Molecular Determinants of Melanocortin 4 Receptor Ligand Binding and MC4/MC3 Receptor Selectivity

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ABSTRACT

The molecular basis of ligand recognition by the melanocortin 4 receptor (MC4R) has not been fully defined. In this study, we investigated the molecular determinants of MC4R ligand binding, employing a large array of ligands, using three approaches. First, molecular modeling of the receptor was used to identify Phe284, in transmembrane (TM) 7, as a potential site of ligand interaction. Mutation of Phe284 to alanine reduced binding affinity and potency of peptides containing L-Phe by up to 71-fold but did not appreciably affect binding of linear peptides containing D-Phe, consistent with a hydrophobic interaction between the Phe of α-melanocyte-stimulating hormone and Phe284. Second, we examined the effect of a naturally occurring mutation in TM3 (I137T) that is linked to obesity. This mutation decreased affinity and potency of cyclic, rigid peptides but not more flexible peptides, consistent with an indirect effect of the mutation on the tertiary structure of the receptor. Third, we examined the residues that support ligand selectivity for the MC4R over the MC3R. Mutation of Ile125 (TM3) of the MC4R to the equivalent residue of the MC3R (phenylalanine) selectively decreased affinity and potency of MC4R-selective ligands. This effect was mirrored by the reciprocal MC3R mutation F157I. The magnitude of this effect indicates that this locus is of major importance. However, it is considered that an isoleucine/phenylalanine mutation may affect the orientation of Asp122, which has been identified as a major determinant of ligand binding affinity. Thus, this study provides further characterization of the MC4R binding pocket.

The melanocortin receptors are members of the seven transmembrane (TM) G protein-coupled receptor family. There are five melanocortin receptor subtypes cloned to date, MC1 through MC5, all of which couple to Gs to stimulate adenylyl cyclase. These receptors are activated by a group of peptides derived from post-translational processing of the pro-opiomelanocortin gene transcript. These peptides include α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH), both of which contain the common amino acid sequence His6-Phe7-Arg8-Trp9 (Sahm et al., 1994; Haskell-Luevano et al., 1997). Melanocortin receptor activation is modulated by two endogenous antagonists/inverse agonists, agouti and agouti-related protein (AGRP) (Lu et al., 1994; Ollmann et al., 1997). AGRP is 132 amino acids in length but is thought to mimic the binding of the much smaller 13-amino acid agonist α-MSH through an Arg-Phe-Phe motif. This motif is found in the C-terminal half of the protein, and commonly, a truncated portion of the protein AGRP(87-132) is used in characterization studies. AGRP(87-132) exhibits the same melanocortin receptor binding selectivity as full-length AGRP and has been reported to act as a competitive antagonist (Yang et al., 1999).

Recently, there has been great interest in the MC4 receptor, as it has been reported to be important in the regulation of feeding. MC4/−/− mice exhibit hyperphagia and accelerated weight gain (Huszar et al., 1997). Ectopically expressed AGRP results in an obese phenotype (Butler et al., 2001). Furthermore, the MC4 receptor agonist MTII inhibits feeding in both mice models of hyperphagia and fasted mice (Fan et al., 1997). It was also demonstrated that this effect could be blocked by the antagonist SHU9119, which when given alone was able to enhance nocturnal feeding. However, both MC3 and MC4 receptors are expressed at high levels in the brain, and MC3 receptor action in the regulation of food intake is unclear. MC3 receptor knockout mice exhibit increased fat mass but are not significantly overweight (Butler et al., 2000; Chen et al., 2000). This indicates that the MC3
and MC4 receptors serve different roles in the regulation of
energy homeostasis. Potential pharmaceutical benefit can be
reaped from development of ligands that can discriminate
between MC3 and MC4 receptors, and therefore, it is impor-
tant to identify the molecular determinants of the receptor
that enable MC4/MC3 receptor discrimination. A number of
MC4-selective ligands have been developed, including the
agonist peptide H1RWK (Bednarek et al., 1999), the antago-
nist peptide M10 (Bednarek et al., 2001), and the nonpeptide
agonist tetrahydroisoquinoline (THIQ) (Van der Ploeg et al.,
2002) (Table 1). Conversely, γ-MSH and a modification of the
dipeptide γtrp9 show some selectivity for the MC3 receptor
over the MC4 receptor.

Earlier mutagenesis studies have already identified a
number of important residues involved in ligand binding to
the MC4 receptor (Yang et al., 2000; Haskell-Luevano et al.,
2001). These include an ionic interaction between melanocor-
tin peptide residue Arg8 and aspartate residues 122 and 126
in TM3 and a hydrophobic interaction between melanocortin
peptide residue Phe7 with aromatic residue Phe261 in TM6.
A further phenylalanine residue in TM4 of the mouse MC4
receptor has also been proposed to interact with melanocor-
tin peptide Phe7 residue (Haskell-Luevano et al., 2001).
However, the binding pocket of the melanocortin peptides
and the endogenous antagonists is still not clearly defined.

In this study, we have taken three approaches to further
define the molecular determinants of the MC4 receptor in-
volved in ligand interaction. In the first approach, we have
used molecular modeling to identify candidate residues of the
receptor involved in ligand binding. Using this method, we
identified Phe284 as a potential ligand binding site. Second,
knowledge of the binding site, we have identified a residue
that may account for some of the differences in the MC4 and
MC3 receptor binding sites. In all cases, a large array of
ligands have been used to define the effect of the mutation,
allowing inferences in regard to effects on specific ligands
and effects on specific residues, regions, or physical prop-
ties of the ligands.

### Materials and Methods

Radiolabeled [125I]NDP-MSH, [125I]AGRP(87-132), and
[125I]SHU9119 were obtained from PerkinElmer Life Sciences (Bos-
ton, MA). α-MSH, NDP-MSH, ACTH, and SHU9119 were obtained
from Peninsula Laboratories (Belmont, CA). γ-MSH, MTII, and
AGRP(83-132) were obtained from Phoenix Pharmaceuticals, Inc.
(Belmont, CA). Peptides, M10 (Bednarek et al., 2001), H1RWK
(Bednarek et al., 1999), and γtrp9 were synthesized by solid-phase
methodology on a Beckman Coulter 990 peptide synthesizer (Fuller-
ton, CA) using t-N-t-butoxycarbonyl-protected amino acids, and
the assembled peptide was deprotected with hydrogen fluoride.

The crude peptide products were purified by preparative high-perfor-
mance liquid chromatography. All primers were synthesized and
desalted by Invitrogen (Carlsbad, CA). The nonpeptide agonist THIQ
(Van der Ploeg et al., 2002) was synthesized by the Chemistry De-
partment at Neurocrine. All other reagents were of the highest
quality available.

### Creation of MC4 and MC3 Receptor-Stable Cell Lines

HEK-293 cells were maintained in DMEM with 10% fetal calf serum.
Wild-type receptor constructs in pcDNA3.1 were transfected using
LipofectAMINE (Invitrogen). Stable receptor populations were gene-
erated using G418 (Invitrogen) selection (1 mg/ml).

### Receptor Mutagenesis

Human MC4 or MC3 receptor cDNAs in
pcDNA3.1 were used as a template for mutagenesis. Mutants were
prepared using the QuikChange site-directed mutagenesis kit
(Stratagene, La Jolla, CA). PCR reactions (95°C, 1 min; 52°C, 1 min;
and 72°C, 16 min) were performed using Pfu polymerase and a
complementary set of primers encoding the nucleotide mutation(s),

### Table 1

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Sequence</th>
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<tr>
<td>α-MSH</td>
<td>Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Arg-Gly-Lys-Pro-Val-NH2</td>
</tr>
<tr>
<td>NDP-MSH</td>
<td>Ac-Ser-Tyr-Ser-Nle-Glu-His-Arg-Phe-Arg-Gly-Lys-Pro-Val-NH2</td>
</tr>
<tr>
<td>MTII</td>
<td>Ac-Nle-c[Asp-His-n-Phe-Arg-Arg-Trp-Lys]-NH2</td>
</tr>
<tr>
<td>SHU9119</td>
<td>Ac-Nle-c[Asp-His-n-Phe-Arg-Arg-Trp-Lys]-NH2</td>
</tr>
<tr>
<td>γ-MSH</td>
<td>Ac-Val-Met-Gly-His-Phe-Arg-Arg-Arg-Phe-Gly-NH2</td>
</tr>
<tr>
<td>γtrp9</td>
<td>Ac-Val-Met-Gly-His-Phe-Arg-Arg-Arg-Phe-Gly-NH2</td>
</tr>
<tr>
<td>M10</td>
<td>Ac-c[Suc-n-Nal-Arg-Trp-Lys]-NH2</td>
</tr>
<tr>
<td>H1RWK</td>
<td>Ac-c[Suc-His-n-Phe-Arg-Arg-Trp-Lys]-NH2</td>
</tr>
<tr>
<td>AGRP(83-132)</td>
<td>SSRRCVRLHESCLGQQVPCCDPCATCRRFNNACFCYCRKLGTAMNPCRT</td>
</tr>
</tbody>
</table>

![THIQ](image-url)
Results

Pharmacological Characterization of the MC4 Receptor Expressed Stably in HEK-293 Cells. Changes in ligand affinity observed in site-directed mutagenesis studies are subject to different interpretation depending on the conformation (i.e., activated or inactivated) of the receptor being studied. The MC4 receptor has not previously undergone a full in vitro pharmacological characterization to determine the potential affinity states present. Therefore, we determined this before proceeding with the mutagenesis experiments.

According to the extended ternary complex model (Sama et al., 1993), ligands display different functional profiles because they differ in affinity for different receptor conformations. Agonists display a preference for the G protein-coupled form of the receptor, inverse agonists display a preference for the inactivated G protein-uncoupled form of the receptor, and antagonists do not discriminate between different receptor conformations. Different receptor states are usually detected using radioligand binding. Three different radioligands, [125I]NDP-MSH, [125I]AGRP(87-132), and [125I]SHU9119, are available that can bind to the MC4 receptor. These ligands all bind specifically to membranes prepared from HEK-293 cells stably expressing the human MC4 (hMC4) receptor but not to membranes prepared from untransfected HEK-293 cells. These ligands all display different pharmacological profiles. In the cell line stably expressing the MC4 receptor used in this study, NDP-MSH is a full agonist (Fig. 6), SHU9119 is a very weak partial agonist ([5 ± 1% of α-MSH response; pEC50, 9.50 ± 0.29 (EC50, 0.316 nM); n = 3], and AGRP(83-132) is an inverse agonist (pEC50, 7.55 ± 0.28 (EC50, 28.2 nM); n = 3) (Fig. 1).

Saturation analysis with [125I]SHU9119 revealed that the MC4 receptor was stably expressed at 435 ± 36 fmol/mg (Kd, 0.70 ± 0.13 nM; n = 3). [125I]NDP-MSH labeled a similar number of sites (391 ± 17 fmol/mg; Kd, 4.10 ± 0.60 nM; n = 3). However, saturation analysis with [125I]AGRP(87-132) resulted in a Bmax value of 217 ± 25 fmol/mg (Kd, 0.87 ± 0.15 nM; n = 3). In all cases, saturation curves fit best to a one binding site model and were fully saturable (Fig. 2).
were unaffected by the inclusion of 30 stants of antagonists and the agonists NDP-MSH and ACTH receptor from G protein (Gilman, 1987). The affinity con-

Analysis against [125I]SHU9119, [125I]NDP-MSH, and Saturation analysis of radioligand binding to MC4 receptors. Data Fig. 2.

were fitted best by a one binding site model and are representative of three independent experiments.

A number of ligands were characterized in competition analysis against [125I]SHU9119, [125I]NDP-MSH, and [125I]AGRP(87-132) (Fig. 3). Affinity values for the competing ligands were similar versus all radioligands (Table 2). Interestingly, whichever radioligand was used, competition binding curves for ligands, including agonists, were best fit by a one binding site model. This was an unexpected observation, since the ternary complex model (De Lean et al., 1980) predicts that agonists display a preference for receptors coupled to G protein, whereas antagonists do not discriminate between uncoupled receptors and those coupled to G protein. Consequently, competition curves involving an agonist and a radiolabeled antagonist commonly exhibit curves characterized by low Hill coefficients, from which two affinity constants can be generated.

The effect of receptor/G protein coupling on ligand binding affinity was further investigated, using GTPγS to uncouple receptor from G protein (Gilman, 1987). The affinity constants of antagonists and the agonists NDP-MSH and ACTH were unaffected by the inclusion of 30 μM GTPγS in the assay buffer, whereas the remaining agonists exhibited up to a 6-fold decrease in affinity in the presence of GTPγS (Table 3). In addition, GTPγS did not consistently increase the Hill coefficients of the agonist/[125I]SHU9119 competition curves. These findings suggest that the MC4 receptor population labeled by [125I]SHU9119 exists predominantly in an “active” conformation in the absence of GTPγS, which is transformed to an inactive state with slightly lower affinity for certain agonists by GTPγS. Thus, it was concluded that a single activated receptor population was being labeled in binding studies.

Mutagenesis Studies. A number of MC4 receptor residues have been mutated in this study and are summarized in Fig. 4A. Putative ligand-receptor interactions (Fig. 4B) were derived from homology molecular modeling of the human MC4 receptor based on the 2.8Å-resolution structure of rhodopsin (Palczewski et al., 2001). This model predicted an ionic interaction between α-MSH and aspartate residues 122 and 126 in TM3 and a hydrophobic interaction between α-MSH and both Phe261 in TM6 and Phe284 in TM7. In addition to molecular modeling studies, further receptor mutations were systematically executed based on both previous characterization of the ligand binding site and sequence analysis looking at the differences between the MC3 and MC4 receptors.

All mutant receptors were transiently transfected into HEK-293 cells, ligand affinity was measured in competition analysis versus [125I]SHU9119, and agonist potency was measured in cAMP accumulation assays. Receptor expression levels were measured by [125I]SHU9119 saturation binding, and although expression levels were found to differ (Table 4), it was considered unlikely that this had any influence on the results obtained. In addition, no marked differences in basal cAMP accumulation were observed in the functional assays, indicating that none of the mutant receptors displayed constitutive activity.

Hydrophobic Interaction with TM4, TM6, and TM7. Both the Phe7 and Trp9 residues of α-MSH have been re-

ported to be important in binding to the MC4 receptor (Yang et al., 2000). To ascertain which residues of the MC4 receptor interact with α-MSH, a number of phenylalanine residues were mutated. First, a F261A mutation was characterized; this exhibited similar binding parameters to those previously reported (Yang et al., 2000), with a large loss of affinity for α-MSH (>-100-fold) but little loss in affinity for NDP-MSH (2-fold) and AGRP(83-132) (3.5-fold) (Fig. 5 and Table 4). Additionally, we discovered that the binding affinity of SHU9119 was not significantly decreased by the F261A mutation (p > 0.05, Student’s t test), but the binding of the small-molecule agonist THIQ was, with a 66-fold decrease in affinity (p < 0.05, Student’s t test). Consistent with previous literature and binding data, the F261A receptor exhibited decreased agonist potency and discriminated between α-MSH and NDP-MSH (Fig. 6 and Table 5). The potency of NDP-MSH was only marginally affected, but an EC50 value could not be determined for α-MSH. Furthermore, the small-molecule agonist THIQ showed a 55-fold decrease in potency (p < 0.05, Student’s t test). The efficacies of the agonists were not significantly affected by this mutation (Table 6).

Our molecular model predicts that Phe284 could interact with α-MSH. Therefore, we mutated Phe284 to an alanine and tested the effect of this mutation on ligand binding and
The effect of guanine nucleotides on the binding of agonists to the MC4 receptor

**TABLE 3**

Affinity of agonists was assessed in competition experiments versus \([125I]SHU9119\). Data were fitted to a sigmoidal dose-response curve with a variable Hill coefficient.

Values for \(K_i\) are expressed as mean ± S.E.M \((K_i\ nM)\) of four independent experiments.

![Image](image.png)

![Image](image.png)

**TABLE 2**

Affinity constants of ligand binding in competition with different radioligands at the MC4 receptor

<table>
<thead>
<tr>
<th>Ligand</th>
<th>(K_i) Mean ± S.E.M</th>
<th>(n_H) Mean ± S.E.M</th>
</tr>
</thead>
<tbody>
<tr>
<td>[125I]SHU9119</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-MSH</td>
<td>7.43 ± 0.07 (32.2)</td>
<td>0.71 ± 0.06</td>
</tr>
<tr>
<td>NDP-MSH</td>
<td>8.47 ± 0.06 (3.39)</td>
<td>0.94 ± 0.11</td>
</tr>
<tr>
<td>THIQ</td>
<td>8.01 ± 0.12 (9.77)</td>
<td>0.94 ± 0.03</td>
</tr>
<tr>
<td>SHU9119</td>
<td>9.71 ± 0.03 (0.195)</td>
<td>0.94 ± 0.03</td>
</tr>
<tr>
<td>AGRP(83–132)</td>
<td>9.75 ± 0.09 (0.178)</td>
<td>1.08 ± 0.13 (0.066)</td>
</tr>
<tr>
<td>[125I]AGRP(87–132)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[125I]NDP-MSH</td>
<td>7.23 ± 0.06 (58.9)</td>
<td>7.30 ± 0.08 (50.1)</td>
</tr>
</tbody>
</table>

**Fig. 3.** Affinity of melanocortin ligands for the MC4 receptor measured against different radioligands. Ligand binding was measured in competition studies against \([125I]NDP-MSH, [125I]AGRP87-132,\) and \([125I]SHU9119\). Ligands are represented by the following symbols: ■, α-MSH; ○, NDP-MSH; □, THIQ; ○, AGRP(83-132); and ×, SHU9119. Data are representative of three independent experiments.

agonist function. Ligand binding to the F284A receptor was severely affected, with a 2.8- to 71-fold decrease in ligand affinity. This reached significance \((p < 0.05,\) Student's \(t\) test) for all ligands except NDP-MSH. In fact, similarly to the D122A and P261A mutant receptors, substitution of phenylalanine 284 with an alanine led to clear discrimination between NDP-MSH and the endogenous peptides α-MSH and ACTH, as the affinities of these ligands showed the least (2.8-fold) and greatest (71- and 33-fold) decrease, respectively. The F284A mutation exhibited the greatest decreases (M10, 16-fold) in antagonist binding affinity observed in this study. Functional assay data obtained with the P284A receptor mimicked binding data in that the potency of all agonists with the exception of NDP-MSH was affected, with significant \((p < 0.05,\) Student's \(t\) test) decreases in potency ranging from 3.5- (MTII) to 21-fold (ACTH). However, the efficacies of the various agonists were largely unaffected by this mutation.

Phe184 is conserved in MC1, 3, 4, and 5 receptors but corresponds to a methionine in the MC2 receptor. The phenylalanine-to-methionine mutation F184M did not reduce the binding affinity of any of the ligands tested \((p > 0.05,\) Student's \(t\) test).
consistent with the binding data, the potency of NDP-MSH inverse agonist AGRP(83-132) and the antagonist SHU9119 was observed. There was also no significant change in the efficacy or potency of any of the agonists tested (p > 0.05, Student’s t-test).

**Interaction with TM3.** MC4 receptors have been implicated in obesity, and a number of studies have suggested that mutations in the MC4 receptor may be one of the causes of human obesity. Therefore, we decided to further characterize a TM3 mutation found in obese subjects (I137T) (Gu et al., 1999). The I137T mutation significantly decreased the binding of most ligands, with the exception of α-MSH and NDP-MSH, with decreases between 1.8- and 4.8-fold compared with wild-type MC4 receptor (p < 0.05, Student’s t-test). The I137T mutation did not significantly affect efficacy but caused a decrease in potency of most agonists, with THIQ, HHRWK, and MTH showing significant differences of between 3.7- and 9.6-fold (p < 0.05, Student’s t-test).

Previous mutagenesis studies (Yang et al., 2000; Haskell-Luevano et al., 2001) have implicated the aspartate residues in TM3 (122 and 126) as being important in melanocortin peptide binding. In this study, the D122A mutant receptor exhibited the expected change in binding parameters, with a much greater loss of affinity for α-MSH (>100-fold) than NDP-MSH (6-fold). Furthermore, the binding affinities of the inverse agonist AGRP(83-132) and the antagonist SHU9119 were all significantly decreased (p < 0.05, Student’s t-test). We also demonstrated that the small-molecule agonist THIQ interacts with Asp122, as substitution of this residue with an alanine decreased the binding affinity 66-fold. Agonist potency was also decreased by the D122A mutation; an EC₅₀ value for α-MSH could not be determined, although a functional response was seen at 10 μM. The potency of THIQ was also significantly decreased (p < 0.05, Student’s t-test), but, consistent with the binding data, the potency of NDP-MSH was not markedly affected.

A number of ligands have been developed that can discriminate between the MC4 and MC3 receptors. Since the con-
served aspartate residues (Asp122 and Asp126 in MC4R and Asp117 and Asp121 in MC1R) are critical for α-MSH binding and function in both MC4 (Yang et al., 2000; Haskell-Luevano et al., 2001) and MC1 receptors (Yang et al., 1997), it is probable that analogous residues in the MC3 receptor (Asp154 and Asp158) play a role in α-MSH binding to the MC3 receptor. It therefore follows that the positioning of the aspartate residues may play a role in determining the MC3/MC4 selectivity of ligands. We observed that between these two key residues, an isoleucine is present in the MC4 receptor (Ile125), and a phenylalanine is present in the MC3 receptor (Phe157). Due to the difference in bulk of these residues, it was hypothesized that they may affect the interaction of ligands with the aspartate residues. Hence, the MC4 receptor mutant I125F and the reverse MC3 receptor mutant F157I were made.

Competition analysis revealed that the I125F mutation in the MC4 receptor resulted in a 2- to 5-fold decrease in the affinity of the MC4 receptor-selective ligands, THIQ, HfRWK, MTII, and SHU9119. This was significant in the cases of THIQ, HfRWK, and SHU9119 (p < 0.05, Student’s t test). The binding affinities of nonselective and MC3 receptor-selective ligands were not significantly affected, indicating that the receptor conformation was not appreciably affected by the mutation. The MC3 receptor mutant F157I showed a significant increase in affinity for the MC4 receptor-selective ligands HfRWK (5.9-fold) and MTII (3.4-fold) (p < 0.05, Student’s t test). The affinity of α-MSH was also significantly increased. Although the affinity of α-MSH is not significantly different between MC3 and MC4 receptors, there is a trend for its affinity to be higher at the MC4 receptor. Functional data mimicked binding data, with the I125F mutation significantly (p < 0.05, Student’s t test) decreasing the potency of the MC4 receptor-selective agonists THIQ (6.2-fold), HfRWK (7.2-fold), and MTII (2.5-fold). MC3 receptor-selective or nonselective agonists showed no change in potency, and none of the agonists showed a marked change in efficacy. The MC3 receptor mutant F157I mirrored these results. Compared with the wild-type MC3 receptor, F157I showed an increase in potency of MC4 receptor-selective agonists. This reached significance in the case of MTII (4.4-fold) and HfRWK (6.4-fold) (p < 0.05, Student’s t test). There was also a 3-fold increase in α-MSH potency. The F157I mutant receptor also exhibited a decrease in the maximal response (E_max) of γ-MSH and γtrp9 compared with the wild-type MC3 receptor (p < 0.05, Student’s t test).

Discussion

In this study, we used site-directed mutagenesis to further define the MC4 receptor binding site and to determine which residues contribute to the selectivity between the MC3 and MC4 receptors. We examined the binding and function of a large range of peptides and a small molecule to define differences in binding between similar compounds. We also investigated some of the pharmacological properties of this receptor, in particular, the sensitivity of ligands to GTPγS, to gain some insight into the process of receptor activation.

In agreement with previous literature (Haskell-Luevano and Monck, 2001; Nijenhuis et al., 2001), AGRP(83-132) was an inverse agonist at the MC4 receptor. NDP-MSH was a full agonist, and SHU9119 was a very weak partial agonist. The MC4 receptor is largely guanine nucleotide insensitive. In [125I]NDP-MSH displacement assays, a one binding site model was preferred in both the absence and presence of GTPγS, and agonists only displayed a minimal decrease in affinity in the presence of GTPγS. In particular, NDP-MSH binding was not detectably affected by GTPγS. This is consistent with the saturation data; the B_max of [125I]NDP-MSH was equivalent...
to that of \(^{125}\text{I}\)SHU9119, indicating that NDP-MSH does not discriminate between G protein-coupled and -uncoupled forms of the receptor. Similar observations have been made for the somatostatin 5 receptor (Siehler et al., 1999) and the D₂ dopamine receptor (Cordeaux et al., 2001); at both of these receptors, agonists differ in their sensitivity to guanine nucleotides. It has been proposed that guanine nucleotide-insensitive agonists are able to stabilize a conformation of the receptor, which is close to the conformation of the active receptor/G protein complex, so that there is little energy gain in coupling to the G protein (Strange, 1999). Overall, it is concluded from these data that a single activated receptor population is being labeled. This simplifies interpretation of the site-directed mutagenesis binding data, as changes in ligand affinity likely affect receptor/ligand interaction, be it directly or indirectly, rather than the receptor state.

The \(B_{\text{max}}\) of \(^{125}\text{I}\)SHU9119 and \(^{125}\text{I}\)NDP-MSH binding was twice that of \(^{125}\text{I}\)AGRP(87-132) binding. These data may indicate that AGRP binds to an MC4 receptor dimer. Previous evidence for MC4 receptor dimerization is seen in a report demonstrating that palmitoylated peptides derived from the third intracellular loop of the MC4 receptor acted as agonists in cells expressing the MC4 receptor (Covic et al., 2002). Furthermore, the high-resolution NMR structure of AGRP implies that there may be more than one site that interacts with the receptor (McNulty et al., 2001). Following this assumption, the complete inhibition of \(^{125}\text{I}\)SHU9119 binding by AGRP(83-132) may imply that the receptors are dimerized. Nevertheless, little evidence for this phenomenon was observed in ligand binding. NDP-MSH, SHU9119, and AGRP all acted competitively against each other, and ligands displayed similar affinity in competition studies against the different radioligands, which is consistent with all three radioligands binding to the same site.

Analysis of the expression levels of the MC4 receptor mutants revealed large variation. However, an increase in receptor expression level did not correlate with an increase in agonist affinity or potency, which may have been predicted. It is considered unlikely that the mutations themselves have affected this correlation, as the F184M, F261A, and D122A

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**Fig. 6.** Potency of agonists at wild-type and mutant melanocortin 3 and 4 receptors. Potency values were obtained from cAMP accumulation assays as described under Materials and Methods. Data are expressed as percentage of the maximal cAMP produced by α-MSH, except D122A, which is expressed as percentage of the maximal cAMP produced by NDP-MSH, due to the reduction in activity of α-MSH. Graphs are representative of three to six independent experiments.
Student

E
the maximal response of NDP-MSH was designated as 100%. Efficacy values are expressed as potency to the wild-type MC4 receptor or that F157I exhibits a significantly different functional potency to the wild-type MC3 receptor (*p < 0.05, Student's t test).

### TABLE 5

<table>
<thead>
<tr>
<th>NPD-MSH</th>
<th>α-MSH</th>
<th>MTII</th>
<th>ACTH</th>
<th>HRWK</th>
<th>γ-MSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC4R</td>
<td>8.86 ± 0.07</td>
<td>7.83 ± 0.06</td>
<td>8.83 ± 0.15</td>
<td>9.73 ± 0.05</td>
<td>8.44 ± 0.09</td>
</tr>
<tr>
<td>D122A</td>
<td>8.35 ± 0.14*</td>
<td>&gt;1000 nM***</td>
<td>7.31 ± 0.11**</td>
<td>(4.47)</td>
<td>N.D.</td>
</tr>
<tr>
<td>I125F</td>
<td>8.57 ± 0.08</td>
<td>7.77 ± 0.03</td>
<td>8.04 ± 0.10*</td>
<td>(9.12)</td>
<td>(0.468)</td>
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<tr>
<td>I137T</td>
<td>8.51 ± 0.15</td>
<td>7.70 ± 0.05</td>
<td>8.26 ± 0.16*</td>
<td>(5.50)</td>
<td>(1.78)</td>
</tr>
<tr>
<td>F184M</td>
<td>8.88 ± 0.18</td>
<td>8.16 ± 0.14</td>
<td>8.49 ± 0.09</td>
<td>9.18 ± 0.20</td>
<td>(0.661)</td>
</tr>
<tr>
<td>F261A</td>
<td>8.83 ± 0.14</td>
<td>6.93 ± 0.14**</td>
<td>7.09 ± 0.14**</td>
<td>(81.3)</td>
<td>N.D.</td>
</tr>
<tr>
<td>F284A</td>
<td>8.85 ± 0.10</td>
<td>7.02 ± 0.01***</td>
<td>7.57 ± 0.20**</td>
<td>9.19 ± 0.13*</td>
<td>(0.646)</td>
</tr>
<tr>
<td>MC3R</td>
<td>8.68 ± 0.10</td>
<td>8.00 ± 0.07</td>
<td>6.45 ± 0.22</td>
<td>8.62 ± 0.01</td>
<td>(2.40)</td>
</tr>
<tr>
<td>F157I</td>
<td>9.16 ± 0.21</td>
<td>8.49 ± 0.08*</td>
<td>6.74 ± 0.14</td>
<td>9.26 ± 0.18*</td>
<td>(0.550)</td>
</tr>
</tbody>
</table>

N.D., not determined.

### TABLE 6

<table>
<thead>
<tr>
<th>NPD-MSH</th>
<th>α-MSH</th>
<th>MTII</th>
<th>ACTH</th>
<th>HRWK</th>
<th>γ-MSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC4R</td>
<td>114 ± 15</td>
<td>100</td>
<td>96 ± 15</td>
<td>132 ± 9</td>
<td>78 ± 16</td>
</tr>
<tr>
<td>D122A</td>
<td>100</td>
<td>50% at 0.1 μM</td>
<td>78 ± 9</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>I125F</td>
<td>93 ± 11</td>
<td>100</td>
<td>100 ± 7</td>
<td>126 ± 18</td>
<td>N.D.</td>
</tr>
<tr>
<td>I137T</td>
<td>102 ± 11</td>
<td>100</td>
<td>109 ± 9</td>
<td>114 ± 15</td>
<td>78 ± 15</td>
</tr>
<tr>
<td>F184M</td>
<td>123 ± 2</td>
<td>100</td>
<td>128 ± 16</td>
<td>115 ± 8</td>
<td>97 ± 36</td>
</tr>
<tr>
<td>F261A</td>
<td>129 ± 24</td>
<td>100</td>
<td>104 ± 15</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>F284A</td>
<td>102 ± 5</td>
<td>100</td>
<td>117 ± 7</td>
<td>121 ± 8</td>
<td>71 ± 13</td>
</tr>
<tr>
<td>MC3R</td>
<td>114 ± 15</td>
<td>100</td>
<td>72 ± 4</td>
<td>85 ± 8</td>
<td>87 ± 5</td>
</tr>
<tr>
<td>F157I</td>
<td>92 ± 9</td>
<td>100</td>
<td>83 ± 13</td>
<td>73 ± 6</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not determined.

### Materials and Methods

Agonist-stimulation of cAMP accumulation was determined as described under Materials and Methods, and potency (EC 50) values were determined. Potency values are expressed as pEC 50 ± S.E.M (IC 50) (n = 3–6). The asterisk (*) indicates that D122A, I125F, I137T, F184M, F261A, or F284A exhibit a significantly different functional potency to the wild-type MC4 receptor or that F157I exhibits a significantly different functional potency to the wild-type MC3 receptor (*p < 0.05, Student's t test).

The findings of Yang et al. (2002), who report that Leu133 (TM3) is a determinant of ligand binding in the MC4 receptor, are in agreement with our laboratory's findings that increasing the expression level of the MC4 receptor does not lead to an increase in agonist affinity. The D122A, I125F, and F184M mutations did not significantly affect ligand binding, while the F261A, F284A, and I137T mutations did. The F261A and F284A mutations decreased ligand binding, while the I137T mutation increased ligand binding.

### Efficacy values of melanocortin agonists at wild-type and mutant MC4 and MC3 receptors measured using cAMP accumulation

Efficacy values were determined as described under Materials and Methods, and potency (EC 50) values were determined. Potency values are expressed as pEC 50 ± S.E.M (IC 50) (n = 3–6). The asterisk (*) indicates that F157I exhibits a significantly different functional potency to the wild-type MC3 receptor (*p < 0.05, Student's t test).
most MC4 receptor ligands. Because this residue is positioned deep within the TM3 helix, we suggest that the effect of this mutation may be a consequence of nonspecific alteration of the receptor tertiary structure. Consistent with this notion, the binding of the more rigid cyclic peptides was affected, whereas that of the more flexible linear peptides was not.

It has previously been proposed that there is an ionic interaction between the Arg8 of melanocortin peptides and the aspartate residues in TM3 (Yang et al., 2000; Haskell-Luevano et al., 2001). The Asp122 mutation made in this study significantly affects the binding of all components except NDP-MSH, which is consistent with the above hypothesis. This study also demonstrated that the spacing between the two aspartate residues plays a small role in determining MC3/MC4 receptor selectivity. Asp122 and Asp126 theoretically both face into the binding pocket, since three to four amino acids is approximately one full turn in the presumed α-helical structure of the TM domains. One would anticipate that the difference in bulk of the isoleucine (MC4) versus phenylalanine (MC3) separating these residues would lead to a slightly different orientation of the aspartate residues in the binding pocket. Accordingly, the MC4 receptor mutant I125F and the MC3 receptor mutant F157I displayed a respective decrease or increase in affinity and potency for most MC4 receptor-selective ligands, although the small shifts in affinity and potency values indicate that this locus is of minimal importance in determining receptor selectivity.

Overall, the data herein highlight potentially important differences between the ligand binding site of the human MC4 receptor and that of other melanocortin receptor subtypes and species variants of the MC4 receptor. Specifically, a mutation analogous to F284A in the hMC1 receptor had no effect on binding or function (Yang et al., 1997). The data presented in this study show that ligand binding and function was generally unaltered by the F184M mutation, which would disrupt any hydrophobic interactions between the phenylalanine and the melanocortin peptides. Conversely, mutation of an analogous residue in the mouse MC4 receptor to a serine drastically decreased peptide binding and agonist function (Haskell-Luevano et al., 2001), leading to the hypothesis that the hydrophobic binding pocket for Phe7 was formed between TMs 6 and 4 rather than TMs 6 and 7, as indicated by our data. It is considered that a F184S mutation repulses the aromatic residues in the melanocortin ligands; in accordance with this reasoning, an analogous mouse F184L mutation did not produce such a drastic decrease in ligand affinity (Haskell-Luevano et al., 2001).

In summary, this study demonstrates some unique pharmacological properties of the MC4 receptor. Agonists exhibit little sensitivity to guanine nucleotides, and the agonist NDP-MSH appears to label all forms of the receptor with the same affinity. We have demonstrated that the MC4 receptor residue Ile125 has a role in determining ligand selectivity between the MC3 and MC4 receptors. This leads to the hypothesis that conserved residues in the melanocortin receptors are involved in ligand binding, but differences in their positioning due to differences in receptor structure lead to ligand selectivity for one receptor over another. We have also determined that Phe284 in TM7 forms a hydrophobic pocket with Phe261 in TM6, in which the Phe7 residue of α-MSH binds.

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References


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