Molecular Characterization of Human Melanocortin-3 Receptor Ligand−Receptor Interaction †

ARTICLE *in* BIOCHEMISTRY · FEBRUARY 2006

Impact Factor: 3.02 · DOI: 10.1021/bi0521792 · Source: PubMed

7 AUTHORS, INCLUDING:

Min Chen
University at Buffalo, The State University of …
47 PUBLICATIONS **1,022** CITATIONS
SEE PROFILE

Ahmet Celik
Ege University
39 PUBLICATIONS **482** CITATIONS
SEE PROFILE

Molecular Characterization of Human Melanocortin-3 Receptor Ligand−Receptor Interaction†

Min Chen,§ Charles J. Aprahamian,§ Ahmet Celik,§ Keith E. Georgeson,§ W. Timothy Garvey,‡ Carroll M. Harmon,§ and Yingkui Yang*,§

Department of Surgery, and Department of Nutrition and Sciences, University of Alabama at Birmingham, Birmingham, Alabama 35233

Received October 25, 2005; Revised Manuscript Received November 30, 2005

ABSTRACT: Melanocortin-3 receptor (MC3R), primarily expressed in the hypothalamus, plays an important role in the regulation of energy homeostasis. MC3R-deficient (*MC3R*$^{-/-}$) mice demonstrate increased fat mass, higher feeding efficiency, hyperleptinaemia, and mild hyperinsulinism. At least one specific mutation of MC3R has been identified to be associated with human obesity. Functional analysis of this altered MC3R (I183N) has indicated that the mutation completely abolishes agonist-mediated receptor activation. However, the specific molecular determinants of MC3R responsible for ligand binding and receptor signaling are currently unknown. The present study is to determine the structural aspects of MC3R responsible for ligand binding and receptor signaling. On the basis of our theoretical model for MC1R, using mutagenesis, we have examined 19 transmembrane domain amino acids selected for these potential roles in ligand binding and receptor signaling. Our results indicate that (i) substitutions of charged amino acid residues E131 in transmembrane domain 2 (TM2), D154 and D158 in TM3, and H298 in TM6 with alanine dramatically reduced NDP-MSH binding affinity and receptor signaling, (ii) substitutions of aromatic amino acids F295 and F296 in TM6 with alanine also significantly decreased NDP-MSH binding and receptor activity, (iii) substitutions of D121in TM2 and D332 in TM7 with alanine resulted in the complete loss of ligand binding, ligand induced receptor activation, and cell surface protein expression, and (iv) interestingly, substitution of L165 in TM3 with methionine or alanine switched antagonist SHU9119 into a receptor agonist. In conclusion: Our results suggest that TM3 and TM6 are important for NDP-MSH binding, while D121 in TM2 and D332 in TM7 are crucial for receptor activity and signaling. Importantly, L165 in TM3 is critical for agonist or antagonist selectivity. These results provide important information about the molecular determinants of hMC3R responsible for ligand binding and receptor signaling.

Obesity is a common and rapidly growing health problem in the United States (*1*−*5*). The condition arises from an improper balance between energy intake and expenditure, and is a risk factor for diabetes mellitus, cardiovascular disorders, and cancer. The rising prevalence of obesity may be due to the multiple effects that result from the interaction of genetic factors with the environment (*6*−*8*). The melanocortin system has been identified to play an important role in the regulation of food intake and body weight in animals and humans. In particular, the melanocortin-3 (MC3R)[1] and -4 receptors (MC4R) are critical in the control of feeding behavior and body weight (*9*−*13*). Genetic studies indicate that both MC3R- and MC4R-deficient mice are obese; however, their obesity phenotypes differ in that MC4R knock-out (KO) mice are hyperphagic, while obese MC3R KO mice are not (*14*, *15*). However, to date, only one specific MC3R mutation has been identified to be associated with human obesity (*16*). Functional analysis of this MC3R mutation (I183N) has indicated that the mutation completely abolishes agonist-mediated receptor activation (*16*). While great effort and progress have been made in determining the role of MC4R in regulating food intake, much still remains to be learned regarding the molecular basis of ligand binding and receptor activation, and the explanation for the different pharmacological profiles of MC3R and MC4R. Alpha melanocyte-stimulating hormone (α-MSH) is equally potent at MC3R and MC4R, but γ-MSH has higher affinity at MC3R than MC4R. Agouti related protein (AGRP) is a potent antagonist at both MC3R and MC4R, but agouti is a potent antagonist only at MC4R (*17*−*19*). These differences indicate that these two receptors, though members of the same system, are likely to possess functional and structural differences. Determination of the molecular basis of ligand binding and receptor signaling should, therefore, provide important insights into the mechanism of MC3R action. In this study, we have found that the TM3 and TM6 of hMC3R

† This work has been funded by NIH Grants R03 HD047312-01A1 (Y.-K.Y.).

* Correspondence to Yingkui Yang, M.D., Ph.D. Division of Pediatric Surgery, University of Alabama at Birmingham, 300 ACC, 1600 7th Avenue South. Birmingham, AL 35233. Phone: (205) 939-9688. Fax: (205) 975-4972. E-mail: ying-kui.yang@ccc.uab.edu.

§ Department of Surgery.

‡ Department of Nutrition and Sciences.

[1] Abbreviations: MCR, melanocortin receptor; hMC3R, human melanocortin-3 receptor; GPCR, G-protein coupled receptor; NDP-MSH, [Nle^4,D-Phe^7]-α-melanocyte stimulating hormone; SHU9119 Ac-Cys-c[Asp-His-D(Nal)-Arg-Trp-Cys-Lys]-NH_2; TM, transmembrane domains; IBMX, 3-isobutyl-methylxanthine; PCR, polymerase chain reaction; FACs, flow cytometry.

10.1021/bi0521792 CCC: $33.50
Published on Web 01/07/2006

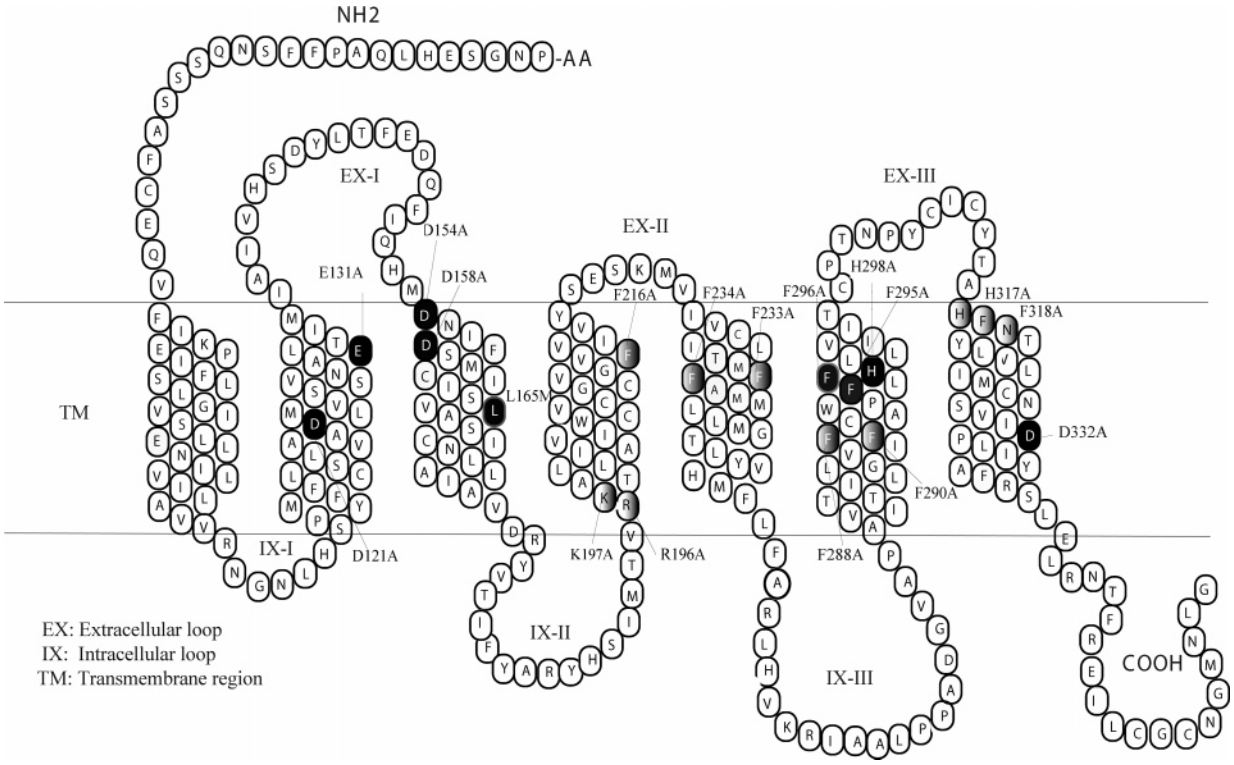

FIGURE 1: Two-dimensional representation of the seven TM structure of the hMC3R. The TM residues mutated in these experiments are denoted by black or gray highlighting. Those TM residues whose mutation significantly affected NDP-MSH binding as determined are highlighted by black.

are important for NDP-MSH binding, while D122 in TM2 and D332 in TM7 are important for receptor expression and activation. The residue L165 in TM3 is critical for agonist or antagonist selectivity.

EXPERIMENTAL PROCEDURES

Peptides. (Nle^4*,* D*-Phe*^{7}*)* α*-MSH* (NDP-MSH), Melanotan II (MTII), and SHU9119 were purchased from Peninsula Laboratories, Inc. (Belmont, CA).

Site-Directed Mutagenesis. Single mutation was constructed using the Quick-Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). We examined the entire coding region of the mutated receptors to confirm that the desired mutation sequences were present and that no sequence errors had been introduced by performing sequencing at the University of Alabama at Birmingham Sequence Core. The mutated receptors are shown in Figure 1. The mutant receptors were subcloned into the eukaryotic expression vector pCDNA 3.1 (Invitrogen; Carlsbad, CA).

Cell Culture and Transfection. The HEK-293 cell line was purchased from ATCC (Manassas, VA) and cultured in DMEM medium containing 10% bovine fetal serum and HEPES. Cells at 80% confluence were washed twice with DMEM, and the receptor constructs were transfected into cells using lipofectamine (Life Technologies, Rockville MD). The permanently transfected clonal cell lines were selected by resistance to the neomycin analogue G418 (*20*).

Binding Assays. After media was removed, cells were incubated with various nonradioligands in 0.5 mL of MEM (Fisher Scientific, Pittsburgh, PA) containing 0.2% BSA and radioligand. Binding experiments were performed using conditions previously described (19). Briefly, 2×10^5 cpm of ^{125}I-NDP-MSH (Amersham, NJ) was used in combination with nonradiolabeled ligands, NDP-MSH, MTII or SHU9119. Binding reactions were terminated by removing the media and washing the cells twice with MEM containing 0.2% BSA. The cells were lysed with 0.2 N NaOH, and the radioactivity in the lysate was quantified in an analytical gamma counter. Nonspecific binding was determined by measuring the amount of ^{125}I-label bound in the presence of 10^{-6} M unlabeled ligand. Specific binding was calculated by subtracting nonspecifically bound radioactivity from total bound radioactivity.

cAMP Assay. cAMP generation was measured using a competitive binding assay (TRK 432, Amersham, Arlington Heights, IL). Briefly, HEK cell lines stably expressing hMC3R were used in these assays (*19*). Cell culture media was removed, and cells were incubated with 0.5 mL of Earle's Balanced Salt Solution (EBSS), containing melanocortin agonist NDP-MSH, MTII, or SHU9119 (10^{-10}−10^{-6} M), for 30 min at 37 °C in the presence of 10^{-3} M isobutylmethylxanthine. The reaction was stopped by adding ice-cold 100% ethanol (500 μL/well). The cells in each well were scraped, transferred to a 1.5 mL tube, and centrifuged for 10 min at 1900*g*, and the supernatant was evaporated in a 55 °C water bath with prepurified nitrogen gas. cAMP content was measured according to instructions accompanying the assay kit. Each experiment was performed a minimum of three times with duplicate wells.

Receptor Expression. For receptor protein expression studies, we utilized PCR to insert a FLAG tag onto the NH_2 terminus of hMC3R to characterize receptor protein cell

Table 1: Effect of NDP-MSH Residue Substitutions 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	5.6 ± 1.0	0.8 ± 0.1
Ala^6-NDP-MSH	Ac- Ser-Tyr-Ser-Nle-Glu- Ala -DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH2	38.5 ± 3.9*	13.9 ± 0.1*
DAla^7-NDP-MSH	Ac- Ser-Tyr-Ser-Nle-Glu-His- DAla -Arg-Trp-Gly-Lys-Pro-Val-NH2	>10^3*	>10^3*
Nle^8-NDP-MSH	Ac- Ser-Tyr-Ser-Nle-Glu-His- DPhe- Nle-Trp-Gly-Lys-Pro-Val-NH2	707.7 ± 112 *	487.6 ± 19.2*
Leu^9-NDP-MSH	Ac- Ser-Tyr-Ser-Nle-Glu-His- DPhe-Arg- Leu -Gly-Lys-Pro-Val-NH2	>10^3*	>10^3*

[a] $n > 3$, mean ± SEM. *$P < 0.01$ compared with NDP-MSH. Nle: norleucine.

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

		Ki (nM)	EC_{50} (nM)
NDP-MSH	Ac-Ser-Tyr-Ser-Nle-Glu-His- DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH2	5.6 ± 1.1	0.8 ± 0.1
HfRWK	His- DPhe-Arg-Trp-Lys-NH2	68 ± 13.7*	61.6 ± 5.7*
HfRW	His- DPhe-Arg-Trp-NH2	164 ± 11.6*	687.7 ± 45.1*
HfR	His- DPhe-Arg-NH2	No	No
fRW	DPhe-Arg-Trp-NH2	> 10^3*	> 10^3*

[a] NR, no response. $n > 3$, mean ± SEM. *$P < 0.01$ compared with NDP-MSH.

surface expression by flow cytometry using fluorescence-activated cell sorting (FACS). The FLAG protein is an eight-amino-acid peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys), useful for immunoaffinity purification of fusion proteins (*21*, *22*). hMC3R or mutant receptor transfected cells were harvested using 0.2% EDTA and washed twice with phosphate buffer saline (PBS). Aliquots of 3×10^6 cells were centrifuged and fixed with 3% paraformaldehyde in PBS (pH 7.4). The cells were incubated with 50 μL of 10 μg/mL murine anti-FLAG M1 monoclonal antibody (Sigma, catalog No. 316, St. Louis, MO) in incubation buffer for 45 min. Under these conditions, the primary antibody binds only to receptors located at the cell surface. The cells were collected by centrifugation and washed three times with incubation buffer. The cell pellets were suspended in 100 μL of incubation buffer containing CY3-conjugated Affinity Pure Donkey Anti-Mouse Ig G (ImmunoResearch Lab, Inc., West Grove, PA) and incubated at room temperature for 30 min. Flow cytometry was performed on a fluorescence-activated cell sorter (Becton Dickinson FACStar plus six parameter cytometer/sorter with a dual Argon ion laser, San Jose, California). The results were analyzed using the software CellQuest (Beckton-Dickinson Immunocytometry Systems, San Jose, California).

Statistical Analysis. Each experiment was performed at three separate times with duplicated wells. Data are expressed as mean ± SEM. The mean value of the dose−response data of binding and cAMP production was fit to a sigmoid curve with a variable slope factor using nonlinear squares regression analysis (Graphpad Prism, Graphpad Software, San Diego, CA). Significant differences were assessed by one-way ANOVA, with $p < 0.05$ considered to be statistically significant.

RESULTS

Role of Amino Acid Residues in NDP-MSH for High Affinity Binding and Potency at the Wild-Type hMC3R. To determine which NDP-MSH residues are essential for high affinity binding and potency at hMC3R, we performed structure−activity studies using the substituted and truncated NDP-MSH peptides. The sequences of the substituted and truncated peptides are shown in Tables 1 and 2. Regarding the substitution experiments, our results indicate that substitution of NDP-MSH His^6 with alanine resulted in a 7-fold decrease in NDP-MSH binding affinity and a 17-fold decrease in potency for receptor activation, as defined by cAMP production (Figure 2 and Table 1). Substitution of NDP-MSH residue D-Phe^7 with the D-stereoisomer of alanine, or residue Arg^8 with norleucine, and residue Trp^9 with leucine also profoundly decreased NDP-MSH binding affinity and potency, as evidenced by K_i and EC_{50} values shown in Table 1 and Figure 2.

Our results with truncated NDP-MSH peptides also reveal that all truncations tested affected binding affinity and potency for hMC3R activation (Table 2). The peptide His^6-D-Phe^7-Arg^8-Trp^9 was the smallest fragment that displayed full agonist efficacy (Figure 3). However, full hMC3R activation required a micromolar concentration of that peptide, and, even at that high concentration, His-D-Phe-Arg-Trp did not completely displace ^{125}I-NDP-MSH. Removal of Trp^9 from the pentapeptide Glu-His-D-Phe-Arg-Trp resulted in a totally inactive tetrapeptide, which further highlighted the importance of this aromatic amino acid residue in NDP-MSH activity.

Effect of Charged Amino Acids Mutation in Transmembrane Domains of hMN3R Responsible for NDP-MSH Binding and Activation. We examined previous data pertaining to MC1R and MC4R (*5*, *23*) to develop a model for MC3R pharmacophore ligand−receptor interactions. On the basis of these data, we have examined the role of MC3R transmembrane domain residues in α-MSH binding and potency. We first mutated hMC3R residues conserved across the MC3R, MC1R, and MC4R subtypes because evolutionary conservation implies that there may be functional

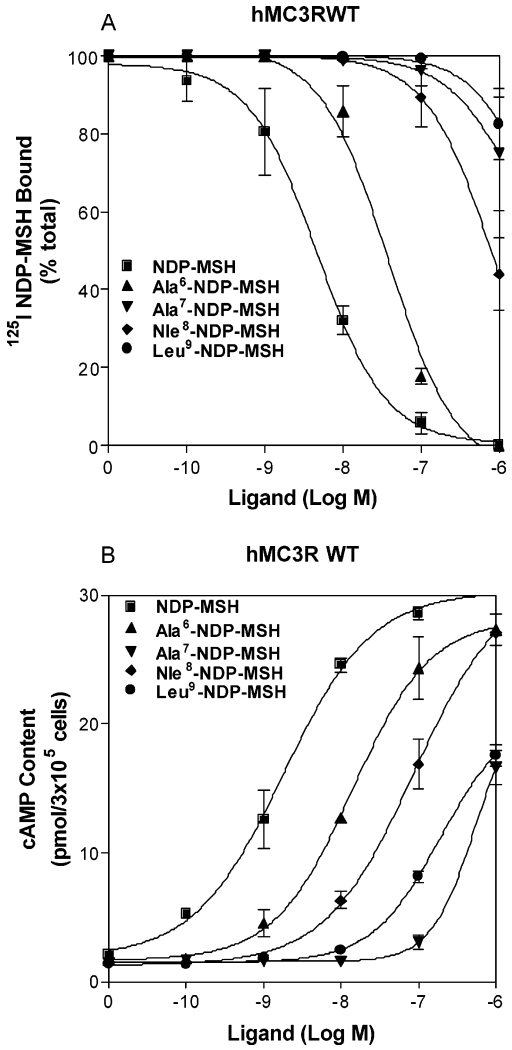

FIGURE 2: Binding affinity and potency of mutated NDP-MSH peptides in HEK-293 cells stably transfected with the wild-type hMC3R. Panel A shows that HEK-293 cells transfected with hMC3R were incubated with 125I-NDP-MSH in the presence of the indicated amounts of unlabeled ligands, and total 125I-NDP-MSH binding was determined. Panel B shows that the cells were incubated with the indicated amounts of peptides, and total cAMP accumulation was determined. ($n = 3$; see Table 1 for K_i and EC_{50} values).

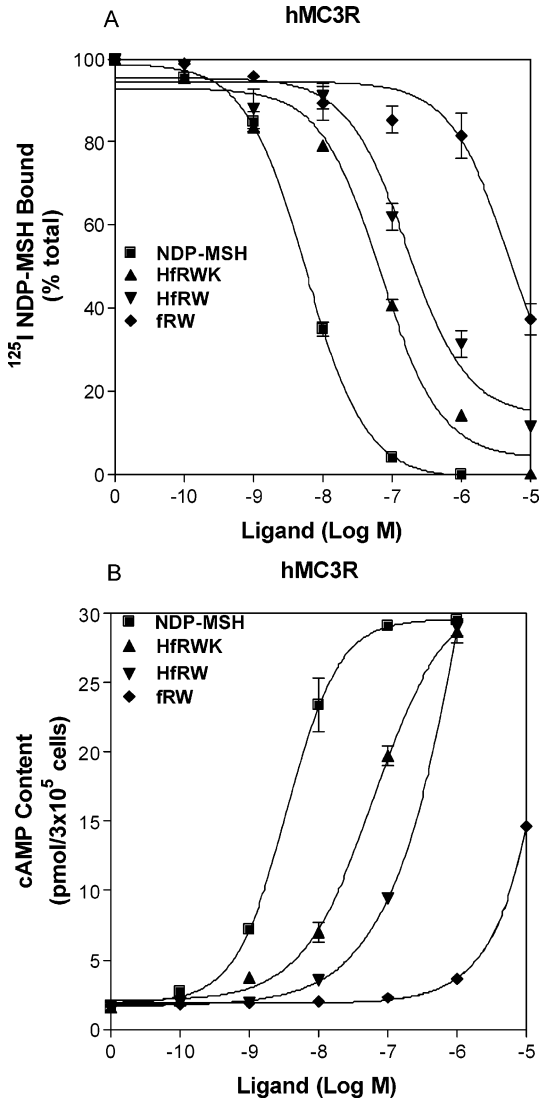

FIGURE 3: Binding affinity and potency of truncated NDP-MSH peptides in HEK-293 cells stably transfected with the wild-type hMC3R. Panel A shows the binding affinities of the truncated peptides to displace ^{125}I NDP-MSH. Panel B shows that the ability of the truncated peptides to stimulate cAMP production. ($N = 3$; see Table 2 for K_i and EC_{50} values) f = D-phenylalanine, R = arginine, W = tryptophan, H = histidine, K = lysine.

significance of the maintained receptor residues. We have examined alanine substitutions for 19 transmembrane domain amino acids that are hypothesized to be involved in ligand binding and receptor signaling. The hMC3R mutations generated in this study are listed in Figure 1. On the basis of the hypothesis that NDP-MSH docks into MC3R transmembrane domains near the surface of the plasma membrane, mutagenesis studies were performed to examine the role of relevant charged amino acids in the upper region of hMC3R transmembrane in NDP-MSH binding and biological activity. Alanine is the generally accepted amino acid of choice for mutagenesis substitution in this type of study since its small neutral nature theoretically makes it unlikely to disturb receptor tertiary structure. Since an ionic interaction between the arginine residue of the key NDP-MSH tetrapeptide and an anionic receptor residue could provide substantial energy for agonist binding. Therefore, all three negatively charged hMC3R TM residues were first mutated. This included several anionic TM residues (E131, D154, and D158) homologous to receptor residues previously implicated in hMC1R and hMC4R agonist binding. The fourth substitution was at the hMC3R TM6 H298 residue homologous to hMC1R H260 and hMC4R H264, which has also been shown to contribute to α-MSH binding affinity (*5*, *20*). Our results indicate that mutations of amino acid residues E131 in TM2, D154, and D158 in TM3 and H298 in TM6 significantly altered NDP-MSH binding affinity and receptor activity (Figure 4). The respective K_i and EC_{50} values are shown in Table 3. However, mutations of R196 and K197 in TM4, and H317 in TM7, did not alter NDP-MDH binding affinity and receptor activity. However, the B_{max} for these mutant receptors is lower that that of hMC3RWT (Table 3).

Role of hMC3R TM Residues in Receptor Expression and Activation. It has been reported that mutations of glutamic acid residues in TM2 and TM7 of hMC4R results in altered agonist mediated receptor activation but no effect on ligand binding (*5*). To determine whether the corresponding residues of hMC3R are also involved in agonist mediated receptor

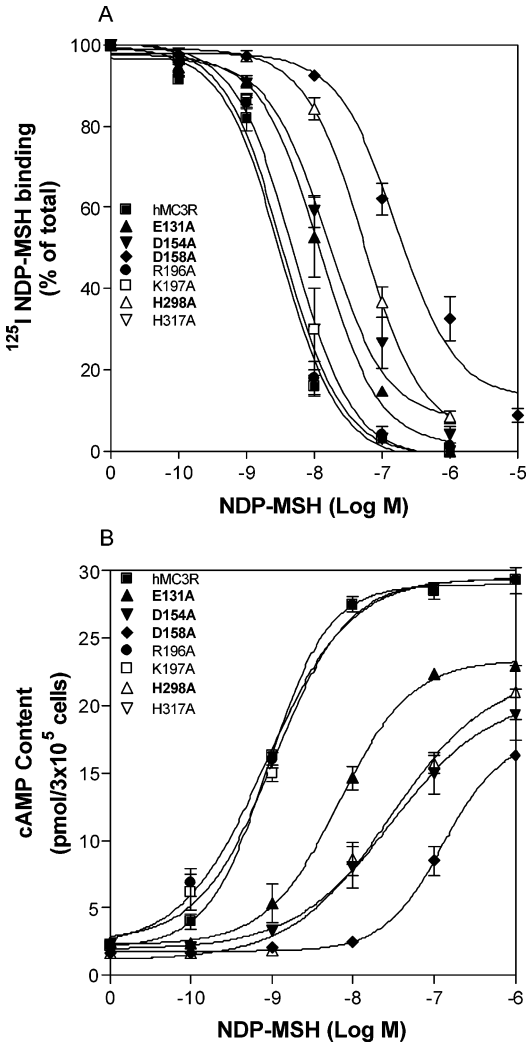

FIGURE 4: Effects of the substitution of charged amino acid hMC3R TM residues with alanine on NDP-MSH binding affinity and receptor activity. Panel A shows the NDP-MSH binding affinity of these mutants. Panel B shows the ability of NDP-MSH stimulated cAMP production at these mutants. ($n = 3$; see Table 3 for actual K_i and EC_{50} values).

Table 3: Effect of NDP-MSH on ^{125}I NDP-MSH Binding and cAMP Production in HEK Cells Transfected with the Mutated Forms of the of hMC3R

	B_{max} (fmol/mg pro)	^{125}I-NDP-MSH binding K_i (nM)	cAMP production EC_{50} (nM)
hMC3RWT	325 ± 5.8	5.6 ± 1.0	0.8 ± 0.2
D121A	no	NB	NR
D121N	235 ± 3.2	11.2 ± 2.1*	7.8 ± 1.1*
E131A	233 ± 16	27.9 ± 7.6*	15.8 ± 3.2 *
D154A	203 ± 13	76.6 ± 12*	33.4 ± 2.3*
D158A	210 ± 11	234 ± 23*	285 ± 43*
R196A	269 ± 7.5	7.4 ± 1.2	1.5 ± 0.9
K197A	238 ± 7.5	6.0 ± 0.9	1.7 ± 0.2
H298A	281 ± 7.5	98 ± 6.9*	216 ± 18*
H317A	287 ± 11.2	8.0 ± 1.7	3.2 ± 0.8*
D332A	no	NB	NR
D332N	218 ± 21	23 ± 2.5*	87 ± 4.3*

[a] *$P < 0.05$ compared with WT receptor.

activation, we mutated residue D121in TM2 and D332 in TM7 by exchanging aspartic acid with alanine. Our results indicate that mutations D121A and D332A resulted in a complete loss of receptor−ligand binding and activation.

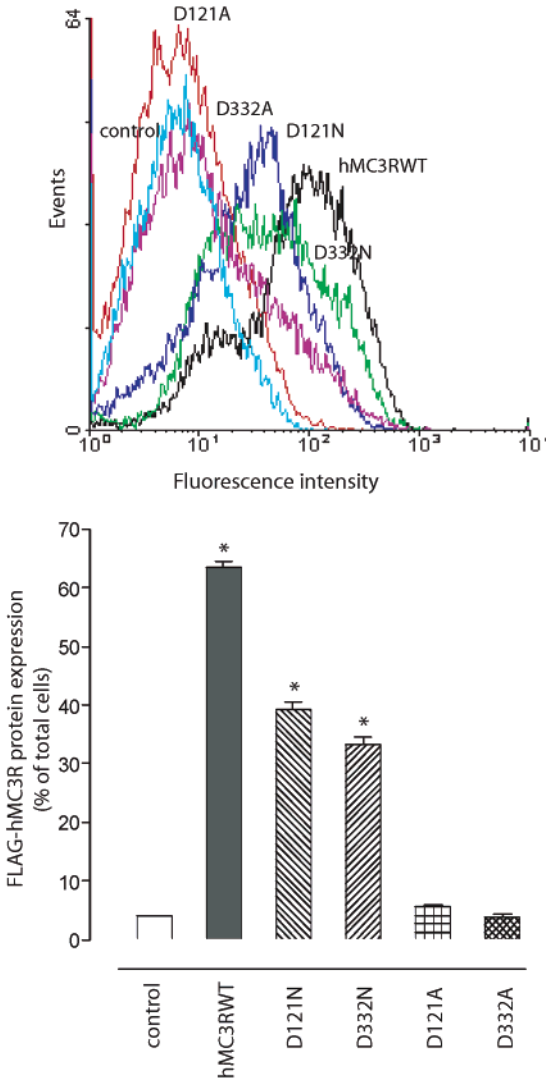

FIGURE 5: Expression of mutant hMC3R in HEK-293 cells. FLAG-tagged hMC3RWT or mutated hMC3Rs were transfected into HEK-293 cells and detected by FACs (A). Ten thousand cells from each transfection were sorted, and the percentage of cells having a fluorescence intensity signal was determined via a basal cutoff value (B). No positive FLAG signals were observed in the mutant D121A and D332A, but positive FLAG signals were observed in the mutant D121N and D332N.

Because of the possibility that these mutations impaired receptor expression, we utilized FACS to determine FLAG-tagged mutant D121A and D332A protein expression on the cell surface (Figure 5). Our results show that strong signal was detected at FLAG tagged MC3R WT but not detected in FLAG-tagged mutants D121A and D332A, suggesting that these two mutants were not properly expressed on the cell surface. Our next step was to substitute these two residues, D131 and D332, with asparagines (D131N, D332N) instead of alanine, and again assess effects on receptor expression and function. We were now able to observe dose-dependent displacement of ^{125}I-NDP-MSH bound to D131N and D332N and dose-dependent induction of cAMP production (Figure 6); however, mutant receptor binding affinity and activation were dramatically reduced compared to the WT receptor.

Effect of Aromatic Amino Acids Mutation in Transmembrane Domains of hMC3R Responsible for NDP-MSH Binding and Activation. It has been proposed that there is

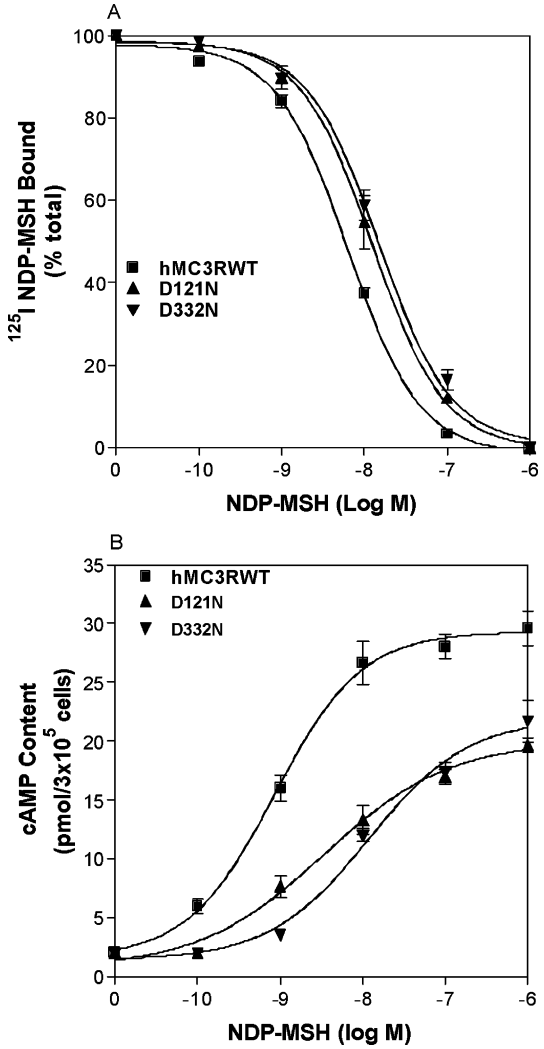

FIGURE 6: Binding affinity and potency of NDP-MSH upon binding to mutant hMC3R D121N and D332N. Panel A shows the binding affinity of different ligands to displace ^{125}I NDP-MSH. Panel B shows the ability of different ligands to stimulate cAMP production upon binding wild-type and mutant receptors. Data points represent the mean ± SE of three independent experiments with duplicate wells.

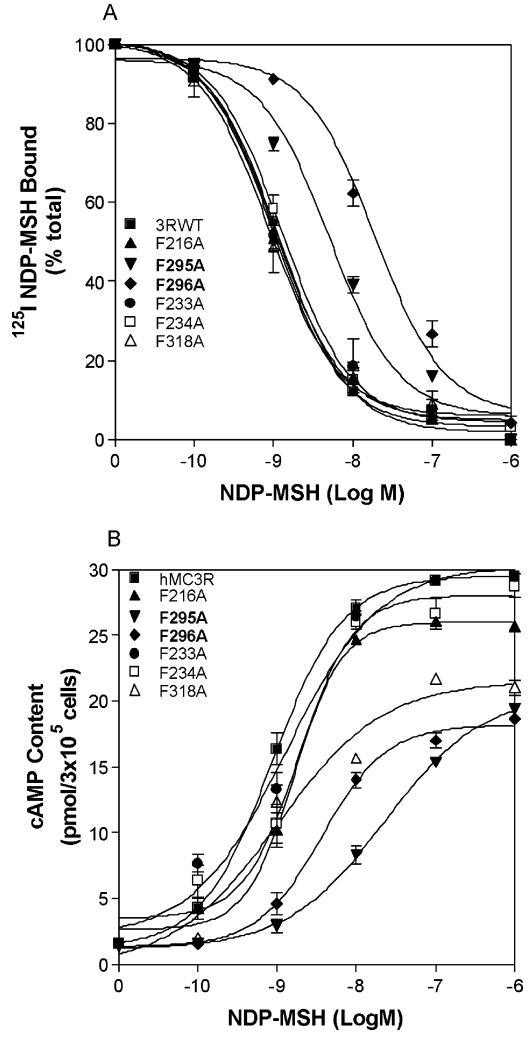

FIGURE 7: Effect of the substituting various aromatic amino acids with alanine in the hMC3R on NDP-MSH binding affinity and receptor activity. Panel A shows the NDP-MSH binding affinity to these mutant receptors. Panel B shows the ability of NDP-MSH to stimulate cAMP production upon binding to the mutant receptors. (Panels A and B) ($n = 3$; see Table 4 for actual K_i and EC_{50} values).

second distinct binding pocket for interaction between α-MSH and both MC1R and MC4R (*5*, *20*, *23*, *24*) mediated by aromatic amino acids in receptor transmembrane domains (*5*, *20*). To examine the role of hMC3R aromatic amino acids, we substituted nine phenylalanine residues in transmembrane domains with alanine and examined whether these residues are involved in NDP-MSH binding and receptor activity. These aromatic residues are located in TM4 (F216), TM5 (F233 and F234), TM6 (F288, F290, F295 and F296), and TM7 (F318). Our results indicate that mutations of F295A and F296A in TM6 significantly reduced NDP-MSH binding affinity and receptor activity while mutations of F216A, F233A, F234A, F288A, F290A, and F318A did not significantly alter NDP-MSH binding affinity and receptor activity (Figure 7 and Table 4). However, we also found that receptor B_{max} was lower for these mutant receptors than that of hMC3RWT.

Substitution Amino Acid L165 in Transmembrane Domains of hMC3R on Ligand Selectivity. SHU 9119 is a cyclic melanocortin analogue that contains D-naphthylalanine instead of D-phenylalanine in the core melanocortin sequence and is an antagonist at hMC3R and hMC4R (*27*). On comparing the sequence of hMC3R with other MCRs, it became apparent that leucine L165 and L133 in TM3 was unique to hMC3R and hMC4R. Interestingly, leucine 133 in TM3 of hMC4R has been reported to play an important role in SHU9119 antagonism (*25*). To determine whether L165 in TM3 of hMC3R is also important for SHU9119

Table 4: Effect of NDP-MSH on ^{125}I NDP-MSH Binding and cAMP Production in HEK Cells Transfected with the Mutated Forms of the hMC3R[a]

	B_{max} (fmol/mg pro)	^{125}I-NDP-MSH binding K_i (nM)	cAMP production EC_{50} (nM)
hMC3RWT	325 ± 5.8	5.6 ± 1.0	0.8 ± 0.1
F216A	278 ± 22.4	5.9 ± 0.6	1.6 ± 0.2
F233A	287 ± 23.4	6.3 ± 1.1	2.1 ± 0.3
F234A	295 ± 12.6	7.1 ± 1.7	1.3 ± 1.0
F288A	283 ± 10.2	6.1 ± 0.6	1.4 ± 0.1
F290A	290 ± 9.2	5.6 ± 0.3	1.9 ± 0.2
F295A	276 ± 12.2	155 ± 11*	28 ± 6.5*
F296A	311 ± 14.5	17.8 ± 13*	7.6 ± 0.3*
F318A	282 ± 12.3	6.3 ± 1.1	1.2 ± 0.2

[a] *$P < 0.05$ compared with WT receptor.

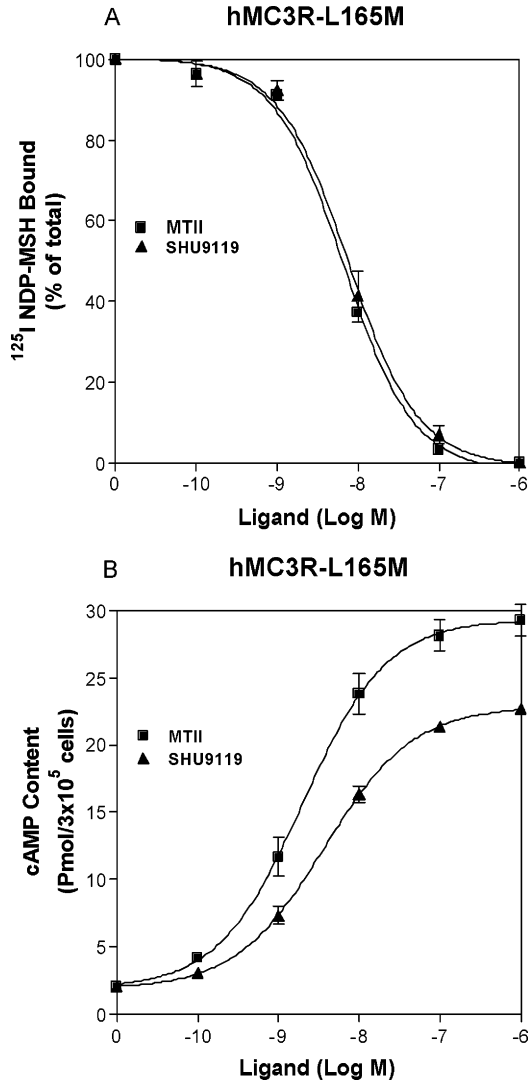

FIGURE 8: Binding affinity and potency of MTII and SHU9119 upon binding to mutant hMC3R L165M. Panel A shows the binding affinity of different ligands to displace ^{125}I NDP-MSH. Panel B shows the ability of the different ligands to stimulate cAMP production. HMC3R mutant L165M did not exhibit altered ligand binding affinity but did switch SHU9119 from antagonist to agonist. Data points represent the mean ± SE of three independent experiments with duplicate wells.

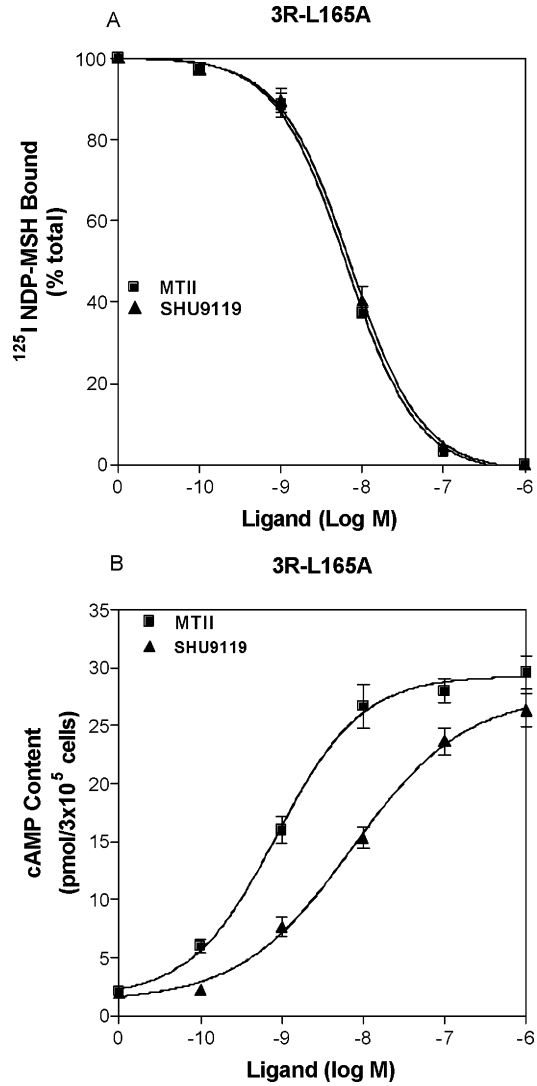

FIGURE 9: Binding affinity and potency of MTII and SHU9119 upon binding to mutant hMC3R L165A. Panel A shows the binding affinity of different ligands to displace ^{125}I NDP-MSH. Panel B shows the ability of the ligands to stimulate cAMP production. HMC3R mutant L165A did not change ligand binding affinity but switched SHU9119 from antagonist to agonist. Data points represent the mean ± SE of three independent experiments with duplicate wells.

action, we replaced this residue in hMC3R with methionine, which corresponds to the amino acid that resides in the homologous residue in hMC1R (methionine 128 in TM3). As shown in Figure 8, the mutant receptor L165M is functionally expressed on the cell surface, and the B_{max} for cAMP stimulation and binding affinity for NDP-MSH, MTII (MSH analogue), and SHU9119 are all in the range of the wild-type receptor (Table 5). However, substituting the residue L165 with methionine in the hMC3R did lead to complete conversion of SHU9119 activity from antagonist to agonist (Figure 8). To further determine whether specific methionine is required for this activity, we substituted residue leucine 165 with alanine (L165A). Our results indicate that L165A also converts SHU9119 from antagonist to agonist (Figure 9 and Table 5).

Table 5: Effect of Melanocortin Ligands on ^{125}I NDP-MSH Binding and cAMP Production in HEK Cells Transfected with the WT hMC1R, hMC3R, and Mutated Forms of the of hMC3R[a]

		^{125}I-NDP-MSH binding K_i (nM)			cAMP production EC_{50} (nM)		
	B_{max} (fmol/mg pro)	NDP	MTII	SHU9119	NDP	MTII	SHU9119
hMC3RWT	325 ± 5.8	5.6 ± 1.0	6.1 ± 1.0	5.9 ± 0.2	0.8 ± 0.1	0.7± 0.1	NR
L165M	307 ± 12.2	6.1 ± 0.5	5.9 ± 0.6	5.7 ± 0.5	1.1 ± 0.1	1.6 ± 0.5	15.3 ± 1.7*
L165A	311 ± 11.1	5.9 ± 0.3	5.7 ± 0.5	6.1 ± 0.1	1.2 ± 0.2	0.9 ± 0.1	21 ± 0.3*
hMC1RWT	295 ± 12.6	3.2 ± 0.3	4.3 ± 0.7	4.3 ± 0.4	0.7 ± 0.1	1.3 ± 1.0	7.8 ± 0.6

[a] NR: no response. *P < 0.05 compared with WT hMC3R.

DISCUSSION

The discovery that melanocortin-3 and -4 receptors are involved in the hypothalamic control of feeding behavior has generated substantial academic and pharmaceutical industry interest because of the possibility of using melanocortin agonists as anti-obesity drugs. In this study, we have confirmed that NDP-MSH residues His^6, Arg^8, D-Phe^7, and Trp^9 are important for ligand binding and biological activities at hMC3R. TM3 and TM6 of hMC3R are important for NDP-MSH binding, while Asp 121 in TM2 and Asp332 in TM7 are crucial for receptor activity and signaling. In addition, L165 in TM3 is critical for agonist or antagonist selectivity.

Our 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, DAla7 and Leu9, greatly decreased binding affinity and potency of substituted NDP-MSH peptides at the hMC3R. Studies with truncated NDP-MSH peptides further highlighted the importance of residues 6−9 by demonstrating that the smallest NDP-MSH fragment that possesses full agonist efficacy is the tetrapeptide His-D-Phe7-Arg8-Trp9, which is consistent with the previous data (*26*). Taken together, these data indicate that amino acids His-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 hMC3R residues, which is different from that of hMC4R (*5*).

Electrostatic and hydrophobic forces have been proposed to be involved in MSH binding and receptor activation at MCRs (*5*, *20*, *23*). An ionic pocket formed by amino acid residues in transmembrane (TM) domains two and three of hMC1R and hMC4R have been proposed to be critical for ligand binding (*5*, *20*). These TM residues have been hypothesized to form an ionic interaction with Arg^8 of NDP-MSH (*23*). A second hydrophobic binding pocket has also been proposed and this binding pocket consisted of a series of hydrophobic receptor residues in TM4, 5, and 6 which are believed to form aromatic−aromatic interactions with Phe^7 and Trp^9 of α-MSH (*5*, *23*). In this study, we have utilized mutagenesis involving 19 selected amino acid residues to examine whether this model also fits for hMC3R. Similar to hMC1R and hMC4R, our results suggest that an ionic binding pocket also existed in hMC3R considering that mutations of TM2 residue Glu 131 and TM3 residues D154 and D158 significantly affected NDP-MSH binding affinity and biological activity. However, unlike MC1R and MC4R, only TM6 of hMC3R seems to be involved in a hydrophobic binding pocket. Mutations of TM6 residues Phe 295, Phe 296, and H298 altered NDP-MSH binding affinity and receptor biological activity. The other aromatic residues in TM4, TM5, and TM7 of hMC3R do not seem to play an important role in NDP-MSH binding and receptor biological activity because mutations of F216 in TM4, F233, F234, and F318 in TM7 did not significantly alter these parameters. Our results suggest that hMC3R shares ionic binding sites with hMC1R and hMC4R. The model for hMC3R ligand receptor interaction is shown in Figure 10. An ionic pocket formed by amino acid residues E131in TM2 and D154, D158 in TM3 of hMC3R and a second hydrophobic binding pocket consisted of F295, F296, and H298 in TM6 of hMC3R.

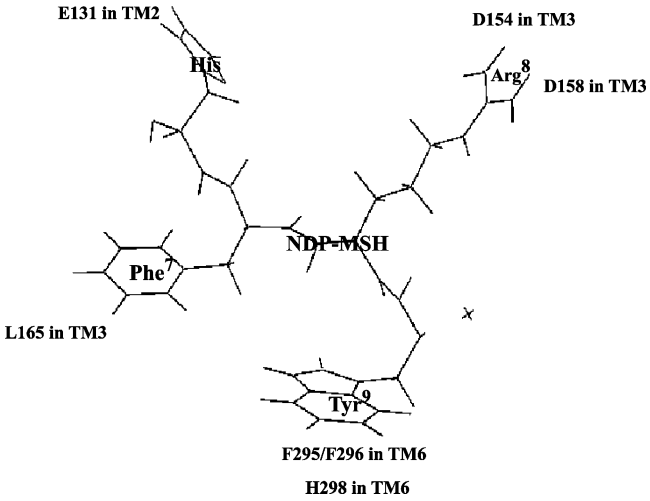

FIGURE 10: Two-dimensional representation of a proposed three-dimensional model illustrating the synthetic melanocortin NDP-MSH docked inside the hMC3R. Two receptor binding pockets are hypothesized. The first is a predominantly ionic pocket formed by Asp154 and Asp 158. The second a hydrophobic pocket formed by aromatic residues in TM6. Phe 257, F258, and H298 are included.

GPCR's activation process has been proposed to involve the rotation of TM domains with outward movement of their cytoplasmic ends (*27*). This theoretically would enable G proteins to interact with some of the intracellular loops as well as the C terminal tail of GPCRs. For melanocortin peptides, extensive studies indicate that the amino acid in position 7 of synthetic melanocortin ligands is important for ligand activity (*28*). Modifications of MTII by substituting D-Phe^7 with different residues result in distinctly different activation profiles at the various melanocortin receptor subtypes. At the mouse MC1R, replacement of MTII D-Phe^7 by DNal(1′) or DNal (2′), retained agonist potency compared with MTII but resulted in decreased potencies, suggesting that for the MC1R, both stereochemistry (D-configuration) and positioning of the bulky aromaticity is important for agonist potencies. However, at hMC3R and hMC4R, replacement of MTII D-Phe7 by DNal (2′)7 (SHU9119), converted the agonist into an antagonist. However, other naphthylalanine derivative modifications resulted only in decreased agonist potencies. These data suggest that both stereochemistry (D-configuration) and the position of the bulky aromatic residue are important for ligand agonist or antagonist selectivity at MC3R and MC4R. After the comparison of the amino acid sequence of hMC3R with other MCRs, we found that leucine in TM3 is unique in hMC3R and hMC4R. Since leucine has been reported to play an important role in SHU9119 antagonism in TM3 of hMC4R (*25*), we determined whether L165 in TM3 of hMC3R was similarly critical for SHU9119 antagonism. We replaced this residue with methionine, which exists in this position of hMC1R, and found that the substitution of leucine 165 to methionine did not alter receptor NDP-MSH binding affinity but led to complete conversion of SHU9119 activity from antagonist to agonist. Our results suggest that this leucine 165 in TM3 of hMC3R is important for agonist or antagonist selectivity. To further determine whether methionine is specifically required for this activity, we further substituted residue leucine 165 with alanine, L165A. We hypothesized that if there was a direct interaction between M165 and DNal

(2′)7 in SHU9119, the mutation of L165A would alter this interaction and SHU9119 would not activate the receptor to induce cAMP production. However, our results indicate that mutation L165A also switches SHU9119 from antagonist to agonist. SHU9119 has the same binding affinity and potency at L165M and L165A, suggesting that there is no direct interaction between DNal of SHU9119 and L165 of hMC3R. One possible explanation is that since SHU9119 has a bulky aromatic residue in position 7, when it docks into the receptor binding pocket, L165 may hinder the receptor conformation change and block receptor activation. When leucine 165 was replaced with methionine or alanine, which is smaller in size than leucine, the hindering amino acid was removed. SHU9119 binds to receptor and rotates both TM domains and allows for outward movement of their cytoplasmic loop. This conformational change would predictably enable G proteins to interact with the third intracellular loop as well as the C terminal tail of the receptor.

The previous studies have indicated that mutations of some residues of hMC4R result in different ligand binding and receptor activation (*5*). For example, mutations of residue D90 and D298 of hMC4R did not alter NDP-MSH binding affinity but completely abolished NDP-MSH signaling (*5*). Our current results indicates that mutations of the homologues residues of hMC3R (D121A and D332A) abolish both NDP-MSH binding and receptor signaling. This suggests that D121 and D332 in hMC3R may have different roles in ligand binding and receptor activation compared to homologues residues in hMC4R. Our FACs results suggest that these two mutated receptors are not fully expressed on the cell surface and that altered receptor function may reflect loss of cell surface receptor expression. Thus, these two amino acids, D121 and D332, may be important determinants for lost ligand binding and receptor expression on plasma membrane.

Three experimental approaches have been used to investigators to study the nature of ligand−receptor interaction: (i) alteration of ligand structure; (ii) alteration of receptor structure (e.g. by mutation or formation of receptor chimeras or receptor truncation); and (iii) physiochemical investigation of the points of contact between the ligand and receptor. The first two approaches cannot precisely define the complementary domains in the opposing partner involved in the interaction. To provide direct information about the nature of the interface between peptides and their respective receptors, a number of laboratories have adopted a photoaffinity scanning approach that relies on the spatially restricted cross-linking of photolabile amino acids within peptide ligands and their cognate receptors. This approach provides a mechanism for directly defining the ligand−receptor interface by using photoactive amino acid derivatives spaced along the peptide to define proximity of particular amino acids in the receptor and ligand. This approach has been used successfully in the study of several GPCRs, including CCK receptor (*29*), PTH1 and PTH2 receptors (*30*), the vasoactive intestinal peptide receptor (*31*), and the motilin receptor (*32*, *33*). Future studies are required utilizing this approach to determine the amino acids involved in the direct physical interaction between NDP-MSH and MC3R.

In summary, Our results indicate that (i) substitutions of charged amino acid residues E131 in transmembrane domain 2 (TM2), D154 and D158 in TM3, and H298 in TM6 with alanine dramatically reduced NDP-MSH binding affinity and receptor signaling, (ii) substitutions of aromatic amino acids F295 and F296 in TM6 with alanine also significantly decreased NDP-MSH binding and receptor activity, (iii) substitutions of D121in TM2 and D332 in TM7 with alanine resulted in the complete loss of ligand binding, ligand induced receptor activation, and cell surface protein expression, and (iv) interestingly, substitution of L165 in TM3 with methionine or alanine switched antagonist SHU9119 into a receptor agonist. In conclusion: Our results suggest that TM3 and TM6 are important for NDP-MSH binding, while D121 in TM2 and D332 in TM7 are crucial for receptor activity and signaling. Importantly, L165 in TM3 is critical for agonist or antagonist selectivity. These results provide important information about the molecular determinants of hMC3R responsible for ligand binding and receptor signaling.

REFERENCES

1. Kopelman, P. G. (2000) Obesity as a medical problem, *Nature 404*, 635−643.
2. Flegal, K. M., Carroll, M. D., Ogden, C. L., and Johnson, C. L. (2002) Prevalence and trends in obesity among U.S. adults, 1999−2000, *JAMA 288*, 1723−1727.
3. Allison, D. B., Fontaine, K. R., Manson, J. E., Stevens, J., and VanItallie, T. B. (1999) Annual deaths attributable to obesity in the United States, *JAMA 282*, 1530−1538.
4. Zimmermann-Belsing, T., and Feldt-Rasmussen, U. (2004) Obesity: the new worldwide epidemic threat to general health and our complete lack of effective treatment, *Endocrinology 145*, 1501−1502.
5. Yang, Y. K., Fong, T. M., Dickinson, C. J., Mao, C., Li, J. Y., Tota, M. R., Mosley, R., Van Der Ploeg, L. H., and Gantz, I. (2000) Molecular determinants of ligand binding to the human melanocortin-4 receptor, *Biochemistry 39*, 14900−14911.
6. Kowalski, T. J. (2004) The future of genetic research on appetitive behavior, *Appetite 42*, 11−14.
7. Damcott, C. M., Moffett, S. P., Feingold, E., Barmada, M. M., Marshall, J. A., Hamman, R. F., and Ferrell, R. E. (2004) Genetic variation in fatty acid-binding protein-4 and peroxisome proliferator-activated receptor gamma interactively influence insulin sensitivity and body composition in males, *Metabolism 53*, 303−309.
8. Geller, F., Reichwald, K., Dempfle, A., Illig, T., Vollmert, C., Herpertz, S., Siffert, W., Platzer, M., Hess, C., Gudermann, T., Biebermann, H., Wichmann, H. E., Schafer, H., Hinney, A., and Hebebrand, J. (2004) Melanocortin-4 receptor gene variant I103 is negatively associated with obesity, *Am. J. Hum. Genet. 74*, 572−581.
9. Huszar, D., Lynch, C. A., Fairchild-Huntress, V., Dunmore, J. H., Fang, Q., Berkemeier, L. R., Gu, W., Kesterson, R. A., Boston, B. A., Cone, R. D., Smith, F. J., Campfield, L. A., Burn, P., and Lee, F. (1997) Targeted disruption of the melanocortin-4 receptor results in obesity in mice, *Cell 88*, 131−141.
10. Fan, W., Boston, B. A., Kesterson, R. A., Hruby, V. J., and Cone, R. D. (1997) Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature 385*, 165−168.
11. Giraudo, S. Q., Billington, C. J., and Levine, A. S. (1998). Feeding effects of hypothalamic injection of melanocortin 4 receptor ligands, *Brain Res. 809*, 302−306.
12. Cone, R. D. (1999) The central melanocortin system and energy homeostasis, *Trends Endocrinol. Metab. 10*, 211−216.
13. Fisher, S. L., Yagaloff, K. A., and Burn, P. (1999) Melanocortin-4 receptor: a novel signalling pathway involved in body weight regulation, *Int. J. Obes. Relat. Metab. Disord. 23 Suppl 1*, 54−58.
14. Chen, A. S., Marsh, D. J., Trumbauer, M. E., Frazier, E. G., Guan, X. M., Yu, H., Rosenblum, C. I., Vongs, A., Feng, Y., Cao, L., Metzger, J. M., Strack, A. M., Camacho, R. E., Mellin, T. N., Nunes, C. N., Min, W., Fisher, J., Gopal-Truter, S., MacIntyre, D. E., Chen, H. Y., and Van der Ploeg, L. H. (2000) Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass, *Nat. Genet. 26*, 97−102.

15. Butler, A. A., Kesterson, R. A., Khong, K., Cullen, M. J., Pelleymounter, M. A., Dekoning, J., Baetscher, M., and Cone, R. D. (2000) A unique metabolic syndrome causes obesity in the melanocortin-3 receptor-deficient mouse, *Endocrinology 141*, 3518−3521.
16. Tao, Y. X., and Segaloff, D. L. (2004) Functional characterization of melanocortin-3 receptor variants identify a loss-of-function mutation involving an amino acid critical for G protein-coupled receptor activation, *J. Clin. Endocrinol. Metab. 89*, 3936−3942.
17. Yang, Y. K., Ollmann, M. M., Wilson, B. D., Dickinson, C., Yamada, T., Barsh, G. S., and Gantz, I. (1997) Effects of recombinant agouti-signaling protein on melanocortin action, *Mol. Endocrinol. 11*, 274−280.
18. Yang, Y. K., Dickinson, C., Lai, Y. M., Li, J. Y., and Gantz, I. (2001) Functional properties of an agouti signaling protein variant and characteristics of its cognate radioligand, *Am. J. Physiol. Regul. Integr. Comp. Physiol. 281*, R1877−1886.
19. Yang, Y. K., Thompson, D. A., Dickinson, C. J., Wilken, J., Barsh, G. S., Kent, S. B., and Gantz, I. (1999) Characterization of Agouti-related protein binding to melanocortin receptors, *Mol. Endocrinol. 13*, 148−155.
20. Yang, Y., Dickinson, C., Haskell-Luevano, C., and Gantz, I. (1997) Molecular basis for the interaction of [Nle4, D-Phe7]melanocyte stimulating hormone with the human melanocortin-1 receptor, *J. Biol. Chem. 272*, 23000−23010.
21. Tritos, N. A., and Maratos-Flier, E. (1999) Two important systems in energy homeostasis: melanocortins and melanin-concentrating hormone, *Neuropeptides 33*, 339−349.
22. Guan, X. M., Kobilka, T. S., and Kobilka, B. K. (1992) Enhancement of membrane insertion and function in a type IIIb membrane protein following introduction of a cleavable signal peptide, *J. Biol. Chem. 267*, 21995−21998.
23. Haskell-Luevano, C., Sawyer, T. K., Trumpp-Kallmeyer, S., Bikker, J. A., Humblet, C., Gantz, I., and Hruby, V. J. (1996) Three-dimensional molecular models of the hMC1R melanocortin receptor: complexes with melanotropin peptide agonists, *Drug Des. Discov. 14*, 197−211.
24. Haskell-Luevano, C., Cone, R. D., Monck, E. K., and Wan, Y. P. (2001) Structure activity studies of the melanocortin-4 receptor by in vitro mutagenesis: identification of agouti-related protein (AGRP), melanocortin agonist and synthetic peptide antagonist interaction determinants, *Biochemistry 40*, 6164−6179.
25. Yang, Y., Chen, M., Lai, Y., Gantz, I., Georgeson, K. E., and Harmon, C. M. (2002) Molecular determinants of human melanocortin-4 receptor responsible for antagonist SHU9119 selective activity, *J. Biol. Chem. 277*, 20328−20335.
26. Holder, J. R., and Haskell-Luevano, C. (2004) Melanocortin ligands: 30 years of structure−activity relationship (SAR) studies, *Med. Res. Rev. 24*, 325−356.
27. Gether, U. (2000) Uncovering molecular mechanisms involved in activation of G protein-coupled receptors, *Endocr. Rev. 21*, 90−113.
28. Sawyer, T. K., Sanfilippo, P. J., Hruby, V. J., Engel, M. H., Heward, C. B., Burnett, J. B., and Hadley, M. E. (1980) 4-Norleucine, 7-D-phenylalanine-alpha-melanocyte-stimulating hormone: a highly potent alpha-melanotropin with ultralong biological activity, *Proc. Natl. Acad. Sci. U.S.A. 77*, 5754−5758.
29. Dong, M., Liu, G., Pinon, D. I., and Miller, L. J. (2005) Differential docking of high-affinity peptide ligands to type A and B cholecystokinin receptors demonstrated by photoaffinity labeling, *Biochemistry 44*, 6693−6700.
30. Macdonald, D., Mierke, D. F., Li, H., Pellegrini, M., Sachais, B., Krause, J. E., Leeman, S. E., and Boyd, N. D. (2001) Photoaffinity labeling of mutant neurokinin-1 receptors reveals additional structural features of the substance P/NK-1 receptor complex, *Biochemistry 40*, 2530−2539.
31. Tan, Y. V., Couvineau, A., Van Rampelbergh, J., and Laburthe, M. (2003) Photoaffinity labeling demonstrates physical contact between vasoactive intestinal peptide and the N-terminal ectodomain of the human VPAC1 receptor, *J. Biol. Chem. 278*, 36531−36536.
32. Matsuura, B., Dong, M., Coulie, B., Pinon, D. I., and Miller, L. J. (2005) Demonstration of a specific site of covalent labeling of the human motilin receptor using a biologically active photolabile motilin analogue, *J. Pharmacol. Exp. Ther. 313*, 1101−1108.
33. Matsuura, B., Dong, M., and Miller, L. J. (2002) Differential determinants for peptide and non-peptidyl ligand binding to the motilin receptor. Critical role of second extracellular loop for peptide binding and action, *J. Biol. Chem. 277*, 9834−9839.

BI0521792