were higher than for MC4R and chimeras 3C and 3D. For example, the affinity of NDP-α-MSH on these chimeras was 6–20-fold higher than for the MC4R, but the same as for the MC3R. All chimeric receptors, except 3D, had the same affinity profile as the MC4R, in which the affinity of NDP-α-MSH ≥ [Nle4]-α-MSH > [Nle4]Lys-γ2-MSH = [Nle4,Gly5,Asp10]-α-MSH. The K values of [Nle4,Gly5,Asp10]-α-MSH (data not shown in Fig. 3) for 3AB (1.9 nM), 3B (5.0 nM), 3C (131 nM), and 4D (11 nM), were not different from [Nle4]Lys-γ2-MSH, but were significantly lower than [Nle4]-α-MSH (p < 0.05). 3D was the only chimera with the same affinity profile as the MC3R, in which [Nle4]Lys-γ2-MSH exhibited higher affinity than [Nle4]-α-MSH. However, the absolute K values were

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**Table I: Inhibition constants (K, in nM) of natural occurring and synthetic MSH peptides for the MC3R and MC4R**

| Peptide            | MC3R | MC4R |
|--------------------|------|------|
| α-MSH              | 4.4  | 37   |
| [Nle4]-α-MSH       | 2.9  | 19   |
| γ2-MSH             | 5.9  | 175  |
| γ1-MSH             | 3.2  | 95   |
| Lys-γ2-MSH         | 3.5  | 75   |
| [Nle4]-γ2-MSH      | 3.3  | 85   |
| [Nle4,Gly5,Asp10]-MSH | 0.9  | 38   |

a N-terminal acetylated and C-terminal amidated peptides.

b Reference peptides used in this study.

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**Fig. 2: Model of the human MC4R.** Gray circles indicate residues identical in the rat MC3R and the human MC4R. Black circles indicate regions with complete homology that were used as boundaries for construction of the chimeric receptors. These were named after the extracellular domain of the MC3R that was placed into the MC4R. Thus, chimera 3AB is the MC4R containing N terminal through TM3 (third transmembrane domain) of the MC3R, chimera 3B is the MC4R containing the IC1 (first intracellular loop) through the C terminus of the MC3R, chimera 3C is the MC4R containing IC2, TM4, EC2 (second extracellular loop) and part of TM5 of the MC3R, chimera 3D is the MC4R containing part of TM5 through the C terminus of the MC3R and vice versa, chimera 4D is the MC4R containing part of TM5 through the C terminus of the MC4R.
Inhibition constants ($K_i$ in nM) and $EC_{50}$ values (in nM) of synthetic α- and γ-MSH analogues for the MC3R and MC4R

All peptides were N-terminal and C-terminal amidated and contain a Nle*. The rat MC3R and the human MC4R were transiently transfected in 293 cells. $K_i$ values were determined by displacement of $[^{125}I]$NDP-α-MSH and calculated with 95% confidence interval derived from 11 duplicate data points. $EC_{50}$ values were determined in the β-galactosidase assay in the same batch of transfected cells to exclude influence of receptor expression levels. For each peptide 12 data points were measured in quadruplicate and $EC_{50}$ values were calculated with a 95% confidence interval. Both $K_i$ and $EC_{50}$ values are representative of at least two independent experiments.

| Peptide                      | MC3R $EC_{50}$ (nM) | MC4R $EC_{50}$ (nM) | MC3R $K_i$ (nM) | MC4R $K_i$ (nM) |
|------------------------------|----------------------|----------------------|-----------------|-----------------|
| NDP-α-MSH                   |                      |                      |                 |                 |
| α-MSH                       | 3.01                 | 9.1                  | 0.94            | 8.1             |
| [Lys1]α-MSH                 | 2.7                  | 11.2                 | 2.93            | 19.4            |
| [Val1]α-MSH                 | 2.75                 | 17.7                 | 2.07            | 23.1            |
| [Gly11]α-MSH                | 3.99                 | 7.22                 | 4.89            | 36.3            |
| [Asp10]α-MSH                | >100                 | >500                 | 5.09            | 19.0            |
| [Gly9,Pro12]α-MSH           | 5.79b,b               | 28.75      | 3.63            | 65.9            |
| [Arg11]α-MSH                | 14.8                 | 39                   | 30.2a           | 79.5g           |
| [Phe12]α-MSH                | 3.5                  | 18                   | 1.54a           | 16.8            |
| [Gly13]α-MSH                | 2.37                 | 4.90                 | 1.69            | 17.3            |
| Lysγγγ-MUSH                  | 5.03                 | 100                  | 0.86            | 38.4            |
| Serγγγ-Lysγγγ-MUSH           | 3.81                 | 128                  | 2.74a           | 228c            |
| Serγγγ-Lysγγγ-MUSH           | 3.56                 | 163                  | 1.73            | 120g            |
| Glyγγγ-Lysγγγ-MUSH           | 9.90                 | 168γγγ              | 0.49γγγ         | 7.57γγγ         |
| Lysγγγ-Lysγγγ-MUSH           | 2.18                 | 57.8                 | 1.12            | 56.5            |
| Proγγγ-Lysγγγ-MUSH           | 3.27                 | 25.2                 | 1.33            | 11.8            |
| Valγγγ-Lysγγγ-MUSH           | 4.59                 | 138                  | 0.86            | 131γγγ          |
| Glyγγγγ; Proγγγγ-Lysγγγ-MUSH | 3.95                 | 14.5y                | 0.67γγγ         | 2.99γγγ         |

Values significantly different ($p < 0.05$) from reference peptide [Nle4]α-MSH.

Data published previously in (24) but were reproduced for this study.

Values significantly different from reference peptide Ac-[Nle4]Lysγγγ-MSH-NH₂-

Fig. 3. $K_i$ values of NDP-α-MSH (○), [Nle4]α-MSH (●), and [Nle4]Lysγγγ-MUSH (■) for the MC3R, MC4R, and chimera 3AB, 3B, 3C, 3D, and 4D. Error bars indicate 95% confidence interval.

More than 10-fold higher than for the MC3R. The affinity of [Nle4,Gly5,Asp10]α-MSH for 3D ($K_i$ of 170 nM) was lower but not significantly different from [Nle4]α-MSH.

No disparity was observed between activity and affinity of [Nle4]Lysγγγ-MUSH, [Nle4]α-MSH, and [Nle4,Gly5,Asp10]α-MSH for the chimeric receptors (data not shown). Thus, the region from TM5 through the C-terminal portion of the MC3R (containing EC3) determined selectivity for [Nle4]Lysγγγ-MUSH. Since peptide hormones presumably interact with their ligands via the extracellular regions and with the binding pocket formed by the transmembrane helices of the receptor (31), we concentrated on the third extracellular loop for [Nle4]Lysγγγ-MUSH selectivity.

The radioligand receptor binding assay was used to further analyze the chimeric and mutant receptors described below. In order to determine in more detail which residues are important for selectivity we first substituted residues 267 to 282 of the MC4R (containing EC3), hereafter named MC4(267–282 MC3), for corresponding residues of the MC3R (Fig. 4). Fig. 5A shows that this chimeric receptor displayed gain of affinity for [Nle4]Lysγγγ-MUSH as compared with [Nle4]α-MSH. Therefore, this region was subdivided into two smaller parts. MC4(278–282 MC3), containing the second half of EC3 of the MC3R, did not display gain of affinity for [Nle4]Lysγγγ-MUSH. In contrast, MC4(267–273 MC3), containing the first half of EC3 of the MC3R, displayed equal affinity for [Nle4]Lysγγγ-MUSH and [Nle4]α-MSH. Thus, [Nle4]Lysγγγ-MUSH selectivity resides within the first half of EC3. The $K_i$ value of [Nle4,Gly5,Asp10]α-MSH for chimera MC4(267–282 MC3) and MC4(267–273 MC3), 128 and 76 nM, respectively, was significantly lower than [Nle4]α-MSH ($p < 0.05$, data not shown in Fig. 4).

Four residues in this region of the MC4R were mutated for the corresponding MC3R residue. Like MC4(267–273 MC3), the double MC4R mutant F267L/Y268I had the same affinity for [Nle4]Lysγγγ-MUSH as for [Nle4]α-MSH (Fig. 5B). Therefore, F267L and the Y268I were also mutated separately. The affin-
ity for [Nle⁴]Lys-γ₂-MSH appeared to be the same as for [Nle⁴]α-MSH on the Y268I mutant. However, the F267L mutant displayed lower affinity for [Nle⁴]Lys-γ₂-MSH than for [Nle⁴]α-MSH. The affinity of [Nle⁴,Gly⁵,Asp¹⁰]α-MSH (124 nM) was significantly lower than [Nle⁴]α-MSH for the Y268I mutant (p < 0.05). [Nle⁴,Gly⁵]Lys-γ₂-MSH exhibited similar affinity for [Nle⁴]α-MSH than for [Nle⁴]α-MSH for the Y268I mutant (8.9 and 7.6 nM, respectively), demonstrating there is no further gain of affinity for [Nle⁴]Lys-γ₂-MSH as compared with [Nle⁴]α-MSH. Thus, mutation of the single residue, Y268I, of the MC4R altered [Nle⁴]Lys-γ₂-MSH selectivity.

**Affinity of NDP-α-MSH Compared with the Cyclic Melanocortin Peptides, MT-II, and [d-Tyr⁴]MT-II, on the MC4R, MC4(Y268I), and the MC3R—**

MT-II (melanotan II) and [d-Tyr⁴]MT-II, are conformationally constrained heptapeptide analogs of α-MSH. The affinity of these peptides was determined on the MC4R and MC3R and the MC4(Y268I) mutant. The competition binding curves in Fig. 6A show that [d-Tyr⁴]MT-II has equal affinity for the MC4R as NDP-α-MSH, but 10-fold lower affinity than MT-II. Thus, the order in affinity is, MT-II > NDP-α-MSH = [d-Tyr⁴]MT-II. In contrast, the competition binding curves for [d-Tyr⁴]MT-II and MT-II on MC4(Y268I) were both shifted to the right, whereas the affinity of NDP-α-MSH was not statistically significantly different from MC4R. Thus, for MC4(Y268I) and the MC3R the order in affinity is, NDP-α-MSH > MT-II > [d-Tyr⁴]MT-II. Fig. 6B summarizes the relative affinity (NDP-α-MSH = 1) of MT-II and [d-Tyr⁴]MT-II on MC4(Y268I) and the MC3R as compared with MC4R.

**Activity of [Nle⁴]α-MSH compared with [Nle⁴]Lys-γ₂-MSH**
and [D-Tyr4]MT-II on MC4R, MC4(267–282 MC3), MC4(267–282 MC3), and MC3R—In order to determine whether differences in binding affinity are reflected by similar differences in activity we determined adenylate cyclase activity in response to two peptides that showed the most pronounced differences in the binding affinity. Thus, we tested [Nle4]Lys-γ2-MSH and [D-Tyr4]MT-II on the MC4R, MC3R, and the key chimeric and mutant receptor MC4(267–282 MC3) and MC4(267–282 MC3) and compared the EC50 values with [Nle4]α-MSH (Table III). The EC50 values of [Nle4]α-MSH for MC4R, MC4(267–282 MC3), MC4(267–282 MC3) and MC3R were 24, 113, 113, and 2.2 nM, respectively. Like for MC3R, [Nle4]α-MSH and [Nle4]Lys-γ2-MSH have equal activity on both MC4(267–282 MC3) and MC4(267–282 MC3) whereas [Nle4]Lys-γ2-MSH has a 15-fold lower activity on MC4R compared with [Nle4]α-MSH on MC4R. Moreover, the selective MC4R agonist, [D-Tyr4]MT-II, displayed significantly lower activity on MC4(267–282 MC3), MC4(267–282 MC3), and MC3R compared with [Nle4]α-MSH while these peptides have similar activity on MC4R.

DISCUSSION

Using a gain of function approach in which systematic exchanges of peptides and receptors were used, we identified the importance of Asp10 in Lys-γ2-MSH in determining MC3R selectivity. Furthermore, Tyr268 in the MC4R hindered interaction with [Nle4]Lys-γ2-MSH. Tyr268 mutated toward the corresponding residue of the MC3R, Ile, not only increased [Nle4]Lys-γ2-MSH affinity but, in addition, decreased affinity for two cyclic melanocortin peptides, MT-II and [D-Tyr4]MT-II. Thus, this receptor residue is critical in determining MC3/MC4 receptor selectivity.

Independent substitutions of Gly10 and Lys11 in [Nle4]α-MSH for Asp10 and Arg11, respectively, led to a significant decrease in activity and affinity for both MC3R and MC4R. This indicates that introduction of these residues in [Nle4]α-MSH prohibited interaction with both receptors. In a previous study (32) the affinity of [Asp10]α-MSH also appeared to be much lower than that of α-MSH for MC3R and MC1R. Still, Gly10, and also Lys11, of [Nle4]α-MSH are probably not important for a direct interaction with the receptor, since Ala substitution on positions 10 and 11 of α-MSH did not affect affinity on the MC3R or the MC1R (23).

Substitution of Pro12 of [Nle4]α-MSH for Phe12 slightly increased MC3R affinity while decreasing activity, but not affinity, for the MC4R. This is in agreement with the observation that substitution of Pro12 for Ala in α-MSH did not affect binding to the MC3R (22). This implies that the residue at this position is not essential for receptor binding but may be important for MC4R activation only. Our data and others (33) indicate that Phe12 in [Nle4]Lys-γ2-MSH may hinder interaction with MC4R. Nevertheless, this residue is probably not involved in a direct interaction with MC3R, since it may substituted for Pro without loss of affinity.

The results show that Asp10 in [Nle4]Lys-γ2-MSH had similar affinity and activity as [Nle4]α-MSH on the MC3R. First, because [Nle4,Gly5,Asp10]α-MSH had similar affinity and activity as [Nle4,Gly10]Lys-γ2-MSH on the MC3R, MC4(267–282 MC3) and MC4R were 2.6, 8.3, and 17 nM, respectively.

![Graph showing competition of [125I]NDP-MSH, MT-II, and [D-Tyr4]MT-II on the MC4R, MC3R, and the key differences in the binding affinity. Curves are representative of two independent experiments.](image)

**Fig. 6. Affinity of NDP-α-MSH, MT-II, and [D-Tyr4]MT-II on the MC3R, MC4(267–282 MC3), and MC4R expressed in HEK 293 cells.** a, the graphs show competition of [125I]NDP-α-MSH by NDP-α-MSH (C), MT-II (C), and [D-Tyr4]MT-II (○). Data points represent mean of duplicate measurements ± S.D. Graphs were fitted with GraphPad Prism, one site competition. Curves are representative of two independent experiments. b, the table shows the relative affinity’s (footnote a indicates a statistically significant difference (p < 0.05) with reference peptide [Nle4]α-MSH for the same receptor.

| Peptide         | MC4R | MC4(267–282 MC3) | MC4(267–282 MC3) | MC3R |
|-----------------|------|-----------------|-----------------|------|
| NDP-α-MSH       | 1    | 1.2             | 1.2             | 1    |
| [Nle4]D-Phe4α-MSH | 1    | 1.2             | 1.2             | 1    |
| MT-II           | 0.75 | 0.75            | 0.75            | 0.75 |
| [D-Tyr4]MT-II   | 1.2  | 1.2             | 1.2             | 1.2  |
| [D-Phe4]MT-II   | 1.7  | 1.7             | 1.7             | 1.7  |

*Footnote (a) indicates a statistically significant different EC50 (p < 0.05) with reference peptide [Nle4]α-MSH for the same receptor.*
Interestingly, it was found that all chimera containing the 3B segment (TM2, EC1, and TM3) of the MC3R displayed a high affinity for MSH peptides, similar to MC3R. In this same region of the MC1R, Yang et al. (35) suggested an important role for residues Glu84 in TM2 and Asp177 or Asp172 in TM3 of the MC1R in forming an ionic binding pocket for the Arg8 residue of NDP-α-MSH, α-MSH, γ12-MSH (Arg8), and MT-II (35). Although these residues are conserved in all MC receptors, other, non-conserved residues may influence the strength of the receptor-ligand interaction, explaining the overall lower affinity of MSH peptides, used in this study, for the MC4R versus the MC3R.

A selective receptor-ligand interaction could occur through a mechanism of exclusion rather than a specific recognition. In that respect, Tyr268 of the MC4R may hinder [Nle4,Lys2]-MSH interaction and the following model may apply. His264 of the MC4R is the equivalent of the His260 in the MC1R and may, as was suggested for the MC1R (36, 37), also be important for interaction with γ-MSH. His264 has been demonstrated to be essential for melanocortin peptide activation of MC4R as well (38). His264 of the MC4R is located only four residues lower in TM6 than Tyr268. Therefore, Tyr268 may mask His264 and thereby exclude an interaction with [Nle4,Lys2]-MSH (Fig. 7).

Using NDP-α-MSH containing Asp10, Arg11, and Phe12 of γ-MSH, Schioth et al. (39) suggested that the γ-MSH C terminal-hinders MC4R binding. However, this peptide showed an equal loss of affinity for the MC3R. Nevertheless, this loss of affinity agrees with our data for [Nle4,Asp10]α-MSH. This suggests that the presence of two acidic amino acid residues on positions 5 and 10 (Glu and Asp, respectively), lead to a significant decrease in MC3R and MC4R affinity, possibly because of repulsion of these two negatively charged residues. This is in agreement with the high affinity of cyclic lactam- (cyclization between residues 5 and 10) and disulfide-bridged (cyclization between residues 4 and 10) melanocortin derivatives (40–44). Taken together, these data suggest that in the active conformation the residues in positions 4/5 and 10 are in close proximity. We here propose that the presence of Asp10 in [Nle4,Lys2]-γ-MSH decreased the chance of the core sequence to be in the optimal conformation necessary to bind to the MC4R.

To investigate whether presentation of a constraint core sequence determined MC4R selectivity, the affinity of cyclic melanocortin peptides with a structurally constrained core sequence was tested. MT-II displayed marked increased and [α-Tyr4]MT-II equal affinity as compared with NDP-α-MSH for the MC4R, but not for the MC3R and MC4(Y268I). These differences in ligand activity appeared to be represented by differences in affinity. Thus, mutation of Tyr268 in MC4R toward the Ile residue present on the corresponding position in MC3R increased both affinity and activity of [Nle4,Lys2]-γ-MSH and decreased affinity and activity for the high affinity MC4R agonist, [α-Tyr4]MT-II. Thus, a single amino acid residue determined MC3/MC4R selectivity for different ligands and MC4R binding and activation increases when the core sequence is presented in a constraint conformation.

Recently, it was shown that cyclic peptides have higher affinity than α-MSH on all MC-Rs (45). Strikingly, for the MC4R, all cyclic peptides displayed higher or similar affinity than for MC3R (45–47), while linear peptides always seemed to exhibit lower affinity on the MC4R than on the MC3R (this study). Therefore, for design of new MC-R selective ligands, cyclization may be appropriate for the MC4R. In contrast, modification in linear MSH peptides may be more valuable for MC3R selectivity, as was suggested by Haskell-Luevano et al. (41). A similar model was proposed for the opioid receptors in which linear dynorphin A analogues were generally more selective for the μ-opioid receptor while cyclic constrained dynorphin A peptides demonstrated slight selectivity for κ-versus δ-opioid receptors or were nonselective (48). Taken together, these data suggests that the message sequence residues do not interact with conserved neuropeptide receptor residues in the same manner. Indeed, it has been shown that the HFRW pharmacophore interacts differently with all MC-R (49).

Here, we propose a general concept for selective receptor-ligand interaction that may apply to all peptide receptors. Ligand residues outside the peptide core sequence direct the conformation of the receptor interacting core sequence presented to the receptor-binding pocket, and thereby determine selectivity. There are several examples that emphasize the critical role of residues positioned outside the core of true contact residues, in determining selectivity of ligands to, for instance, opioid (36) and neuropeptide Y receptors (50).

This study provides, for the first time, a detailed analysis of the structural requirements for selective MC3R versus MC4R recognition. Using a gain of function approach we have demonstrated that the Asp10 of [Nle4,Lys2]-γ-MSH and Tyr268, near the extracellular face of TM6, of the MC4R, determine the low affinity and activity of [Nle4,Lys2]-MSH for the MC4R. The large aromatic residue Tyr268 of the MC4R may change the

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**Fig. 7. Helical wheel model of the MC4 receptor.** Residues in black indicate MC receptor conserved residues. The bulky aromatic Tyr268 of the MC4R may hinder interaction of γ-MSH with His264 which is positioned lower in TM6. Figure template used with permission from Dr. T. W. Schwartz, Laboratory of Molecular Pharmacology, Copenhagen, Denmark.
MC4R-binding pocket unfavorable for [NE+]Lys-γ2-MSH but favorable for the cyclic peptides MT-II and [d-Tyr3]MT-II.

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