Molecular Determinants of Ligand Binding to the Human Melanocortin-4 Receptor†

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ABSTRACT: To elucidate the molecular basis for the interaction of ligands with the human melanocortin-4 receptor (hMC4R), agonist structure−activity studies and receptor point mutagenesis were performed. Structure−activity studies of [Nle^4,D-Phe^7]-α-melanocyte stimulating hormone (NDP-MSH) identified D-Phe7-Arg8-Trp9 as the minimal NDP-MSH fragment that possesses full agonist efficacy at the hMC4R. In an effort to identify receptor residues that might interact with amino acids in this tripeptide sequence 24 hMC4R transmembrane (TM) residues were mutated (the rationale for choosing specific receptor residues for mutation is outlined in the Results section). Mutation of TM3 residues D122 and D126 and TM6 residues F261 and H264 decreased the binding affinity of NDP-MSH 5-fold or greater, thereby identifying these receptor residues as sites potentially involved in the sought after ligand−receptor interactions. By examination of the binding affinities and potencies of substituted NDP-MSH peptides at receptor mutants, evidence was found that core melanocortin peptide residue Arg8 interacts at a molecular level with hMC4R TM3 residue D122. TM3 mutations were also observed to decrease the binding of hMC4R antagonists. Notably, mutation of TM3 residue D126 to alanine decreased the binding affinity of AGRP (87−132), a C-terminal derivative of the endogenous melanocortin antagonist, 8-fold, and simultaneous mutations D122A/D126A completely abolished AGRP (87−132) binding. In addition, mutation of TM3 residue D122 or D126 decreased the binding affinity of hMC4R antagonist SHU 9119. These results provide further insight into the molecular determinants of hMC4R ligand binding.

The melanocortin peptides α-, *β*-, and *γ*-MSH and adrenocorticotropic hormone are a group of neuropeptides derived from the pro-opiomelanocortin prohormone that contain the common amino acid sequence His-Phe-Arg-Trp (*1*). The physiological actions of these peptides are mediated through five seven-transmembrane (TM)[1] G-protein-coupled receptors that couple to the stimulatory G-protein G_s (*2*−*6*). Activation of the melanocortin receptors (MCRs) can be modulated by two endogenous molecules, agouti and agouti-related protein (AGRP), that are thought to act pharmacologically as antagonists of the melanocortin peptides by binding to sites on the MCRs (*7*−*12*).

A heightened interest in the physiology and pharmacology of the melanocortin peptides has resulted from the relatively recent recognition that α-MSH, AGRP, and the MC4R are important hypothalamic elements that control feeding behavior (*13*−*17*). α-MSH has been shown to be a potent satiety-inducing factor synthesized by neurons in the arcuate nucleus that also produce the anorexigenic cocaine and amphetamine responsive transcript (*18*). AGRP, on the other hand, is a hunger-inducing factor synthesized by a different subset of neurons in the arcuate nucleus that also produce the orexigenic neuropeptide Y (*17*). Within this context, identification of the molecular interactions that are responsible for high-affinity α-MSH and AGRP binding to the MC4R could potentially assist antiobesity drug development. While existing information about the structures of α-MSH and AGRP and the molecular pharmacology of other MCR subtypes would be expected to provide some insight into hMC4R ligand−receptor interactions, to date, little has been specifically published about those hMC4R interactions.

The structures of α-MSH and AGRP are believed to be substantially different (*19*−*22*). α-MSH is a tridecapeptide without cysteine residues that available evidence indicates forms a *β*-turn that orients Arg8 away from His6, Phe7, and Trp9 (*18*). In contrast, human AGRP is transcribed as a 132 amino acid protein that has a cysteine-rich C-terminal domain. Although it is not known if AGRP is posttranslationally processed, a chemically synthesized 46 amino acid C-terminal AGRP fragment that is used in these studies, AGRP (87−132), has been demonstrated to have the same binding affinity and inhibitory potency as full-length recombinant AGRP when those parameters are examined in HEK-293 cells transfected with the cloned human (h) MCRs (*12*). With regard to those two specific parameters, AGRP (87−132) may represent the minimal AGRP sequence capable of high-affinity MCR binding and full antagonist potency.

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[1] Abbreviations: hMC4R, human melanocortin-4 receptor; AGRP, agouti-related protein; NDP-MSH, [Nle^4,D-Phe^7]-α-melanocyte stimulating hormone; TM, transmembrane.

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Biochemical studies of AGRP (87−132) indicate that it is a globular protein consisting of three major loops without helices or sheets whose structure is maintained by well-defined disulfide bonds (*20*, *21*). However, despite their apparent structural dissimilarity it has been suggested that AGRP residues Arg111-Phe112-Phe113, which reside on that molecule's external surface, biochemically resemble and functionally mimic residues Phe7-Arg8-Trp9 of the melanocortin sequence (*22*).

While scientific understanding of the molecular interactions involved in AGRP-MC4R binding is incomplete, previous pharmacological studies of chimeric hMC1R and hMC4R receptors have suggested that the exo loop domains of the hMC4R are important to high-affinity AGRP (87−132) binding (*23*). Other studies, however, have found that cyclic octapeptides containing AGRP residues Arg111-Phe112-Phe113 demonstrate some inhibitory activity at the hMC4R (*21*). In view of their small size it is conceivable that those octapeptides bind to receptor domains closer to or within the cell membrane rather than to exo loops.

Point mutagenesis and computer modeling of the hMC1R, the MCR subtype expressed by the melanoctye, suggest that important determinants for α-MSH and NDP-MSH binding may reside in the TM domains of that MCR (*24*−*26*). Since the hMC1R and hMC4R belong to the same receptor subfamily, it might be anticipated that residues homologous to those utilized by the hMC1R for high-affinity agonist binding would serve a similar hMC4R function. On the other hand, the hMC1R and hMC4R have only 54.9% amino acid sequence similarity, and several functional differences distinguish these two MCR subtypes. One difference, undoubtedly of great physiological significance, is that AGRP is able to antagonize α-MSH action at the hMC4R but is not able to antagonize α-MSH action at the hMC1R. In addition, peptide compounds such as SHU 9119 are antagonists of the hMC4R and agonists of the hMC1R (*27*). These observations suggest that differences must exist in the ligand binding pockets of the hMC1R and hMC4R.

Upon this background we performed structure−activity studies of NDP-MSH and hMC4R mutagenesis to gain further insight into the molecular interactions involved in ligand (agonist and antagonist) binding to that receptor.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. In these studies the assignment of receptor residues to TM domains was determined by hydropathy plot of the sequences of the MCRs (GCG program, Madison, WI) and a manual comparison of MCR sequences with a previously published alignment of seven TM G-protein-coupled receptor α-helices (*28*). Superimposition of the sequence of the hMC4R onto the recently published three-dimensional X-ray crystal structure of bovine rhodopsin supports our assignment of mutated hMC4R residues to TM helices (*29*). The cDNA fragment of the hMC4R gene beginning at the start codon and ending 63 bp distal to the stop codon was subcloned into M13mp18 in the correct orientation for single-stranded site-directed mutagenesis using the Muta-Gene T4 in vitro mutagenesis kit (Bio-Rad Laboratories, Hercules, CA). Alanine, which has been widely used in receptor mutagenesis because its small nonpolar side chain is unlikely to alter receptor tertiary structure, was the amino acid used to replace receptor residues in the majority of the mutant constructs. In several instances where aspartic acid was the receptor residue asparagine mutations were also constructed since asparagine more closely approximates the size of aspartic acid yet, like alanine, lacks a positive charge. The presence of desired mutations was confirmed by single-strand sequencing using Sequenase Version 2.0 (Amersham Pharmacia Biotech, Piscataway, NJ). The mutant receptors were then excised from M13 and subcloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA). Large-scale plasmid preparations were then performed after the receptor-containing plasmids were amplified in *Escherichia coli* strain XL1-Blue (Stratagene, La Jolla, CA) using an alkaline lysis method followed by purification of closed circular DNA over a CsCl gradient (*30*). The fidelity of the entire receptor sequence was then confirmed by double-strand nucleotide sequencing using an ABI Prism Model 377 automated sequencer (Foster City, CA) to ensure that no unplanned mutations were present in the finished mutant constructs. The sequence of the "wild-type" hMC4R used in these studies can be found in Genbank under accession number L08603 (polymorphisms of this receptor are recognized). The hMC4R TM residues that were mutated are schematically depicted in Figure 1.

Receptor Expression. Cell transfection was performed using lipofectamine reagent (Life Technologies, Rockville, MD). For assays receptor mutants were transiently expressed in HEK-293 cells. However, to have a greater number of cells readily available for experiments involving receptor mutants D126A and H264A, in those two instances experiments were also performed on stably transfected pools.

cAMP Assays. cAMP assays were performed as previously described using a competitive binding assay (Amersham Pharmacia Biotech, cAMP assay kit TRK 432) (*23*). Substituted and truncated NDP-MSH molecules were synthesized by Research Genetics (Huntsville, AL) except peptide IX, which was synthesized at Merck, and peptide VII, which was synthesized by Dr. Victor Hruby (University of Arizona). The long-acting superpotent α-MSH analogue [Nle^4,D-Phe^7]-α-MSH (NDP-MSH) and the synthetic hMC4R antagonist SHU 9119 were obtained from Peninsula Laboratories (Belmont, CA) (*27*, *31*). Human AGRP (87−132) was obtained from Gryphon Sciences (South San Francisco, CA). Data were analyzed using Graphpad Prism (Graphpad Software, San Diego, CA).

Binding Assays. ^{125}I-NDP-MSH and ^{125}I-AGRP (87−132) were prepared by simple oxidative methods using chloramine-T and $Na^{125}I$ (Amersham Pharmacia Biotech) as previously described, followed by HPLC purification over a C18 column (*12*). ^{125}I-SHU 9119 was obtained from NEN Life Science Products (Boston, MA). Binding experiments were performed on whole cells using conditions previously described with some modification (*12*). Briefly, 12 h prior to the experiments 0.3 million cells were plated on 24 well plates. Before the binding experiments were initiated, cells were washed twice with MEM medium. Cells were then incubated with different concentrations of unlabeled ligand containing 0.2% BSA and 2×10^5 cpm of either ^{125}I-NDP-MSH (200 000 cpm = 0.166 nM NDP-MSH), ^{125}I-AGRP (87−132) (200 000 cpm = 0.152 nM AGRP), or ^{125}I-SHU 9119 (200 000 cpm = 0.152 nM SHU 9119). In the cases

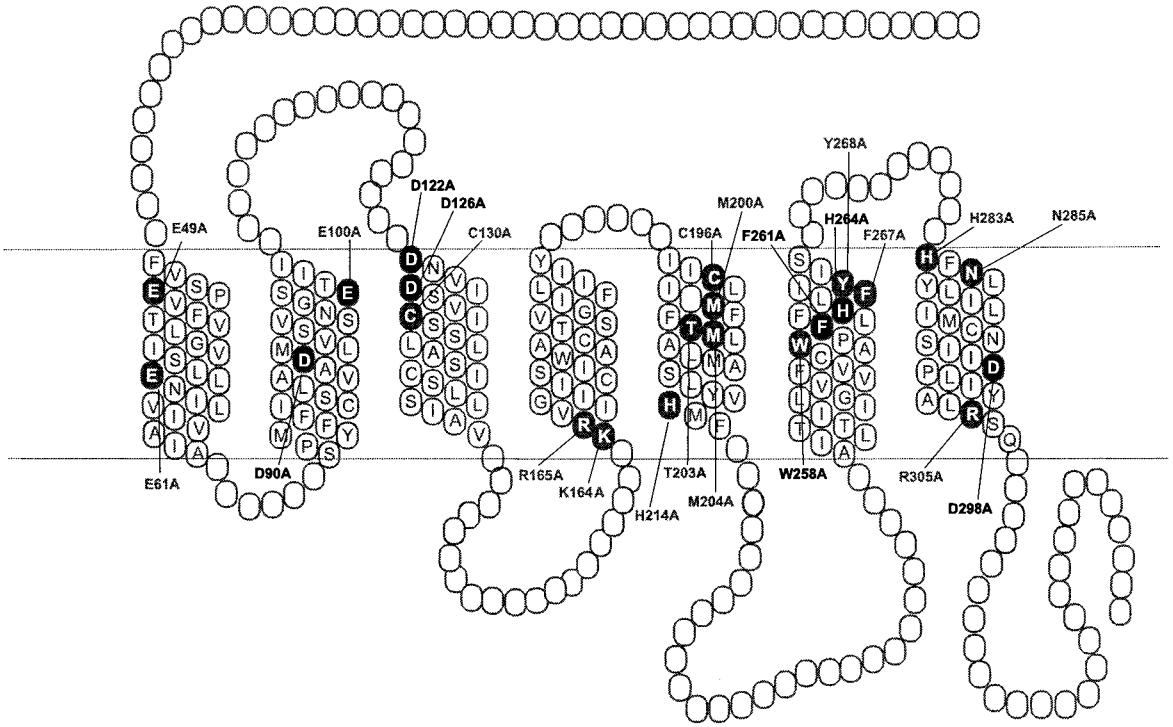

FIGURE 1: Two-dimensional representation of the seven TM structure of the hMC4R. The TM residues mutated in these experiments are denoted by gray or black highlighting. Those TM residues whose mutation significantly affected NDP-MSH binding as determined by a paired T-test are highlighted in black.

of D126A and H264A, binding experiments were performed with 600 000 cpm of ^{125}I-NDP-MSH (600 000 cpm = 0.501 nM NDP-MSH), and in the case of D126A, 400 000 cpm of ^{125}I-AGRP (87−132) (400 000 cpm = 0.303 nM AGRP) was used. After 1 h incubation the cells were again washed twice with MEM medium, and the experiment was terminated by lysing the cells with 0.1 N NaOH and 1% Triton X-100. Radioactivity present in the lysate was quantified using an analytical γ counter. Nonspecific binding was determined by measuring the amount of ^{125}I-label remaining bound in the presence of 10^{-5} M unlabeled ligand, and specific binding was obtained by subtracting nonspecific bound radioactivity from total bound radioactivity.

Control experiments were performed using the maximal counts per minute of radioligand stipulated above and HEK-293 cells transfected with the pcDNA3.1 vector alone. In the cases of D126A and H264A, control experiments were performed at room temperature in the presence of 0.5% sodium azide, conditions that partially inhibit receptor internalization, to determine whether any of the specific binding detected at these mutants was due to internalization of ^{125}I-NDP-MSH within the assayed cells. Data were analyzed using Graphpad Prism. K_i values for NDP-MSH were calculated using the equation $K_i = K_d = IC_{50} -$ [radioligand] (*32*). For α-MSH and substituted and truncated NDP-MSH peptides the equation $K_i = IC_{50}/(1 +$ [radioligand]/K_i of NDP-MSH]) was used. Statistical significance of cAMP and radioligand binding assays was determined by analyzing experimental means ± SEM using a paired T-test with $P < 0.01$ considered significant.

RESULTS

Control Experiments. Specific NDP-MSH, AGRP (87−132), and SHU 9119 binding could not be detected at HEK-293 cells transfected with the pcDNA3.1 vector alone using the maximal concentrations of radioligand used in these experiments. Similarly, agonist-stimulated cAMP generation could not be detected with HEK-293 cells transfected with the pcDNA3.1 vector alone, even with NDP-MSH concentrations as high as 10^{-5} M. In addition, no differences in binding affinity and potency were observed between the transiently transfected and stably transfected pools of D126A and H264A that were used in these studies.

Role of NDP-MSH Residues in High-Affinity Binding and Potency at the Wild-Type hMC4R. To determine which NDP-MSH residues are essential for high-affinity binding and potency at the hMC4R, structure−activity studies were performed with substituted and truncated NDP-MSH peptides. The sequences of the substituted and truncated peptides and their K_i and EC_{50} values at the hMC4R are listed in Tables 1 and 2.

Substitution of NDP-MSH residues D-Phe7 with the D-stereoisomer of alanine (substituted peptide III), Arg8 with norleucine (substituted peptide IV), and Trp9 with leucine (substituted peptide V) profoundly decreased binding affinity and potency. Substitution of Trp9 with another aromatic amino acid, phenylalanine (substituted peptide VI), decreased peptide binding affinity to a much lesser degree than substitution with leucine, and Phe9 substitution was not associated with a change in peptide potency. Substitution of His6 with alanine (substituted peptide II) had a lesser effect on binding affinity and potency.

Studies with truncated NDP-MSH peptides (Table 2) revealed that all truncations tested affected binding affinity and potency. The tripeptide D-Phe7-Arg8-Trp9 (truncated peptide IX) was the smallest fragment that displayed full agonist efficacy (Figure 2); however, full hMC4R activation required a micromolar concentration of that tripeptide and

Table 1: Effect of NDP-MSH Residue Substitution on ^{125}I-NDP-MSH Binding and cAMP Generation[a]

		K_i (nM)	EC_{50} (nM)
NDP-MSH	Ac- Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH2	1.32 ± 0.07	0.27 ± 0.04
I	Ac- Ser-Tyr-Ser-Nle-**Ala**-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH2	7.1 ± 0.14*	0.98 ± 0.01*
II	Ac-Ser-Tyr-Ser-Nle-Glu-**Ala**-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH2	20.3 ± 0.03*	1.1 ± 0.03*
III	Ac- Ser-Tyr-Ser-Nle-Glu-His-**DAla**-Arg-Trp-Gly-Lys-Pro-Val-NH2	1592 ± 88.8*	321 ± 19*
IV	Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-**Nle**-Trp-Gly-Lys-Pro-Val-NH2	269.7 ± 14.5*	15 ± 3.4*
V	Ac- Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-**Leu**-Gly-Lys-Pro-Val-NH2	130 ± 18.4*	3.45 ± 0.87*
VI	Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-**Phe**-Gly-Lys-Pro-Val-NH2	8.6 ± 1.0*	0.31 ± 0.06

[a] $n > 3$, mean ± SEM. An asterisk indicates $P < 0.01$. Nle = norleucine.

Table 2: Effect of NDP-MSH Truncation on ^{125}I-NDP-MSH Binding and cAMP Generation[a]

		K_i (nM)	EC_{50} (nM)
NDP-MSH	Ac-Ser-Tyr-Ser-Nle-Glu-His-**DPhe-Arg-Trp**-Gly-Lys-Pro-Val-NH2	1.32 ± 0.07	0.27 ± 0.04
I	Met-Glu-His-**DPhe-Arg-Trp**-Gly-Lys-Pro-Val	5.87 ± 0.12*	0.78 ± 0.11*
II	Glu-His-**DPhe-Arg-Trp**-Lys-NH2	975 ± 73.7*	3.65 ± 0.65*
III	His-**DPhe-Arg-Trp**-Lys-NH2	788 ± 81.6*	4.27 ± 0.09*
IV	Glu-His-**DPhe-Arg-Trp**-NH2	611.8 ± 30.1*	3.44 ± 0.12*
V	**DPhe-Arg-Trp**-Lys-NH2	4406 ± 239*	13.2 ± 1.4*
VI	**DPhe-Arg-Trp**-Gly-Lys	3881 ± 60.5*	31.4 ± 3.2*
VII	His-**DPhe-Arg-Trp**-NH2	415.8 ± 9.6*	8.65 ± 0.9*
VIII	Glu-His-**DPhe-Arg**-NH2	$>10^{-6}$*	NR
IX	**DPhe-Arg-Trp**-NH2	6135.5 ± 291*	17.7 ± 4.5*

[a] $n > 3$, mean ± SEM. An asterisk indicates $P < 0.01$. NR = no response. Nle = norleucine.

even at that high concentration D-Phe-Arg-Trp did not completely displace ^{125}I-NDP-MSH. The smaller EC_{50} value than K_i value is possibly due to the use of a transient expression system for determination of EC_{50} since activation properties are dependent on expression level per cell. Alternatively, a nonlinearity may exist between peptide binding and receptor signal transduction activation. Removal of Trp9 from the pentapeptide Glu-His-DPhe-Arg-Trp (peptide IV) resulted in a totally inactive tetrapeptide (peptide VIII), which further highlighted the importance of this aromatic amino acid residue. Even though substitution of His6 with alanine had only relatively minor effects on binding affinity and potency within the context of a substituted NDP-MSH peptide, the addition of histidine to the N-terminus of the minimal tripeptide sequence (truncated peptide VII) was observed to substantially increase binding affinity and potency.

Rationale for Choosing hMC4R TM Residues for Mutagenesis. In view of the fact that hMC4R does not have an extended amino terminus that might overtly implicate that receptor domain in ligand binding (like the seven TM G-protein-coupled glycoprotein receptors), that previous mutagenesis studies have implicated a number of TM residues in agonist binding to the hMC1R, and that a three amino acid fragment of NDP-MSH is able to elicit full biological activity, we hypothesized that D-Phe7-Arg8-Trp9 would likely bind to the hMC4R in a pocket formed by its TM domains. Therefore, the present studies exclusively targeted hMC4R TM residues for mutagenesis.

In addition to mutating hMC4R residues homologous to those previously demonstrated to affect hMC1R agonist binding affinity and potency (*24*, *25*), the choice of which TM residues were to be mutated was guided by the biochemical characteristics of amino acids in the key NDP-MSH tripeptide and by an assumption that conservation of a TM residue among all MCR subtypes might indicate a role for that residue in ligand binding.

Since an ionic interaction between the arginine residue of the key NDP-MSH tripeptide and an anionic receptor residue would be expected to provide substantial energy for agonist binding, all seven negatively charged hMC4R TM residues were mutated. This included several anionic TM residues homologous to receptor residues previously implicated in hMC1R agonist binding (E100, D122, and D126) (*24*, *25*).

On the basis of our assumption that receptor residues conserved among the MCRs might participate in ligand binding, all six basic TM residues and several aromatic-, hydroxyl-, and sulfhydryl-containing residues were mutated. This included two conserved residues that have previously been implicated as determinants of agonist binding affinity at the hCM1R (TM6 residues F261 and H264) (*24*, *25*). Conversely, several residues without homologous counterparts in other MCR subtypes, TM5 residue T204 and TM6 residues F267 and Y268, were mutated. Residues homolo-

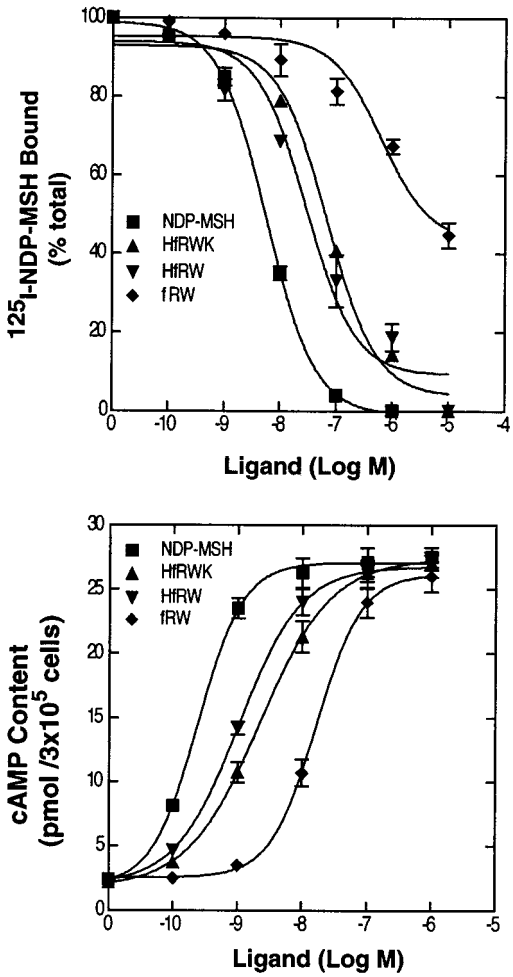

FIGURE 2: Binding affinity and potency of truncated NDP-MSH peptides at HEK-293 cells stably transfected with the wild-type hMC4R ($n \geq 3$; see Table 2 for K_i and EC_{50} values). Abbreviations: f = D-phenylalanine, R = arginine, W = tryptophan, and H = histidine.

gous to these latter TM6 residues have been implicated in neurokinin receptor ligand binding (*33*).

Role of Acidic hMC4R TM Residues in High-Affinity Agonist Binding and Potency. Mutagenesis of all seven acidic TM residues in the sequence of the hMC4R (TM 1 residues E49 and E61, TM2 residues D90 and E100, TM3 residues D122 and D126, and TM7 residue D298) significantly affected α-MSH and/or NDP-MSH binding affinity as analyzed by a paired T-test. However, mutagenesis of only TM3 residues D122 and D126 affected NDP-MSH binding affinity greater than 5-fold. K_i and EC_{50} values for these mutations are reported in Tables 3 and 4, and selected mutations are depicted in Figure 3. Mutagenesis of acidic TM1 residues E49 and E61 to alanine had no significant effect on NDP-MSH agonist binding affinity or potency but did have a 2-fold effect on α-MSH binding and a 4-fold effect on α-MSH potency.

Mutagenesis of TM2 residue D90 to alanine caused a 3-fold decrease in NDP-MSH and a 1 log decrease in α-MSH binding affinity. Notably, agonist binding to mutant D90A did not stimulate cAMP production. Mutagenesis of TM2 residue E100 to alanine decreased NDP-MSH and α-MSH binding affinity approximately 4-fold and decreased their potency 2.5- and 3-fold, respectively. Mutation of acidic TM7 residue D298 to alanine decreased NDP-MSH and α-MSH binding affinity 2−3-fold and similar to mutation D90A abolished cAMP generation.

Mutagenesis of acidic TM3 residue D122 to alanine caused a 6-fold decrease in NDP-MSH binding affinity and a greater than 1 log decrease in α-MSH binding affinity. These decreases in binding affinity were accompanied by proportionate losses in NDP-MSH and α-MSH potency. Asparagine mutation of D122 had a lesser effect on agonist binding affinity and potency than the alanine mutation of that residue.

Mutation of acidic TM3 residue D126 to alanine or asparagine completely abolished the biological activity of α-MSH. In competition binding studies using the radioligand ^{125}I-NDP-MSH, no α-MSH binding could be demonstrated at D126A or D126N, and in second messenger assays of these mutants, α-MSH did not stimulate cAMP production. These mutations also dramatically decreased the binding affinity and potency of NDP-MSH. The D126A and D126N mutants only exhibited marginal ^{125}I-NDP-MSH binding. Therefore, in Table 3 the IC_{50} values for NDP-MSH at these mutations are listed as $\geq 10^3$ nM. Consistent with this markedly diminished NDP-MSH binding, the dose−response curves of NDP-MSH in cAMP assays at mutants D126A and D126N were markedly shifted to the right. In the case of D126A the cAMP response was submaximal compared to the wild-type hMC4R and did not reach a plateau (Figure 3B). Therefore, an EC_{50} for NDP-MSH at D126A could not be calculated and in Table 4 is listed as $\geq 10^3$ nM. In the case of D126N a maximal cAMP response was observed.

In an attempt to find mutations of D126 with binding characteristics that would be favorable for our planned studies with substituted NDP-MSH peptides (i.e., an NDP-MSH binding affinity intermediate between wild-type hMC4R and D126A), leucine and serine mutations of this receptor residue were made. However, both mutations D126L and D126S were characterized by little, if any, demonstrable NDP-MSH binding, and both had markedly rightward-shifted NDP-MSH dose−response curves in cAMP assays (data not shown).

As might be anticipated from the results of the individual mutations of D122 and D126, double mutants D122A/D126A and D122N/D126N had no detectable α-MSH or NDP-MSH binding. Nonetheless, a dose-dependent cAMP response were observed upon stimulation of both double mutants with higher concentrations of NDP-MSH ($\geq 10^{-7}$ M), indicating some level of mutant receptor expression and function. The E_{max} of these double mutants was approximately one-third of the wild-type hMC4R at 10^{-5} M NDP-MSH. In the presence of 10^{-7}, 10^{-6}, and 10^{-5} M NDP-MSH, the cAMP content (in picomoles per 3×10^5 M cells) for D122A/D126A was 1.5 ± 0.01, 2.43 ± 0.67, and 9.7 ± 0.4, respectively. The cAMP content for D122N/D126N was 1.05 ± 0.05, 2.75 ± 0.05, and 6.5 ± 0.2, respectively.

In the cases of hMC4R mutants D122A and D126A no differences in agonist binding affinities were observed when experiments were performed at room temperature in the presence of 0.5% sodium azide, conditions that partially inhibit receptor internalization. These results helped to support our interpretation that measured radioactivity in binding-displacement assays was due to specific receptor binding.

Role of Basic hMC4R TM Residues in High-Affinity Agonist Binding and Potency. Of the six basic TM residues

Table 3: K_i of ^{125}I-NDP-MSH and ^{125}I-AGRP (87−132) Binding at hMC4R Mutants Expressed in HEK-293 Cells[a]

mutation		B_{max} (fmol/mg of protein)	K_i (nM)		
			^{125}I-NDP-MSH binding		^{125}I-AGRP binding
			NDP-MSH	α-MSH	AGRP (87−132)
hMC4R	WT	331 ± 6.86	1.54 ± 0.09	34.3 ± 4.4	2.95 ± 0.19
TM1	E49A	228 ± 8.3	2.20 ± 0.10	83.7 ± 10.7*	3.87 ± 0.09
TM1	E61A	253 ± 9.4	4.04 ± 0.04	71.9 ± 8.1*	4.3 ± 0.12
TM2	D90A	258 ± 26.1	5.61 ± 0.21*	364 ± 43*	3.12 ± 0.2
TM2	E100A	232 ± 9	6.71 ± 0.65*	132 ± 18.7*	3.97 ± 0.11
TM3	D122A	364 ± 36.2	9.32 ± 2.1*	722.7 ± 28*	4.9 ± 1.2
TM3	D122N	243 ± 41	5.56 ± 1.3*	129.3 ± 16.3*	3.98 ± 0.87
TM3	D126A	180 ± 9.2	$>10^3$*	ND	26.4 ± 7.43*
TM3	D126N	116 ± 12.4	$>10^3$*	ND	15.9 ± 3.2*
TM3	D122N/D126N		ND	ND	ND
TM3	D122A/D126A		ND	ND	ND
TM3	C130A	227 ± 11	2.82 ± 0.25	28.3 ± 2.2	3.3 ± 0.41
TM4	K164A	243 ± 19.3	1.73 ± 0.31	35.1 ± 3.6	3.89 ± 0.43
TM4	R165A	216 ± 21	2.03 ± 0.12	54.5 ± 8.9	3.12 ± 0.31
TM5	C196A	324 ± 19	3.10 ± 0.33	30.9 ± 12.4	3.18 ± 0.33
TM5	T199A	198 ± 19	1.6 ± 0.1	42.6 ± 10.6	2.5 ± 0.9
TM5	M200A	209 ± 17	3.02 ± 0.54	579.7 ± 22.6*	3.1 ± 0.1
TM5	T203A	289 ± 23	2.11 ± 0.13	45.3 ± 4.3	2.14 ± 0.3
TM5	M204A	211 ± 6.5	2.31 ± 0.3	$>10^3$*	2.6 ± 0.2
TM5	H214A	306 ± 17	1.41 ± 0.54	42.8 ± 6.1	3.3 ± 0.23
TM5	W258A	394 ± 14	4.01 ± 0.13	782.7 ± 16.5*	2.31 ± 0.08
TM6	F261A	293 ± 24	8.31 ± 1.32*	160 ± 11.3*	3.98 ± 0.13
TM6	H264A	210 ± 12.8	$>10^3$*	ND	4.1 ± 0.43
TM6	F267A	215 ± 23	1.65 ± 0.3	22 ± 7.5	1.9 ± 0.3
TM6	Y268A	203 ± 11	1.87 ± 0.9	16 ± 3.1	3.6 ± 0.8
TM7	H283A	250 ± 16	1.11 ± 0.3	27 ± 3.4	2.26 ± 0.3
TM7	N285A	277 ± 34	2.52 ± 0.01	36.3 ± 4.5	2.64 ± 0.1
TM7	D298A	204 ± 15.2	4.54 ± 0.05*	79.4 ± 5.6*	2.98 ± 0.34
TM7	R305A	333 ± 32	1.9 ± 0.32	90.4 ± 17.3*	2.7 ± 0.27

[a] ND = specific binding is not detectable. $n > 3$, mean ± SEM. WT = wild-type. An asterisk indicates $P < 0.01$. B_{max} values of hMC4R WT and mutants were determined with ^{125}I-NDP-MSH except for D126A, D126N, and H264A where ^{125}I-AGRP (87−132) was used.

mutated in the sequence of the hMC4R (TM4 residues K164 and R165, TM5 residue H214, TM6 residue H264, and TM7 residues H283 and R305), only TM6 residue H264 dramatically affected α-MSH and NDP-MSH binding affinity or potency. Only marginal NDP-MSH binding was detectable at mutant H264A. Consistent with this decreased binding affinity, the potency of NDP-MSH at mutant H264A was decreased 3 logs, and the ability of α-MSH to stimulate cAMP was nearly abolished. In contrast, mutation R305A had no effect on NDP-MSH binding affinity and only a 3-fold effect on α-MSH binding affinity. Mutation R305A had no effect on the potency of either agonist. Mutations of basic TM3 residues K164 and R165, TM5 residue H214, and TM7 residue H283 to alanine had no significant effect on agonist binding affinity or potency (see Tables 3 and 4 for K_i and EC_{50} values).

Role of Hydroxyl, Sulfhydryl, and Aromatic hMC4R TM Residues in High-Affinity Agonist Binding and Potency (See Tables 3 and 4 for K_i and EC_{50} Values). Mutagenesis of hydroxyl-containing TM5 residues T199 and T203 and TM7 residue Y268 to alanine had no significant effect on α-MSH or NDP-MSH binding affinity or potency.

Four sulfhydryl-containing TM residues, TM3 residue C130, TM5 residue C196, and TM5 residues M200 and M204, were mutated. Mutations C130A and C196A had no effect on α-MSH or NDP-MSH binding affinity or potency. Mutations M200A and M204A had a differential effect on agonist binding affinity and potency. While neither mutation affected NDP-MSH binding affinity or potency, M200A caused a 1 log decrease in the binding affinity and a 7-fold decrease in the potency of α-MSH, and M204A caused a 2 log decrease in the binding affinity and a 4-fold loss in the potency of α-MSH.

Mutations of aromatic TM6 residues W258 and F261 to alanine both significantly affected α-MSH binding affinity and potency. However, only F261A had a significant effect on NDP-MSH binding affinity and potency. Mutation F261A caused a 0.5 log decrease in the binding affinity and potency of both NDP-MSH and α-MSH (NDP-MSH data depicted in Figure 4). Mutation of F267A had no effect on agonist binding affinity or potency.

SHU 9119 Binding to hMC4R Mutants. SHU 9119 is a cyclic melanocortin analogue that contains D-naphthalene instead of Phe in the core melanocortin sequence and is an antagonist at the hMC3R and hMC4R (*27*). It has been frequently used in studies examining the effects of melanocortins on feeding behavior and ^{125}I-SHU 9119 and provided us with another radioligand with which to examine the effects of selected hMC4R TM mutations (D122A, D126A, H264A) on ligand binding. As depicted in Figure 6, SHU 9119 binding affinity was decreased 1 log (26 ± 15 nM) at D122A compared to the wild-type hMC4R. The K_i values of SHU 9119 at hMC4R mutants D126A and H264A were greater than or equal to 1 μM.

^{125}I-AGRP (87−132) Binding to hMC4R Mutations. D126 was the only individual receptor residue of those mutated whose mutation significantly affected AGRP (87−132) binding affinity. The single mutation D126A decreased binding affinity 8-fold, and simultaneous mutations of hMC4R residues D122 and D126 to alanine (mutant D122A/

Table 4: EC_{50} of NDP-MSH and α-MSH at hMC4R Mutants Expressed in HEK-293 Cells[a]

mutation		cAMP EC_{50} (nM)	
		NDP-MSH	α-MSH
hMC4R	WT	0.27 ± 0.05	1.34 ± 0.11
TM1	E49A	0.42 ± 0.10	2.37 ± 0.12
TM1	E61A	0.51 ± 0.12	6.12 ± 0.54*
TM2	D90A	NR	NR
TM2	E100A	0.65 ± 0.16*	4.45 ± 0.08*
TM3	D122A	1.67 ± 0.1*	$>10^3$*
TM3	D122N	0.69 ± 0.1*	155 ± 32*
TM3	D126A	$>10^3$*	NR
TM3	D126N	342 ± 22*	NR
TM3	D122A/D126A	$>10^3$*	NR
TM3	D122N/D126N	$>10^3$*	NR
TM3	C130A	0.47 ± 0.15	1.23 ± 0.12
TM4	K164A	0.44 ± 0.12	2.5 ± 12
TM4	R165A	0.33 ± 0.11	3.1 ± 0.4
TM5	C196A	0.42 ± 0.02	2.36 ± 0.13
TM5	T199A	0.29 ± 0.02	2.5 ± 0.1
TM5	M200A	0.45 ± 0.16	9.1 ± 1.2*
TM5	T203A	0.33 ± 0.08	1.76 ± 0.21
TM5	M204A	0.54 ± 0.16	5.7 ± 1.1*
TM5	H214A	0.18 ± 0.13	2.4 ± 0.2
TM6	W258A	0.54 ± 0.03	16 ± 1.4*
TM6	F261A	1.06 ± 0.07*	5.43 ± 0.76*
TM6	H264A	134 ± 7.2*	$>10^3$*
TM6	F267A	0.37 ± 0.1	2.9 ± 0.1
TM6	Y268A	0.5 ± 0.12	90 ± 0.8*
TM7	H283A	0.27 ± 0.01	2.54 ± 0.01
TM7	N285A	0.23 ± 0.03	2.37 ± 0.09
TM7	D298A	NR	NR
TM7	R305A	0.49 ± 0.14	1.31 ± 0.3

[a] NR = no response. $n > 3$, mean ± SEM. An asterisk indicates $P < 0.01$.

D126A) completely abolished AGRP (87−132) binding. The mutation D126N had a lesser effect on AGRP (87−132) binding affinity, but the double mutation D122N/D126N displayed no demonstrable binding. Because the mutations D126L and D126S did not increase NDP-MSH binding affinity compared to D126A, AGRP (87−132) binding studies were not performed with these mutations. Notably, the mutation H264A had no effect on AGRP (87−132) binding despite the fact this mutation abolished NDP-MSH binding (Figure 4, panel C).

Binding Affinity and Potency of Substituted NDP-MSH Peptides at Receptor Mutants. To find evidence of an interaction between a specific ligand group and receptor residue, the binding affinity and potency of NDP-MSH peptides substituted at positions 6−9 (peptides II, III, IV, and V in Table 1) were examined at hMC4R mutants found to affect the binding affinity of NDP-MSH 5-fold or more, including mutants D122A, D126A, F261A, and H264A. In the cases of mutant D126A and H264A these studies ultimately proved to be inconclusive because of the greatly diminished NDP-MSH binding affinity. In addition, no concordance in the binding affinity or potency of NDP-MSH and substituted NDP-MSH peptides was observed at mutation F261A.

However, in studies that compared the binding affinity of substituted peptides at the wild-type hMC4R and mutant D122A, it was observed that Nle8-substituted NDP-MSH and NDP-MSH had identical K_i values (Table 5). Notably, K_i values were similar when either ^{125}I-NDP-MSH or ^{125}I-SHU 9119 was used as the radioligand. In binding experi-

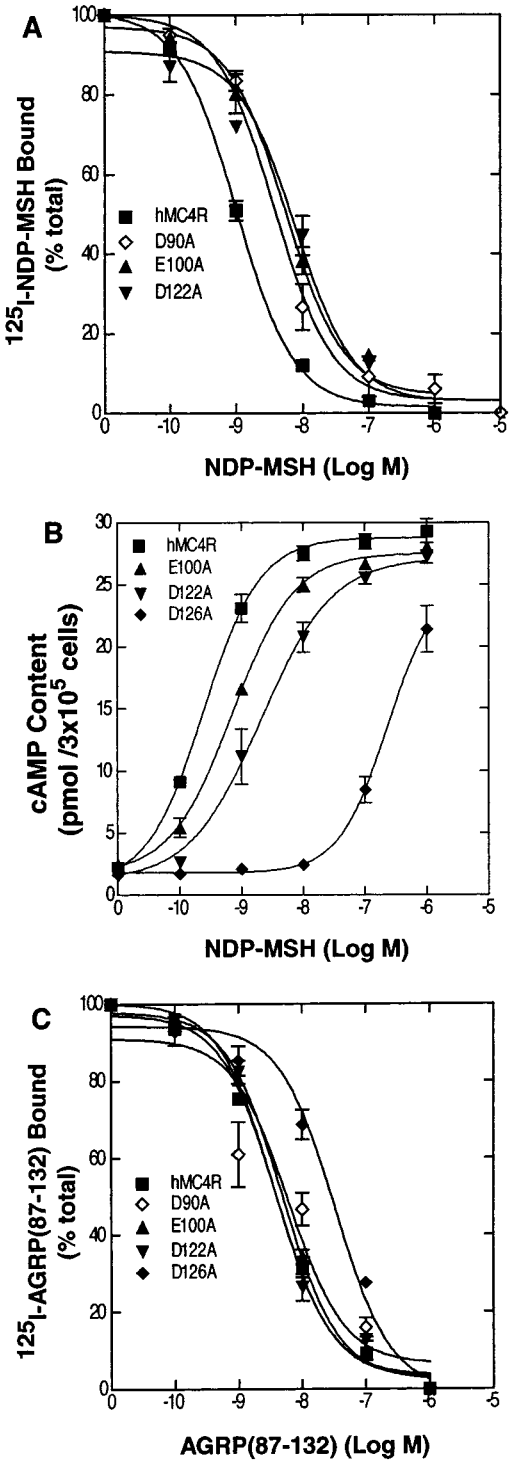

FIGURE 3: Effects of alanine mutation of acid hMC4R TM residues on the binding affinity and potency of NDP-MSH (panels A and B) and on the binding affinity of AGRP (87−132) (panel C) (n = 3; see Tables 3 and 4 for actual K_i and EC_{50} values). The marginal NDP-MSH binding observed at D126A is not depicted in panel A. The D90A mutation did not generate cAMP. Total cpm and nonspecific cpm for ^{125}I-NDP-MSH binding were as follows: wild-type hMC4R = 37 530 ± 1027 and 3963 ± 250; D90A = 19 429 ± 719 and 5035 ± 482; E100A = 26 767 ± 972 and 5940 ± 714; D122A = 16 244 ± 449 and 4076 ± 137. Total cpm and nonspecific cpm for ^{125}I-AGRP binding were as follows: wild-type hMC4R = 23 941 ± 1936 and 3824 ± 532; D90A = 23 669 ± 2151 and 6675 ± 521; E100A = 14 619 ± 388 and 3602 ± 319; D122A = 28 515 ± 313 and 4588 ± 389; D126A = 17 614 ± 1016 and 4679 ± 408.

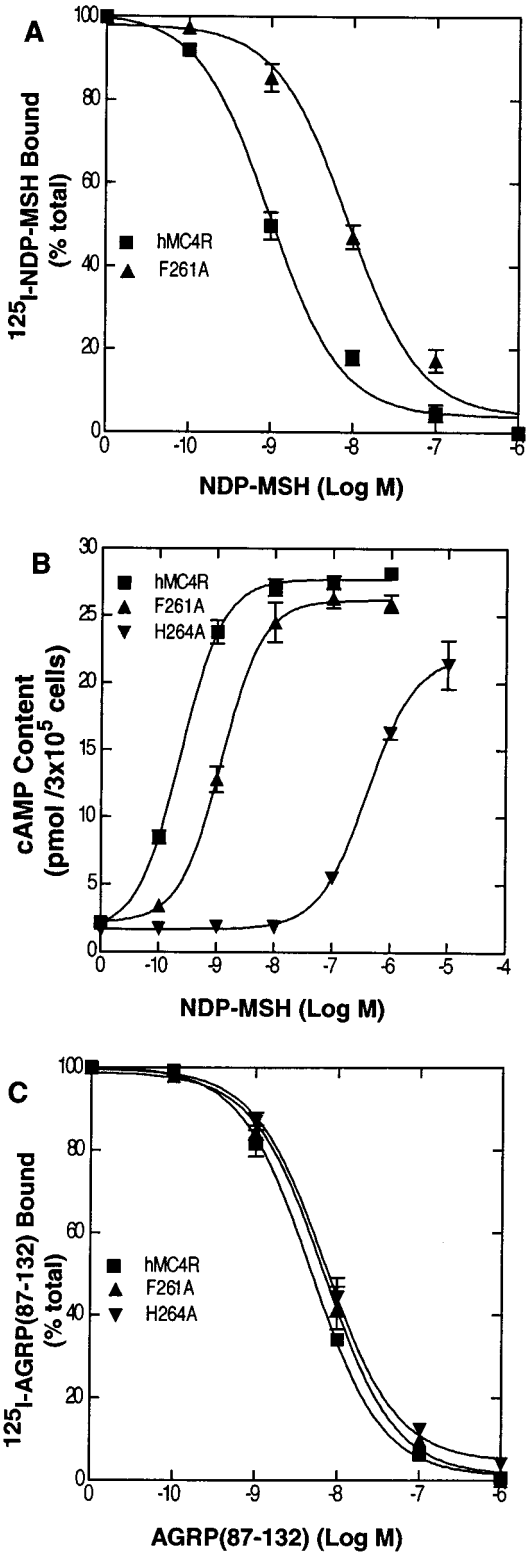

FIGURE 4: Effect of alanine mutation of basic hMC4R TM6 residues F261 and H264 on binding affinity and potency of NDP-MSH (panels A and B) and on the binding affinity of AGRP (87−132) (panel C) ($n = 3$; see Tables 3 and 4 for actual K_i and EC_{50} values). The marginal NDP-MSH binding to H264A is not depicted in panel A. Total cpm and nonspecific cpm for ^{125}I-NDP-MSH binding were as follows: wild-type hMC4R = 38 797 ± 1265 and 5423 ± 398; F261A = 16 503 ± 506 and 3494 ± 322. Total cpm and nonspecific cpm for ^{125}I-AGRP binding were as follows: wild-type hMC4R = 21 435 ± 765 and 4021 ± 321; F261A = 16 116 ± 632 and 4295 ± 334; H264A = 19 755 ± 1089 and 3320 ± 163.

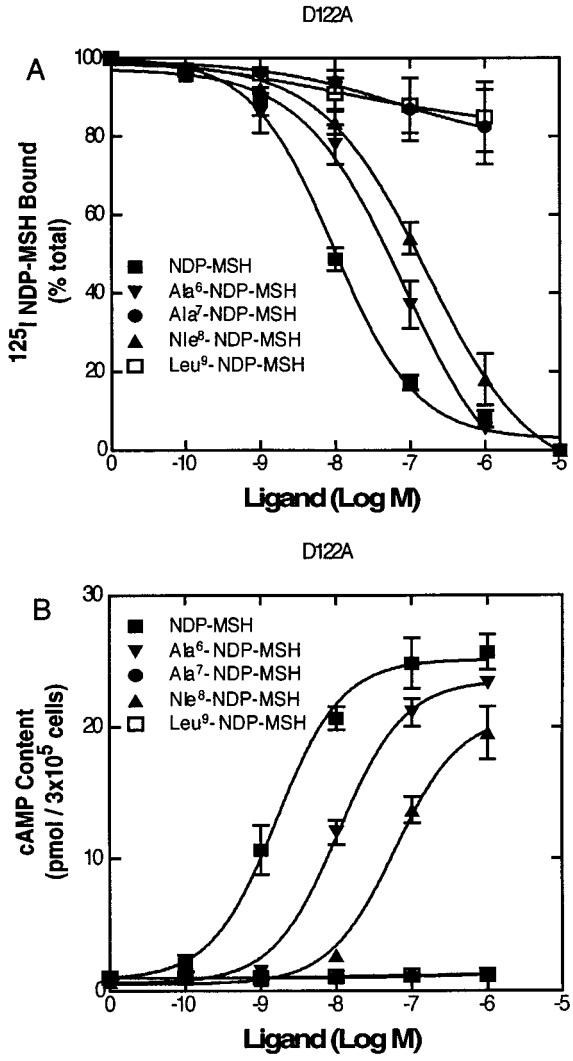

FIGURE 5: Comparison of the binding affinity (A) and potency (B) of NDP-MSH and substituted NDP-MSH peptides at hMC4R mutant D122A ($n = 3$; data depicted are mean ±SE). Total cpm and nonspecific cpm for ^{125}INDP-MSH binding at D122A were 16 121 ± 1519 and 4770 ± 338 when NDP-MSH was used to displace ^{125}I-NDP-MSH, 17 654 ± 1232 and 3654 ± 567 when Ala6-substituted NDP-MSH was used to displace ^{125}I-NDP-MSH, and 16 875 ± 765 and 5463 ± 232 when Nle8-substituted NDP-MSH was used to displace ^{125}I-NDP-MSH.

ments using ^{125}I-NDP-MSH the K_i values for NDP-MSH and Nle8-substituted NDP-MSH were 273 ± 14.6 nM and 264 ± 9.6 nM, respectively. In binding experiments using ^{125}I-SHU 9119 the K_i values for NDP-MSH and Nle8-substituted NDP-MSH were 517 ± 180 nM and 547.5 ± 202 nM, respectively.

In other studies that compared the binding affinity and potency of NDP-MSH and substituted peptides at mutation D122A, it was observed that the competition binding and dose−response curves of NDP-MSH, Ala6-substituted NDP-MSH, and Nle8-substituted NDP-MSH had the same rank order of potency in binding and functional assays (Figure 5). The K_i values for NDP-MSH, Ala6-substituted NDP-MSH, and Nle8-substituted NDP-MSH were 9.3 ± 0.2 nM, 76.8 ± 1.6 nM, and 264 ± 9.6 nM, respectively. The EC_{50} values for NDP-MSH, Ala6-substituted NDP-MSH, and Nle8-substituted NDP-MSH were 1.67 ± 0.1 nM, 10 ± 0.1 nM, and 57 ± 0.1 nM, respectively.

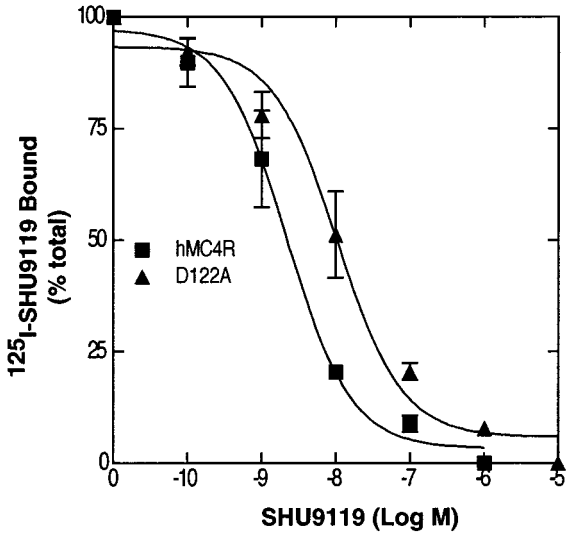

FIGURE 6: SHU 9119 binding to hMC4R mutation D122A ($n = 3$; data depicted are mean ±SE). Total cpm and nonspecific cpm for ^{125}I-SHU 9119 binding were as follows: wild-type hMC4R = 46 155 ± 464 and 4444 ± 642; D122A = 10 435 ± 304 and 3146 ± 198.

DISCUSSION

The hypothalamic control of feeding behavior is a process involving the complex interaction of multiple neuropeptides and biogenic amines. Among opposing neuropeptides the anorexigenic agent α-MSH and the orexigenic agent AGRP are uniquely associated by their use of the same receptor, the MC4R. AGRP is thought to produce its orexigenic effects by competitively inhibiting α-MSH binding and activation of MC4R-expressing neurons in the hypothalamic paraventricular nucleus. To explore the nature of the molecular interaction of α-MSH and AGRP with the hMC4R, structure−activity studies of the synthetic melanocortin agonist NDP-MSH and point mutagenesis of the hMC4R were performed.

Identification of NDP-MSH Residues Critical for hMC4R Binding and Activation. Structure−activity studies of NDP-MSH demonstrated that substitution of residue Arg8 with an uncharged amino acid, Nle8, or substitution of residues D-Phe7 and Trp9 with nonaromatic amino acids, D-Ala7 and Leu9, greatly decreased binding affinity and potency of substituted NDP-MSH peptides at the hMC4R (Table 1, substituted peptides III, IV, and V). Studies with truncated NDP-MSH peptides further highlighted the importance of residues 7−9 by demonstrating that the smallest NDP-MSH fragment that possesses full agonist efficacy is the tripeptide D-Phe7-Arg8-Trp9 (Table 2). Taken together these data indicate that amino acids D-Phe7-Arg8-Trp9 are critical for the biological activity of NDP-MSH, presumably due to their key role in maintaining NDP-MSH structure and/or their role in binding hMC4R residues.

Of the 24 hMC4R TM residues that were mutated, four residues, TM3 residues D122 and D126 and TM6 residues F261 and H264, were identified whose mutations affected the binding affinity of NDP-MSH 5-fold or greater (Tables 3 and 4). Although mutations D90A, E100A, and D298A significantly affected NDP-MSH binding affinity as analyzed by a paired T-test, the effects of these mutations were less than 5-fold. In contrast, D90A affected α-MSH binding affinity 1 log, and alanine mutations of TM5 residues M200 and M204 and TM6 residue W258 constituted a group of mutations that selectively decreased α-MSH binding affinity 1 log or greater without having any significant effect on NDP-MSH binding affinity.

Examination of the Mechanism Underlying Changes in Agonist Binding Affinity and Potency. In an attempt to distinguish whether the observed pharmacological changes associated with mutagenesis were due to the disruption of molecular interactions between ligand and receptor residues or simply a consequence of the nonspecific alteration of receptor tertiary structure, experiments were performed with substituted NDP-MSH peptides.

Since one possible reason for the importance of amino acids belonging to the key NDP-MSH tripeptide D-Phe7-Arg8-Trp9 is their participation in molecular interactions with hMC4R residues, the binding affinities of NDP-MSH peptides substituted at positions 7−9 were examined at hMC4R mutants found to have altered NDP-MSH binding affinity greater than 5-fold, which was a threshold we had set for performing these additional studies. Mutants of D122, D126, F261, and H264 met this criterion; however, marginal ^{125}I-NDP-MSH binding at D126A and H264A made it impossible to obtain consistent results with those mutants in these binding-displacement assays. In addition, we also examined the Ala6-substituted NDP-MSH peptide in these studies since the His6 residue is present in the sequence of all naturally occurring melanocortin peptide, and addition of histidine to D-Phe7-Arg8-Trp9 was found to significantly increase potency (Table 2, peptide VII). We reasoned two types of analyses might provide evidence of a direct interaction between ligand and receptor residues.

In the first type of analysis (type 1 analysis), the binding affinities of the substituted NDP-MSH peptides were compared at the wild-type hMC4R and mutant hMC4Rs. If a specific NDP-MSH residue interacted at a molecular level with a particular receptor residue, one might expect that the binding affinity of that substituted NDP-MSH peptide would be identical at the wild-type and mutant hMC4R. This outcome would be anticipated since the specific ligand−receptor interaction involved should be disrupted to the same extent at both receptors. However, a more complex outcome would occur if the mutated receptor residue interacted with more than one ligand residue.

In the second type of analysis (type 2 analysis), the binding affinities of the substituted NDP-MSH peptides were compared with the binding affinity of NDP-MSH at a specific receptor mutant. In this type of analysis if a specific NDP-MSH residue and a specific receptor residue interacted at a molecular level, the binding affinity of the substituted NDP-MSH peptide and the binding affinity of NDP-MSH would be expected to correlate in one of two ways. If the substituted agonist residue interacted with only the mutated receptor residue, then the binding affinity of the substituted NDP-MSH peptide and wild-type NDP-MSH would be expected to be identical. This outcome would be anticipated since removal of both the ligand and receptor components of the ligand−receptor interaction (by ligand residue substitution and receptor mutagenesis) would not be expected to diminish peptide binding affinity any more than removing only the receptor component of the interaction (by receptor mutagenesis). If, however, one ligand residue interacted with several receptor residues, a more complex pattern of relative binding

Table 5: Binding Affinity of NDP-MSH Analogues[a]

	K_i (nM) of peptides against			
	^{125}I-NDP-MSH		^{125}I-SHU 9119	
	WT	D122A	WT	D122A
NDP-MSH	1.3 ± 0.1 (5)	9.3 ± 0.5 (5)	1.2 ± 0.3 (8)	26 ± 15 (3)
Ala6-substituted NDP-MSH	20 ± 0.3 (3)	76.8 ± 1.6 (3)	2.7 ± 0.98 (7)	900 ± 322 (3)
Ala7-substituted NDP-MSH	1613 ± 90 (3)	$>10^3$ (3)	NP	NP
Nle8-substituted NDP-MSH	273 ± 14.6 (3)	264 ± 9.6 (3)	517 ± 180 (5)	547.5 ± 202 (2)
Phe9-substituted NDP-MSH	8.7 ± 1.1 (3)	136 ± 28 (3)	15.75 ± 3.75 (2)	10000 (2)

[a] NP = not performed.

affinity would be anticipated for NDP-MSH versus the substituted NDP-MSH peptides.

Data obtained from our analyses of the binding affinity of substituted NDP-MSH peptides at mutant receptors appear to provide evidence of a direct molecular interaction between Arg8 of the NDP-MSH sequence and TM3 aspartic acid residue D122. Data supporting the existence of an Arg8−D122 ligand−receptor interaction were found in both type 1 and type 2 analysis (Figure 5A, Table 5). The existence of this interaction is further supported by an examination of potency of substituted peptides at D122 (Figure 5B).

In type 1 analysis (a comparison of the binding affinity of substituted NDP-MSH peptides at the wild-type hMC4R and mutant receptors) Nle8-substituted NDP-MSH was observed to have an identical binding affinity at the wild-type hMC4R and mutant D122A (Table 5). Similar K_i values were observed when either ^{125}I-NDP-MSH or ^{125}I-SHU 9119 was used as the radioligand. In contrast, the binding affinities of other substituted NDP-MSH peptides were more divergent when compared at the wild-type hMC4R and mutant receptor D122A. Since these results are compatible with a direct interaction between a specific ligand and receptor residue as outlined for type 1 analysis, we interpreted these results to indicate that Arg8 of the NDP-MSH sequence interacts at a molecular level with hMC4R residue D122. As discussed above, these results also imply that receptor residue D122 only interacts with one agonist residue, Arg8.

In type 2 analysis (a comparison of the binding affinity of NDP-MSH and substituted NDP-MSH peptides at hMC4R mutants) the fact that NDP-MSH was observed to have a greater binding affinity and potency than Nle8-NDP-MSH indicates that an Arg8−D122 interaction alone is unlikely to account for the entire Arg8−hMC4R interaction (Figure 5). These observations suggested to us that melanocortin residue Arg8 is likely to interact with one or more additional hMC4R residue(s) in addition to D122.

Within the conceptual framework of an Arg8−hMC4R interaction where melanocortin residue Arg8 interacts with D122 and a second (or multiple) hMC4R residue(s), the greater binding affinity and potency of NDP-MSH versus Nle8-substituted NDP-MSH would be anticipated. When binding to receptor mutant D122, Nle8-substituted NDP-MSH is disadvantaged compared to NDP-MSH by having two ligand−receptor interactions disrupted, the Arg8−D122 interaction and the interaction of Arg8 with another hMC4R residue. In contrast, NDP-MSH has only one ligand−receptor interaction disrupted, the Arg8−D122 interaction.

A similar rationale can also be used to explain the greater binding affinity and potency of Ala6-substituted NDP-MSH versus Nle8-substituted NDP-MSH at mutant D122A (Figure 5). When binding to mutant D122A, the Ala6-substituted NDP-MSH peptide essentially represents a peptide with two ligand−receptor interactions disrupted, the His6−hMC4R interaction and the Arg8−D122 component of the Arg8−hMC4R interaction. However, Ala6-substituted NDP-MSH, in contrast to Nle8-substituted NDP-MSH, is still capable of participating in the rest of the Arg8−hMC4R interaction. In addition, data listed in Table 1 indicate that the His6−hMC4R interaction only provides a modest amount of energy to NDP-MSH−hMC4R binding. Consequently, as observed in these studies, Ala6-substituted NDP-MSH has a greater binding affinity than Nle8-substituted NDP-MSH. These results also suggest that the His6−hMC4R interaction contributes less energy to binding than the Arg8−D122 interaction.

The present studies, however, do not identify the other receptor residue(s) that in addition to D122 is (are) involved in the Arg8−hMC4R interaction. D126, another negatively charged hMC4R residue, must be considered a potential candidate for participation in the Arg8−hMC4R interaction because it is negatively charged and theoretically faces the same receptor surface as D122 (since 3−4 amino acids is approximately one full turn in the presumed α-helical structure of seven TM G-protein-coupled TM domains). However, the marked disruption of agonist binding associated with mutations of D126 (D126A, D126L, D126N, and D126S) made it impossible to analyze this receptor residue with substituted NDP-MSH peptides, an analysis that might have provided insight into the mechanism underlying the effect of D126 mutations on binding affinity. It is important to point out that there is clear evidence of the functional expression of D126 mutants as evidenced by their ability to bind AGRP (87−132) (Table 3) and their ability to stimulate the production of cAMP in response to NDP-MSH (Table 4).

The use of substituted NDP-MSH peptides also did not provide insight into the identity of the hMC4R residue(s) that interact(s) with NDP-MSH residue D-Phe7. The role of D-Phe7 in agonist binding is of considerable importance since it is the introduction of this residue into the sequence of α-MSH that confers superpotency (*27*).

Insights into AGRP (87−132) hMC4R Binding Determinants. Another important aspect of these studies is the additional insight that it provides into the diminished AGRP (87−132) binding affinity, 8-fold. Furthermore, simultaneous mutation of hMC4R residues D122 and D126 to alanine (D122A/D126A) completely abolished AGRP (87−132) binding (Table 3). While these results only constitute circumstantial evidence that an AGRP (87−132) residue interacts with either D122 or D126, in view of our evidence

supporting an NDP-MSH−D122 interaction it is interesting to speculate about the total loss of AGRP (87−132) binding affinity at D122A/D126A. That is, regardless of one's biases regarding the mechanism underlying decreased AGRP (87−132) binding affinity at D126A [disruption of a molecular interaction between an AGRP (87−132) residue and D126 versus changes in receptor tertiary structure], the double mutant D122A/D126A could be interpreted to have "uncovered" an AGRP (87−13)−D122 interaction. In other words, the contribution of D122 to AGRP (87−132) binding was not apparent when the isolated D122 mutations D122A or D122N were examined because AGRP (87−132) has many other sites on the hMC4R with which it can interact.

These data, therefore, could be viewed as providing further support for the analogy between AGRP residue Arg111 and α-MSH residue Arg8 (*20*). If this were the case, D122 would be a receptor residue that directly interacts with α-MSH, NDP-MSH, and AGRP (87−132) residues. This, in conjunction with previously published data on the contribution of hMC4R exo loops to AGRP (87−132) binding (*23*), would invoke a model of AGRP (87−132) binding to the hMC4R in which both exo loops and TM domains participate.

A second notable observation arising from the ^{125}I-AGRP binding-displacement studies is the dramatically divergent effect of the TM6 mutation of H264A on the binding affinity of NDP-MSH and AGRP (87−132). This mutation had no effect on AGRP (87−132) binding affinity but markedly diminished NDP-MSH binding affinity. This result further highlights the differences in the receptor binding surfaces of these two ligands.

Comparison of hMC4R and hMC1R Ligand Binding Pockets. The present data indicate that several similarities exist between the hMC1R and hMC4R. First, both MCR subtypes are fully activated by the same minimal NDP-MSH tripeptide sequence, D-Phe-Arg-Trp. Second, mutation of homologous residues in TM2, TM3, and TM6 of the hMC1R and hMC4R are found to affect agonist binding affinity (hMC1R residues E94, D117, D121, and H260 are homologous to hMC4R residues E100, D122, D126, and H264, respectively) (*24*, *25*). Finally, Wikberg et al. have examined the interaction of Glu5-, His6-, and Phe7-substituted α-MSH peptides and concluded that residues Glu5 and His6 do not interact with hMC1R residues D117 and H260 (*34*). If parallels can be drawn between hMC1R and hMC4R, our data concur with those studies; i.e., α-MSH residues His6 and Phe7 do not interact with the corresponding hMC4R residues D126 and H264.

Despite the enumerated similarities between the hMC1R and hMC4R, the present studies did identify a potentially important subtype-specific difference between the hMC1R and hMC4R. In the present studies mutation of TM6 residue H264A affects both α-MSH and NDP-MSH binding affinity. In the case of the hMC1R the homologous TM6 mutation H260A only affects the binding affinity of α-MSH (*24*, *25*). Since NDP-MSH binding affinity is unaltered by the homologous hMC1R mutation H260A, this observation may indicate that NDP-MSH is oriented substantially differently in its hMC4R binding pocket.

In the present studies aromatic residues were not as extensively examined as in previous studies of the hMC1R (*25*). Nonetheless, several aromatic mutants were found that significantly affected agonist binding to the hMC4R (i.e., TM6 W258 and F261). Therefore, the present data are not inconsistent with the existence of aromatic−aromatic interactions between ligand residues Phe7 and Trp9 of the melanocortin sequence and the hMC4R, interactions that we previously hypothesized exist in the case of the hMC1R (*25*, *26*).

Summary. In summary, these studies have identified multiple TM receptor residues that affect agonist and antagonist binding affinity to the hMC4R. Importantly, data derived from our use of substituted NDP-MSH peptides strongly suggest that a direct molecular interaction exists between hMC4R acidic TM3 aspartic acid residue 122 and residue Arg8 of the melanocortin core sequence. These data also suggest that Arg8 of the melanocortin sequence likely interacts with one or more additional receptor residues.

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