BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

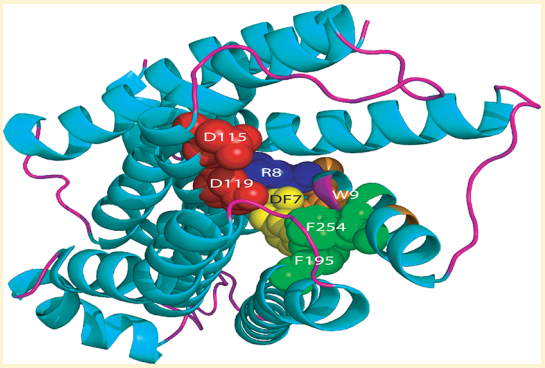
pubs.acs.org/biochemistry

Molecular Characterization of Human Melanocortin-5 Receptor Ligand−Receptor Interaction

Yingkui Yang,*[,†] Vinod K. Mishra,[‡] Min Chen,[§] Elaine Duffee,[∥] Reed Dimmitt,[∥] and Carroll M. Harmon[†]

[†]Surgery, [‡]Medicine, [§]Genetics, and [∥]Pediatric, University of Alabama at Birmingham, Birmingham, Alabama 35233, United States

ABSTRACT: The melanocortin-5 receptor (MC5R) is a subtype receptor of the melanocortin receptor (MCR) family, which is expressed centrally, as well as in a variety of peripheral tissues. MC5R has been implicated in many different physiological fields such as lipid metabolism and exocrine function. However, the specific molecular determinants of MC5R responsible for ligand binding and receptor signaling are currently unknown. The aim of this study is to determine the molecular basis of human MC5R (hMC5R) responsible for ligand binding and receptor signaling. Twenty-four single mutations of hMC5R were created and tested. Our results indicate that (1) substituting charged amino acid residue E92 in transmembrane domain 2 (TM2), aspartic acid 115 (D115) and D119 in TM3, and histidine (H) 257 in TM6 with alanine dramatically reduced NDP-α-MSH binding affinity and receptor signaling and (2) substituting aromatic amino acids phenylalanine (F) 195 in TM5, F254 in TM6, and H276 in TM7 with alanine also significantly decreased NDP-α-MSH binding and receptor activity. Combining pharmacological results and computer modeling, our results suggest that D115 and D119 in TM3, F195 in TM5, and F254 in TM6 may form a binding pocket for NDP-α-MSH binding. Our results provide important information about the structural aspects of hMC5R responsible for ligand binding and receptor signaling.



The melanocortin-5 receptor (MC5R) belongs to a class of seven-transmembrane (TM) domain proteins that are coupled to G proteins and signal through intracellular cyclic adenosine monophosphate (cAMP).[1] The most ubiquitous of the MCRs, MC5R has been identified in the pituitary, adrenal gland, fat cells, kidney, leukocytes, lung, mammary gland, ovary, skeletal muscle, bone marrow, and liver,[1,2] and in vivo studies indicate that MC5R is involved in the regulation of lipid metabolism in muscle and liver.[3] Additionally, α-MSH, a MCR agonist, dose-dependently increases the extent of carnitine palmitoyltransferase-1 and fatty acid oxidation.[4] A synthetic MSH analogue, SHU9119, a MC3R and MC4R antagonist and MC1R and MC5R agonist, increases the effect of NDP-α-MSH binding on fatty acid oxidation in primary muscle cells, suggesting that MC5R plays an important role in lipid metabolism.[4] Unlike MC3R and MC4R, MC5R is not thought to be involved in the regulation of food intake and body weight as fasting did not induce up- or downregulation of hypothalamic MC5R expression.[3] In addition to its role in lipid metabolism, MC5R plays a role in the control of exocrine glands, such as the Harderian gland, preputal gland, lacrimal gland, and sebaceous gland; transgenic mice lacking the MC5R gene demonstrate an increased level of absorption of water through their skin during a test swim versus control.[5]

The melanocortin peptides α-, β-, and γ-MSH and adrenocorticotropic hormone, a group of neuropeptides derived from the pro-opiomelanocortin prohormone, are all endogenous agonists for MC5R. Many studies indicated that these endogenous agonists are equipotent at MC5R and contain a common amino acid sequence, H-F-R-W, suggesting that they may also utilize the same binding sites at hMC5R.[6−8] Protein sequence comparisons of MCRs indicate that MCRs share many conserved amino acid residues, including MC5R. On the basis of five different types of GPCRs whose structures have been determined by X-ray crystallography,[9−19] we have aligned five melanocortin receptors (Figure 1). Previously, we and other groups have identified the NDP-α-MSH binding region at hMC1R, hMC2R, hMC3R, and hMC4R;[20−23] it was therefore anticipated that residues homologous to those utilized by other MCRs for high-affinity agonist binding would serve a similar function at MC5R. The goal of this study was therefore to determine the molecular aspects of hMC5R responsible for ligand binding and signaling. Our results demonstrate that the TM3, TM5, and TM6 regions of the hMC5R are important for NDP-α-MSH binding.

EXPERIMENTAL PROCEDURES

Peptides. [Nle4,D-Phe7]-α-MSH (NDP-α-MSH) was purchased from Peninsula Laboratories, Inc. (Belmont, CA).

Site-Directed Mutagenesis. A single mutation was constructed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The entire coding region of the MC5R mutants was sequenced at the University of Alabama at

Received: October 4, 2012
Revised: January 25, 2013
Published: February 17, 2013

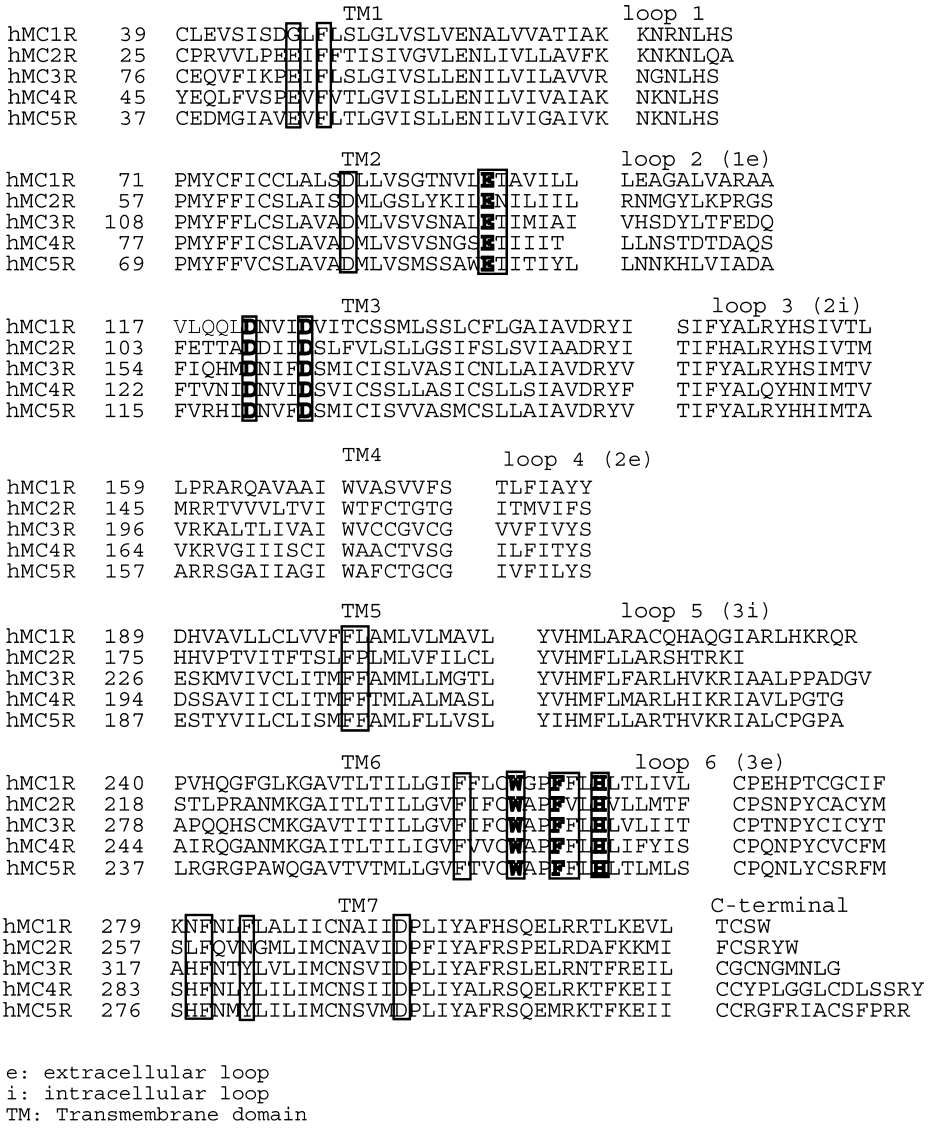


Figure 1. Primary sequences of human melanocortin receptors. The conserved TM residues in these receptors are boxed. The residues involved in ligand binding and signaling are shown in bold.

Birmingham Sequence Core to confirm that the desired mutation sequences were present and that no sequence errors had been introduced. The mutant receptors (shown in Figure 2) were subcloned into 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 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 had been removed, cells were incubated with a nonradioligand in 0.5 mL of MEM (Fisher Scientific. Pittsburgh, PA) containing 0.2% BSA and radioligand. Binding experiments were performed using conditions described previously.[23] Briefly, 2×10^5 cpm of [^{125}I]NDP-α-MSH was used in combination with nonradiolabeled ligands of NDP-α-MSH. 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 assessed 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.[23] Briefly, HEK cell lines stably expressing hMC5R were used in these assays.[23] Cell culture medium was removed, and cells were incubated with 0.5 mL of Earle's Balanced Salt Solution (EBSS), containing melanocortin agonist NDP-NDP-α-MSH (10^{-10} to 10^{-6} M), for 30 min at 37 °C in the presence of 10^{-3} M isobutylmethylxanthine. The reaction was stopped via addition of 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 1900g, and the supernatant was evaporated in a 55 °C water bath with prepurified nitrogen gas. The cAMP content was measured, and each experiment was performed a minimum of three times with duplicate wells.

dx.doi.org/10.1021/bi3013593 | *Biochemistry* 2013, 52, 1737−1745

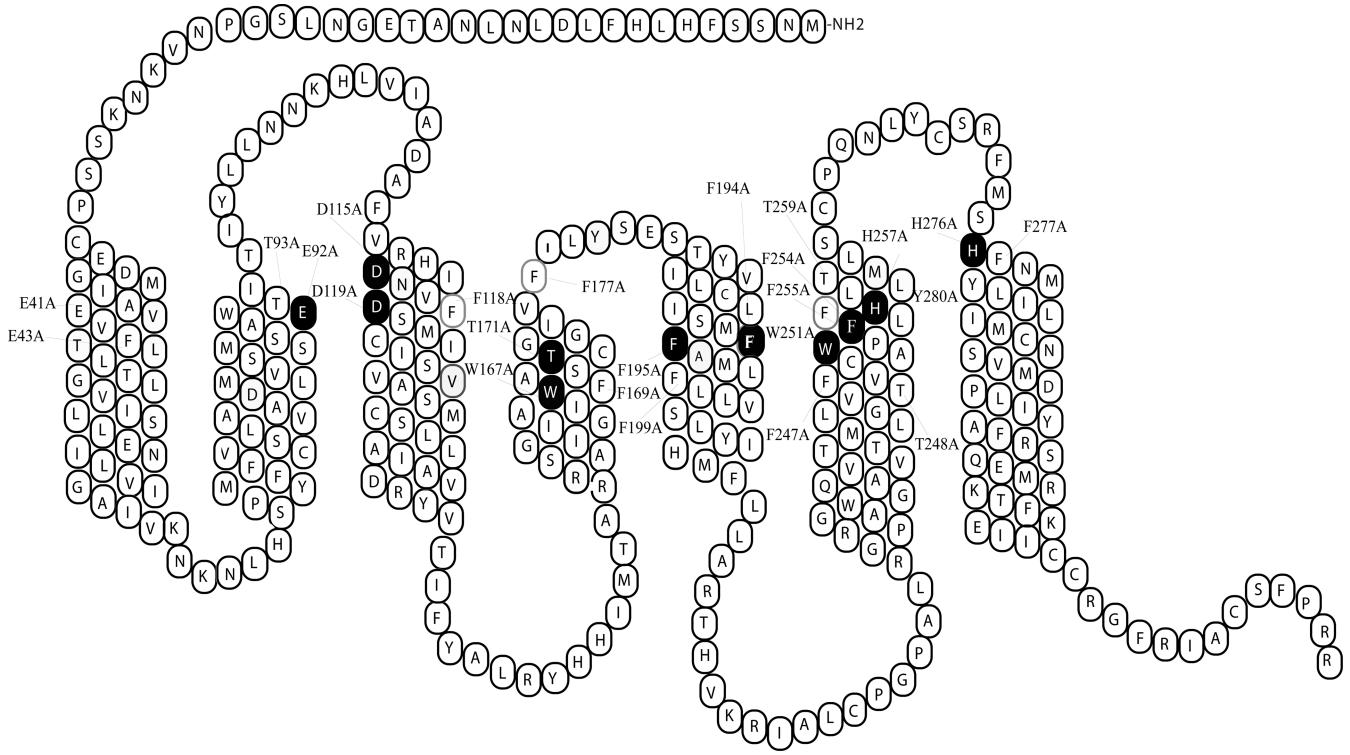


Figure 2. Two-dimensional representation of the seven-TM structure of hMC5R. The TM residues mutated in these experiments are denoted by black or gray highlighting. Those TM residues whose mutation significantly affected NDP-MSH binding as determined are shown with a black background.

Receptor Expression. For receptor protein expression studies, we utilized polymerase chain reaction to insert a FLAG tag onto the NH_2 terminus of hMC5R to characterize receptor protein cell 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.[24] hMC5R or mutant receptor-transfected cells were harvested using 0.2% EDTA and washed twice with phosphate-buffered 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 (catalog no. 316, Sigma, 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 IgG (ImmunoResearch Lab, Inc., West Grove, PA) and incubated at room temperature for 30 min. Flow cytometry was performed on a fluorescence-activated cell sorter (FACStar plus six parameter cytometer/sorter with a dual-argon ion laser, Becton-Dickinson Immunocytometry Systems, San Jose, CA). The results were analyzed using CellQuest (Beckton-Dickinson Immunocytometry Systems).

Statistical Analysis. Each experiment was performed three separate times with duplicate wells. Data are expressed as means ± the standard error of the mean. 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). A Student's t test was used to determine the statistical significance of changes in affinity and potency ($p < 0.05$ was considered significant).

MC5R Modeling. G protein-coupled receptors (GPCRs) represent the single largest class of membrane proteins in the human genome. GPCRs share a common structural signature of seven hydrophobic transmembrane (TM) segments, with an extracellular amino terminus and an intracellular carboxyl terminus. GPCRs share the most homology within the TM segments. So far, several different types of GPCR structures have been determined by X-ray crystallography: β2 adrenergic receptor, β1 adrenergic receptor, adenosine receptor, rhodopsin, CXCR4 chemokine receptor, dopamine D3 receptor, histamine receptor, and the nociceptin/orphanin FQ receptor.[9,11−14,25] Because experimentally elucidated structures are available for only this very limited number of receptors, homology modeling is still used widely for the construction of GPCR models intended to study the structure−function relationships of the receptors and aid the discovery and development of ligands capable of modulating their activity. MC5R belongs to the GPCR family and consists of a single polypeptide featuring seven α-helical TM domains, an extracellular N-terminus, and an intracellular C-terminus. A homology model of hMC5R was therefore generated on the basis of the structure of the nociceptin/orphanin FQ receptor (opioid-like receptor) as a template by using SWISS-MODEL.[26,27] Homology modeling of hMC5R consists of the following steps: selection of the nociceptin/orphanin FQ receptor as a template, alignment of the hMC5R sequence and the nociceptin/orphanin FQ receptor structure, model building, energy minimization, and/or refinement and model quality assessment. PATCHDOCK was used to dock the ligand (NDP-α-MSH) to the receptor.[28,29] The ligand was docked manually into the binding pocket of hMC5R.

dx.doi.org/10.1021/bi3013593 | *Biochemistry* 2013, 52, 1737−1745

RESULTS

Effects of Mutating Conserved Charged Amino Acids in Transmembrane Domains of hMC5R on NDP-α-MSH Binding and Activation. MC5R shares many conserved amino acid residues with other MCRs. Electrostatic and hydrophobic forces have been proposed to be involved in NDP-α-MSH binding and receptor activation at MCRs.[20,21,30] An ionic pocket formed by amino acid residues in transmembrane (TM) domains 2 and 3 of hMC1R, hMC2R, hMC3R, and hMC4R has been proposed to be critical for ligand binding;[20−22,31] furthermore, these TM residues have been hypothesized to form an ionic interaction with Arg8 of NDP-α-MSH.[30] We compared the MC5R sequence with other MCRs and determined that MC5R is 45% similar with other MCRs. This evolutionary conservation implies that there may be functional significance for these maintained receptor residues. We examined the effect of mutating conserved charged amino acids in transmembrane domains of the hMC5R on NDP-α-MSH binding and activation. The hMC5R mutations generated in this study are listed in Figure 2. On the basis of the hypothesis that NDP-α-MSH docks into the MC5R transmembrane domains near the surface of the plasma membrane, we performed mutagenesis studies in the upper region of the hMC5R transmembrane to examine the role of the charged amino acids on NDP-α-MSH binding and biological activity. Given its small neutral nature, alanine is the generally accepted amino acid of choice for mutagenesis substitution in this type of study because it is theoretically unlikely to disturb receptor tertiary structure. The binding affinities of NDP-α-MSH at wild-type hMC5R and mutated receptors were first examined. NDP-α-MSH possessed high-affinity binding at wild-type hMC5R as indicated by dose-dependent displacement of [^{125}I]NDP-α-MSH binding, but mutations of some receptor residues resulted in the alteration of ligand binding affinity (Figure 3A). To examine the ability of NDP-α-MSH to activate wild-type hMC5R and mutated receptors, second-message cAMP production was assessed. NDP-α-MSH dose-dependently increased the level of generation of cAMP at hMC5R and mutated receptors, but ligand potency was altered at some mutated receptors (Figure 3B). As shown in Figure 3, all three negatively charged residues in TM2 and TM3 (glutamic acid E92, D115, and D119) were examined as they are homologous to receptor residues previously implicated in other MCR agonist binding and signaling. One substitution was made at TM6 H257, which is homologous to hMC1R H260 and hMC4R H264. These residues have been shown to contribute to NDP-NDP-α-MSH specific binding.[20,21] Our results indicate that mutating residues E92 and D115 in TM2 and D119 in TM3 significantly altered NDP-α-MSH binding affinity and receptor activity [$p < 0.05$ (Figure 3)], which is similar to that of hMC1R and hMC4R. Another substitution was made at TM7 H276, which is homologous to hMC1R N279 and hMC4R H283, which was not contributed to NDP-α-MSH binding at hMC1R and hMC4R. Our results indicate that mutating this residue significantly decreased NDP-α-MSH binding affinity and receptor activity [$p < 0.05$ (Figure 3)], which is different from what is seen with hMC1R and hMC4R. The respective K_i and EC_{50} values are listed in Table 1. We also examined other charged amino acids, specifically residues E41 and E53, and our results indicated that mutations of these amino acid residues did not alter NDP-α-MSH binding or signaling.

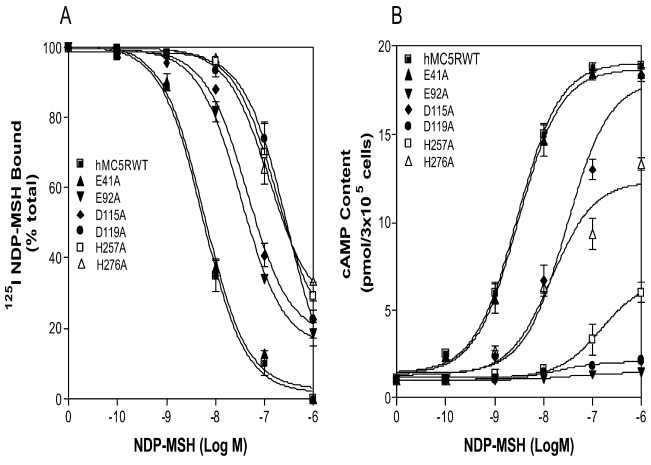


Figure 3. Effects of substitution of the conserved charged amino acid hMC5R TM residues with alanine on NDP-MSH binding affinity and receptor activity. (A) NDP-MSH binding affinities of these mutants. (B) Extents of NDP-MSH-stimulated cAMP production of these mutants. For panels A and B, $n = 3$ (see Table 1 for actual IC_{50} and EC_{50} values).

Table 1. Effects of Substitution of Charged Amino Acid Residues of hMC5R on [^{125}I]NDP-MSH Binding and cAMP Production

	receptor expression (% of WT)	[^{125}I]NDP-MSH binding IC_{50} (nM)	cAMP production EC_{50} (nM)
WT hMC5R	100	5.3 ± 1.0	1.8 ± 0.2
E41A	89 ± 11	8.2 ± 1.6	5.3 ± 0.8
E92A	75 ± 12.3	110 ± 12.1[a]	90 ± 10.1[a]
D115A	68 ± 14	63.7 ± 7.7[a]	45 ± 1.2[a]
D119A	66 ± 11	>10^3 [a]	>10^3 [a]
H257A	67 ± 8.7	551 ± 29[a]	451 ± 27[a]
H276A	84 ± 6.8	332 ± 46[a]	387 ± 32[a]

[a] $p < 0.05$ compared with the WT receptor.

Effects of Mutating Conserved Aromatic Amino Acids in Transmembrane Domains of hMC5R Responsible for NDP-α-MSH Binding and Activation. On the basis of our mutagenesis studies of other MCRs, we have proposed that there is a second distinct binding pocket for the MC5R ligand−receptor interaction,[20−23,30] which consists of a series of hydrophobic receptor residues in TM5 and TM6 that are believed to form aromatic−aromatic interactions with Phe7 and Trp9 of NDP-α-MSH.[21,30] To determine whether aromatic amino acids in TM5 and TM6 of MC5R play important roles in ligand binding and signaling, we tested 13 amino acid residues (TM1 F43, TM2 T93, TM4 W167, T171, and F177, TM5 F194 and F195, TM6 F247, W251, F254, and F255, and TM7 F277 and Y280) for ligand binding and signaling. Our results indicated that mutations of F43, W167, T171, F194, F195, W251, and F254 in TM6 significantly reduced NDP-α-MSH binding affinity and receptor activity ($p < 0.05$) (Figure 4 and Table 2) while mutations of T93, T177, F247, F255, F277, and Y280 did not significantly alter receptor ligand binding or signaling.

Effects of Mutating Nonconserved Charged Amino Acids in Transmembrane Domains of hMC5R Responsible for NDP-α-MSH Binding and Activation. Receptor

dx.doi.org/10.1021/bi3013593 | Biochemistry 2013, 52, 1737−1745

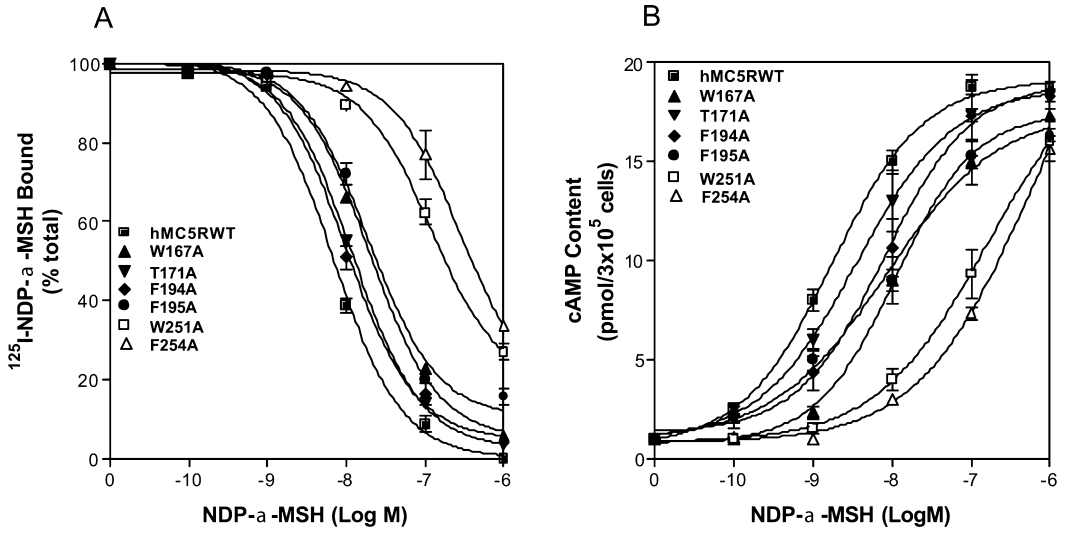


Figure 4. Effects of substitution of conserved aromatic amino acids with alanine in hMC5R on NDP-MSH binding affinity and receptor activity. (A) NDP-MSH binding affinities at these mutant receptors. (B) Ability of NDP-MSH to stimulate cAMP production at these mutant receptors. For panels A and B, $n = 3$ (see Table 2 for actual IC_{50} and EC_{50} values).

Table 2. Effects of Substitution of Conserved Aromatic Amino Acid Residues of hMC5R on [^{125}I]NDP-MSH Binding and cAMP Production

	receptor expression (% of WT)	[^{125}I]NDP-MSH binding IC_{50} (nM)	cAMP production EC_{50} (nM)
WT hMC5R	100	5.3 ± 1.0	1.8 ± 0.2
F43A	95 ± 6.3	34.5 ± 2.5[a]	41.6 ± 3.2[a]
T93A	92 ± 8.3	6.2 ± 0.9	2.7 ± 0.5
W167A	90 ± 8.3	21.2 ± 6.9[a]	21 ± 4.5[a]
T171A	93 ± 6.8	11.3 ± 2.9[a]	8.7 ± 0.6[a]
F177A	92 ± 8.3	4.2 ± 0.9	1.4 ± 0.5
F194A	94 ± 7.3	8.2 ± 2.2[a]	6.2 ± 3.4[a]
F195A	95 ± 5.3	16.1 ± 3.9[a]	13.4 ± 4.6[a]
F247A	92 ± 7.6	6.7 ± 3.7	2.6 ± 1.7
W251A	87 ± 23	174 ± 32[a]	125 ± 23[a]
F254A	66 ± 11	>10^3 [a]	>10^3 [a]
F255A	96 ± 6	6.8 ± 1.8	2.3 ± 0.7
F277A	98 ± 14.7	7.5 ± 1.7	3.1 ± 1.6
Y280A	98 ± 14.7	5.7 ± 1.7	2.4 ± 1.7

[a] $p < 0.05$ compared with the WT receptor.

amino acid sequence analysis also indicates that several charged or aromatic amino acid residues in hMC5R are not conserved among the MCRs. These residues include T45 in TM1, W91 in TM2, F118 in TM3, F169 and F199 in TM5, and T248 and T259 in TM6. To determine whether these nonconserved aromatic residues in the TM of the hMC5R are involved in ligand binding and signaling, we mutated these aromatic residues and examined whether mutation would result in subsequent changes in ligand binding affinity and potency at this receptor. Our results indicate that only mutating F169 significantly altered NDP-α-MSH binding affinity and receptor activity ($p < 0.05$) (Figure 5 and Table 3).

DISCUSSION

The melanocortin receptors consist of a single polypeptide featuring seven α-helical TM domains, an extracellular N-terminus, and an intracellular C-terminus. These receptors are involved in a diverse number of physiological functions, including pigmentation, steroidogenesis, energy homeostasis, exocrine secretion, sexual function, analgesia, and inflammation. Many clinical studies indicate that melanocortin receptor gene variants play important roles in the development of different diseases. MC1R gene variants determine the risk of melanoma skin cancer.[32] MC2R gene variants have been identified in familial glucocorticoid deficiency (FGD), a potentially fatal disease.[33] MC3R and MC4R gene variants are involved in the development of obesity.[34,35] The melanocortin peptides belong to a group of neuropeptides derived from the pro-opiomelanocortin prohormone and contain the common His-Phe-Arg-Trp amino acid sequence.[36] One super potent α-MSH analogue has been developed by altering the chirality of the phenylalanine in the core sequence and replacing methionine with norleucine [Nle4,D-Phe7]MSH (NDP-MSH). This agonist is widely used for melanocortin receptor study because it is stable and potent. Truncated NDP-MSH peptides or singly mutated NDP-MSH was also used for melanocortin receptor ligand binding and signaling studies. The His-Phe-Arg-Trp amino acid residues in NDP-α-MSH were identified as being important for ligand binding and biological activities at hMC1R, hMC3R, hMC4R, and hMC5R.[37−39] The lead peptide γ_2-MSH, Tyr-Val Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH, has been previously reported to possess agonist activity at hMC5R.[40,41] A new study indicates that γ_2-MSH is not selective for mouse MC3R over mouse MC5R by γ_2-melanocyte stimulation hormone (γ_2-MSH) truncation studies, and there appears to be a significant species difference resulting in a 68-fold decrease in potency at hMC5R when compared to mMC5R with peptide **1**.[42]

Similarity of hMC5R with Other hMCRs in Ligand Binding Pockets. Extensive studies have been performed to examine the molecular basis of MC1R, MC2R, MC3R, and MC4R responsible for ligand binding and signaling.[20−22] These studies indicate that conserved residues in TM of the melanocortin receptors are involved in NDP-α-MSH or ACTH binding and signaling. Electrostatic and hydrophobic forces have been proposed in NDP-α-MSH or ACTH binding and receptor activation.[21,22,30,31] Melanocortin receptor subtypes are believed to have the same basic molecular

dx.doi.org/10.1021/bi3013593 | Biochemistry 2013, 52, 1737−1745

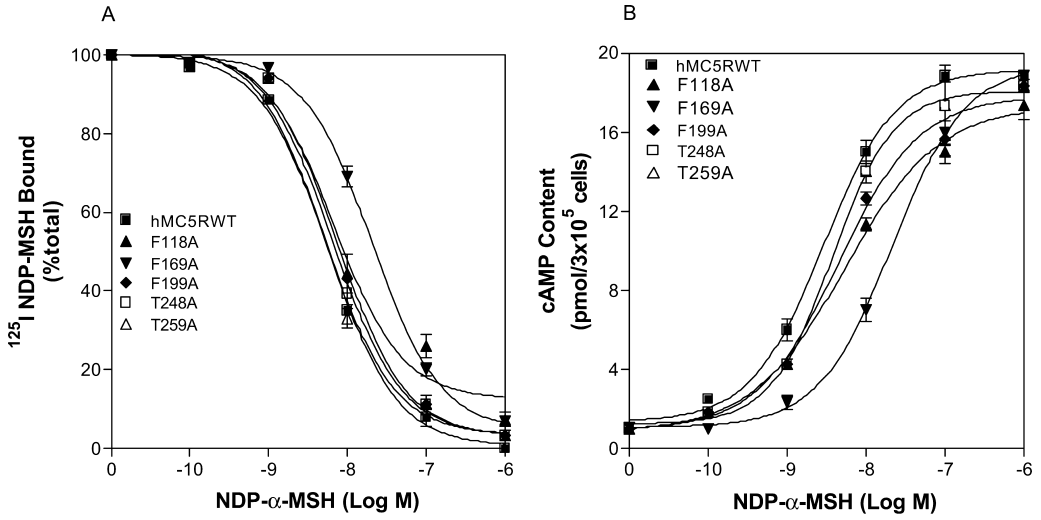


Figure 5. Effects of substitution of nonconserved aromatic amino acids with alanine in hMC5R on NDP-MSH binding affinity and receptor activity. (A) NDP-MSH binding affinity at these mutant receptors. (B) Ability of NDP-MSH to stimulate cAMP production at these mutant receptors. For panels A and B, $n = 3$ (see Table 3 for actual IC_{50} and EC_{50} values).

Table 3. Effects of Substitution of Nonconserved Aromatic Amino Acid Residues of hMC5R on [^{125}I]NDP-MSH Binding and cAMP Production

	receptor expression (% of WT)	[^{125}I]NDP-MSH binding IC_{50} (nM)	cAMP production EC_{50} (nM)
WT hMC5R	100	5.3 ± 1.0	1.8 ± 0.2
T45A	97 ± 1.6	5.4 ± 0.6	1.7 ± 0.7
W91A	98 ± 1.8	5.2 ± 0.9	1.6 ± 0.3
F118A	95 ± 8.8	6.3 ± 2.1	1.7 ± 0.6
F169A	94 ± 5.5	25.3 ± 4.1[a]	29.1 ± 3.1[a]
F199A	98 ± 4.0	4.5 ± 0.7	1.2 ± 0.6
T248A	93 ± 6.4	5.1 ± 0.5	1.5 ± 0.4
T259A	98 ± 2.0	4.9 ± 0.3	1.9 ± 0.3

[a] $p < 0.05$ compared with the WT receptor.

architecture, and hMC5R is nearly 50% identical with other MCRs in the putative TM regions.[1,43−45] Given that MC5R shares common endogenous melanocortin ligands with other MCRs, it was proposed that the conserved MC5R residues may have common orthosteric binding sites with other MCRs; we therefore tested all conserved basic and negative TM residues, as well as aromatic-, hydroxyl-, and sulfhydryl-containing residues in MC5R. We examined the roles of the highly conserved residues across MC5R, MC1R, MC2R, MC3R, and MC4R subtypes that have been identified to be crucial in other MCRs. We examined eight conserved amino acid residues of hMC5R that have previously been identified to be involved in ligand binding and activity in MC1R, MC2R, MC3R, and MC4R.[6,20−22,46−48] It was subsequently found that glutamine acid, E94 in TM2, and aspartic acid, D115 and D119 in TM3 of the MC5R, are conserved among all five melanocortin receptor subtypes; furthermore, mutating either of these amino acids to alanine resulted in significantly less endogenous agonist binding and receptor signaling, suggesting that these residues are important for ligand binding and signaling. Amino acid residue H257 in TM6 of MC5R is also conserved in all five melanocortin receptors. Changing these residues to alanine resulted in a significant decrease in the level of endogenous agonist binding and receptor signaling, further supporting their involvement in orthosteric binding.[6,21,48−50] Our results demonstrate that conserved residues E92 in TM2 and D115 and D119 in TM3 are involved in NDP-α-MSH binding and are similar to those of other MCRs.[6,20−22,48] These data indicate that several similarities exist between hMC5R and other MCRs. First, MC5R and MC1R, MC3R, and MC4R subtypes are activated by the same minimal NDP-α-MSH tripeptide sequence, Ac-D-Phe-Arg-Trp-NH_2.[51] Second, mutations of homologous residues in TM2, TM3, and TM6 of hMC1R, hMC3R, hMC4R, and hMC5R 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] Furthermore, in our study, aromatic residues were extensively examined as in previous studies of other MCRs.[20−22,31] Several aromatic mutants that significantly affected binding of agonist to hMC5R were found (i.e., TM5 F195 and TM6 F254). Therefore, these data are consistent with the existence of aromatic−aromatic interactions between ligand residues Phe7 and Trp9 of the melanocortin sequence and hMC5R, interactions that were previously hypothesized to exist in the case of other MCRs.[22,23,31]

Disparity between hMC5R with Other hMCRs in Ligand Binding Pockets. Despite the enumerated similarities between hMC5R and other MCRs, these studies also identified a potentially important subtype-specific difference between hMC5R and other hMCRs. Receptor amino acid sequence analysis indicates that some charged or aromatic amino acid residues in hMC5R are conserved among MCRs. These residues include W167 and T171 in TM4, F194 and F195 in TM5, and H276 in TM7. Mutations of these residues significantly reduced ligand binding affinity and potency. However, in other MCRs, these residues have not been found to significantly alter ligand binding and signaling. Receptor amino acid sequence analysis also indicates that several charged or aromatic amino acid residues in hMC5R are not conserved among MCRs. These residues include T45 in TM1, W91 in TM2, F118 in TM3, F169 and F199 in TM5, and T248 and T259 in TM6. Mutations of these residues did not significantly reduce ligand binding affinity or potency except at F169. Mutation of this residue (F169A) significantly reduced ligand binding affinity and potency, demonstrating that MC5R

dx.doi.org/10.1021/bi3013593 | Biochemistry 2013, 52, 1737−1745

has a broad binding site for ligand NDP-α-MSH that includes not only conserved amino acid residues but also nonconserved residues.

Modeling of the MC5R NDP-α-MSH Complex. Modeling of complexes of human MC5R with receptor-specific agonists improved our understanding of the structural requirements for ligand binding and signaling. Our results suggest that hMC5R may share similar binding sites with hMC1R, hMC2R, hMC3R, and hMC4R. We incorporated our results into the model for the hMC5R ligand receptor interaction that is shown in Figure 6. As shown in panels A and B of Figure 7, an ionic pocket was formed by amino acids D115 and D119 in TM3 of hMC5R, as well as an aromatic binding pocket that consisted of F195 in TM5 and F254 in TM6 of hMC5R. In this model complex, arginine 8 is in contact with D115 and D119; the central aromatic ring of D-F7 is in contact with F195, while aromatic ring W9 interacts with F254. This computational approach defined the structural details of the receptor-bound conformation of NDP-α-MSH and confirmed their proposed arrangement inside the receptor. Although mutations of W167 and T171 in TM4, W251 and H257 in TM6, and H276 in TM7 significantly decreased ligand binding affinity and potency, these residues are not included in the ligand binding pocket of our computer model. On the basis of the model, these residues do not face the ligand binding pocket. Mutations of these residues may result in a change in the receptor conformation and then alter ligand binding affinity and potency. Although MCR subtypes share sequence homology, ranging from 60% identical (hMC4R and hMC5R) to 45% identical (hMC3R and hMC1R, and hMC3R and hMC2R) to 38% identical (hMC2R and hMC4R),[34,43] our results suggest that they may utilize similar orthosteric binding sites for endogenous ligand binding and signaling.

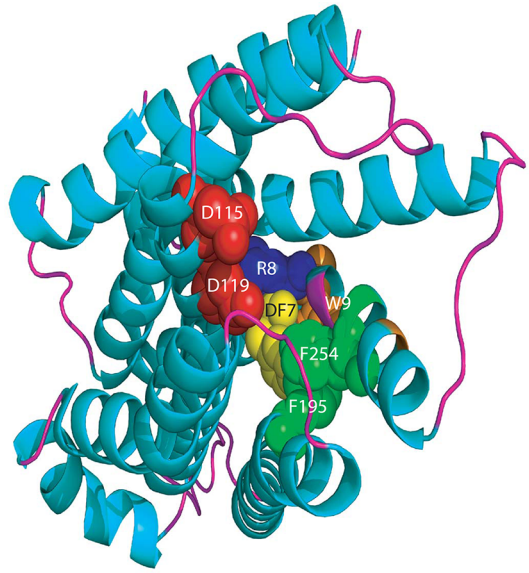


Figure 6. Three-dimensional model of a proposed NDP-α-MSH docked inside hMC5R. Red residues represent D115 and D119 in TM3 of hMC5R. Green residues represent F195 and F254 in TM5 and TM6, respectively, of hMC5R. The blue residue represents R8 in NDP-α-MSH. The yellow residue represents D-F7 in NDP-α-MSH. The brown residue represents W9 in NDP-MSH.

In summary, we propose three-dimensional models of complexes of hMC5R with a linear peptide (NDP-α-MSH) that are consistent with our structure−activity studies. Key findings are that TM3, TM5, and TM6 of hMC5R are important for ligand binding and signaling. These results provide important insight into the molecular aspects of hMC5R responsible for ligand binding and receptor signaling.

AUTHOR INFORMATION

Corresponding Author

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

Funding

This work has been supported by the Alabama Children's Hospital Research fund (C.M.H.) and National Institutes of Health Grant R03 HD047312-01A1 (Y.Y.).

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

MCR, melanocortin receptor; hMC5R, human melanocortin-5 receptor; GPCR, G protein-coupled receptor; NDP-α-MSH,

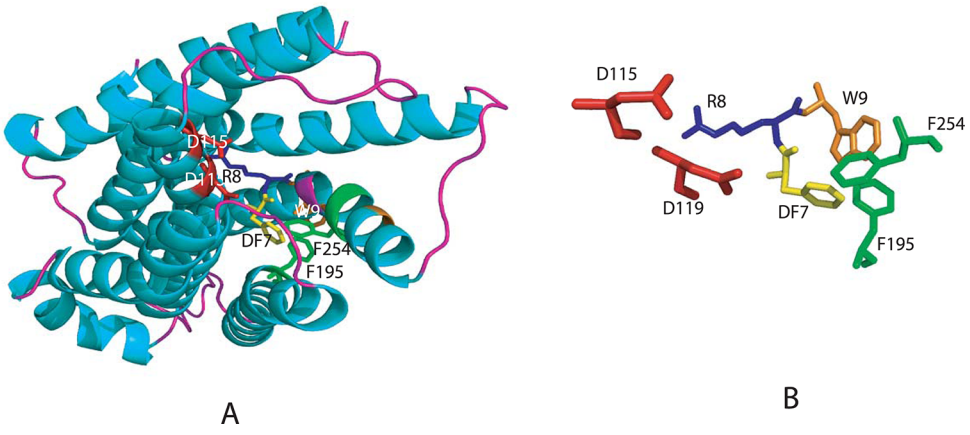


Figure 7. Two main receptor binding pockets. In panel A, the ligand binds to receptor residues. In panel B, the ligand and the receptor side chains make direct contact. The first is a predominantly ionic pocket formed by D115 and D119 in TM3 with R8 in NDP-α-MSH. The second one is a hydrophobic pocket formed by aromatic residues F195 in TM5 and F254 in TM6 with D-F7 and W9 in NDP-α-MSH.

dx.doi.org/10.1021/bi3013593 | Biochemistry 2013, 52, 1737−1745

[Nle4,D-Phe7]-α-melanocyte stimulating hormone; TM, transmembrane domain; IBMX, 3-isobutylmethylxanthine; PCR, polymerase chain reaction; FACs, flow cytometry; WT, wild type.

REFERENCES

(1) Gantz, I., Shimoto, Y., Konda, Y., Miwa, H., Dickinson, C. J., and Yamada, T. (1994) Molecular cloning, expression, and characterization of a fifth melanocortin receptor. *Biochem. Biophys. Res. Commun. 200*, 1214−1220.

(2) Boston, B. A., and Cone, R. D. (1996) Characterization of melanocortin receptor subtype expression in murine adipose tissues and in the 3T3-L1 cell line. *Endocrinology 137*, 2043−2050.

(3) Sanchez, E., Rubio, V. C., and Cerda-Reverter, J. M. (2009) Characterization of the sea bass melanocortin 5 receptor: A putative role in hepatic lipid metabolism. *J. Exp. Biol. 212*, 3901−3910.

(4) An, J. J., Rhee, Y., Kim, S. H., Kim, D. M., Han, D. H., Hwang, J. H., Jin, Y. J., Cha, B. S., Baik, J. H., Lee, W. T., and Lim, S. K. (2007) Peripheral effect of α-melanocyte-stimulating hormone on fatty acid oxidation in skeletal muscle. *J. Biol. Chem. 282*, 2862−2870.

(5) Chen, W., Kelly, M. A., Opitz-Araya, X., Thomas, R. E., Low, M. J., and Cone, R. D. (1997) Exocrine gland dysfunction in MC5-R-deficient mice: Evidence for coordinated regulation of exocrine gland function by melanocortin peptides. *Cell 91*, 789−798.

(6) 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.

(7) Haskell-Luevano, C., Holder, J. R., Monck, E. K., and Bauzo, R. M. (2001) Characterization of melanocortin NDP-MSH agonist peptide fragments at the mouse central and peripheral melanocortin receptors. *J. Med. Chem. 44*, 2247−2252.

(8) Haskell-Luevano, C., Lim, S., Yuan, W., Cone, R. D., and Hruby, V. J. (2000) Structure activity studies of the melanocortin antagonist SHU9119 modified at the 6, 7, 8, and 9 positions. *Peptides 21*, 49−57.

(9) Cherezov, V., Rosenbaum, D. M., Hanson, M. A., Rasmussen, S. G., Thian, F. S., Kobilka, T. S., Choi, H. J., Kuhn, P., Weis, W. I., Kobilka, B. K., and Stevens, R. C. (2007) High-resolution crystal structure of an engineered human β2-adrenergic G protein-coupled receptor. *Science 318*, 1258−1265.

(10) Lebon, G., Warne, T., Edwards, P. C., Bennett, K., Langmead, C. J., Leslie, A. G., and Tate, C. G. (2011) Agonist-bound adenosine A2A receptor structures reveal common features of GPCR activation. *Nature 474*, 521−525.

(11) Warne, T., Moukhametzianov, R., Baker, J. G., Nehme, R., Edwards, P. C., Leslie, A. G., Schertler, G. F., and Tate, C. G. (2011) The structural basis for agonist and partial agonist action on a β_1-adrenergic receptor. *Nature 469*, 241−244.

(12) Chien, E. Y., Liu, W., Zhao, Q., Katritch, V., Han, G. W., Hanson, M. A., Shi, L., Newman, A. H., Javitch, J. A., Cherezov, V., and Stevens, R. C. (2010) Structure of the human dopamine D3 receptor in complex with a D2/D3 selective antagonist. *Science 330*, 1091−1095.

(13) Wu, B., Chien, E. Y., Mol, C. D., Fenalti, G., Liu, W., Katritch, V., Abagyan, R., Brooun, A., Wells, P., Bi, F. C., Hamel, D. J., Kuhn, P., Handel, T. M., Cherezov, V., and Stevens, R. C. (2010) Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. *Science 330*, 1066−1071.

(14) de Graaf, C., Kooistra, A. J., Vischer, H. F., Katritch, V., Kuijer, M., Shiroishi, M., Iwata, S., Shimamura, T., Stevens, R. C., de Esch, I. J., and Leurs, R. (2011) Crystal structure-based virtual screening for fragment-like ligands of the human histamine H_1 receptor. *J. Med. Chem. 54*, 8195−8206.

(15) Shimamura, T., Shiroishi, M., Weyand, S., Tsujimoto, H., Winter, G., Katritch, V., Abagyan, R., Cherezov, V., Liu, W., Han, G. W., Kobayashi, T., Stevens, R. C., and Iwata, S. (2011) Structure of the human histamine H1 receptor complex with doxepin. *Nature 475*, 65−70.

(16) Hanson, M. A., Roth, C. B., Jo, E., Griffith, M. T., Scott, F. L., Reinhart, G., Desale, H., Clemons, B., Cahalan, S. M., Schuerer, S. C., Sanna, M. G., Han, G. W., Kuhn, P., Rosen, H., and Stevens, R. C. (2012) Crystal structure of a lipid G protein-coupled receptor. *Science 335*, 851−855.

(17) Deupi, X., Edwards, P., Singhal, A., Nickle, B., Oprian, D., Schertler, G., and Standfuss, J. (2012) Stabilized G protein binding site in the structure of constitutively active metarhodopsin-II. *Proc. Natl. Acad. Sci. U.S.A. 109*, 119−124.

(18) Hino, T., Arakawa, T., Iwanari, H., Yurugi-Kobayashi, T., Ikeda-Suno, C., Nakada-Nakura, Y., Kusano-Arai, O., Weyand, S., Shimamura, T., Nomura, N., Cameron, A. D., Kobayashi, T., Hamakubo, T., Iwata, S., and Murata, T. (2012) G-protein-coupled receptor inactivation by an allosteric inverse-agonist antibody. *Nature 482*, 237−240.

(19) Worth, C. L., Kleinau, G., and Krause, G. (2009) Comparative sequence and structural analyses of G-protein-coupled receptor crystal structures and implications for molecular models. *PLoS One 4*, e7011.

(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) 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.

(22) Chen, M., Aprahamian, C. J., Celik, A., Georgeson, K. E., Garvey, W. T., Harmon, C. M., and Yang, Y. (2006) Molecular characterization of human melanocortin-3 receptor ligand-receptor interaction. *Biochemistry 45*, 1128−1137.

(23) Chen, M., Cai, M., Aprahamian, C. J., Georgeson, K. E., Hruby, V., Harmon, C. M., and Yang, Y. (2007) Contribution of the conserved amino acids of the melanocortin-4 receptor in [corrected] [Nle4,D-Phe7]-α-melanocyte-stimulating [corrected] hormone binding and signaling. *J. Biol. Chem. 282*, 21712−21719.

(24) 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.

(25) Thompson, A. A., Liu, W., Chun, E., Katritch, V., Wu, H., Vardy, E., Huang, X. P., Trapella, C., Guerrini, R., Calo, G., Roth, B. L., Cherezov, V., and Stevens, R. C. (2012) Structure of the nociceptin/orphanin FQ receptor in complex with a peptide mimetic. *Nature 485*, 395−399.

(26) Arnold, K., Bordoli, L., Kopp, J., and Schwede, T. (2006) The SWISS-MODEL workspace: A web-based environment for protein structure homology modelling. *Bioinformatics 22*, 195−201.

(27) Kiefer, F., Arnold, K., Kunzli, M., Bordoli, L., and Schwede, T. (2009) The SWISS-MODEL Repository and associated resources. *Nucleic Acids Res. 37*, D387−D392.

(28) Schneidman-Duhovny, D., Inbar, Y., Nussinov, R., and Wolfson, H. J. (2005) PatchDock and SymmDock: Servers for rigid and symmetric docking. *Nucleic Acids Res. 33*, W363−W367.

(29) Schneidman-Duhovny, D., Inbar, Y., Polak, V., Shatsky, M., Halperin, I., Benyamini, H., Barzilai, A., Dror, O., Haspel, N., Nussinov, R., and Wolfson, H. J. (2003) Taking geometry to its edge: Fast unbound rigid (and hinge-bent) docking. *Proteins 52*, 107−112.

(30) 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. Discovery 14*, 197−211.

(31) Chen, M., Aprahamian, C. J., Kesterson, R. A., Harmon, C. M., and Yang, Y. (2007) Molecular identification of the human melanocortin-2 receptor responsible for ligand binding and signaling. *Biochemistry 46*, 11389−11397.

(32) Wong, S. S., Ainger, S. A., Leonard, J. H., and Sturm, R. A. (2012) MC1R variant allele effects on UVR-induced phosphorylation of p38, p53, and DDB2 repair protein responses in melanocytic cells in culture. *J. Invest. Dermatol. 132*, 1452−1461.
(33) Selva, K. A., LaFranchi, S. H., and Boston, B. (2004) A novel presentation of familial glucocorticoid deficiency (FGD) and current literature review. *J. Pediatr. Endocrinol. Metab. 17*, 85−92.
(34) Yang, Y. K., and Harmon, C. M. (2003) Recent developments in our understanding of melanocortin system in the regulation of food intake. *Obes. Rev. 4*, 239−248.
(35) Tao, Y. X., and Segaloff, D. L. (2005) Functional Analyses of Melanocortin-4 Receptor Mutations Identified from Patients with Binge Eating Disorder and Non-obese or Obese Subjects. *J. Clin. Endocrinol. Metab. 90*, 5632−5638.
(36) Haskell-Luevano, C., Hendrata, S., North, C., Sawyer, T. K., Hadley, M. E., Hruby, V. J., Dickinson, C., and Gantz, I. (1997) Discovery of prototype peptidomimetic agonists at the human melanocortin receptors MC1R and MC4R. *J. Med. Chem. 40*, 2133−2139.
(37) Holder, J. R., Xiang, Z., Bauzo, R. M., and Haskell-Luevano, C. (2002) Structure-activity relationships of the melanocortin tetrapeptide Ac-His-D-Phe-Arg-Trp-NH_2 at the mouse melanocortin receptors. 4. Modifications at the Trp position. *J. Med. Chem. 45*, 5736−5744.
(38) Holder, J. R., Xiang, Z., Bauzo, R. M., and Haskell-Luevano, C. (2003) Melanocortin tetrapeptide Ac-His-DPhe-Arg-Trp-NH_2 at the mouse melanocortin receptors. Part 3: Modifications at the Arg position. *Peptides 24*, 73−82.
(39) Holder, J. R., Bauzo, R. M., Xiang, Z., and Haskell-Luevano, C. (2002) Structure-activity relationships of the melanocortin tetrapeptide Ac-His-DPhe-Arg-Trp-NH_2 at the mouse melanocortin receptors. 1. Modifications at the His position. *J. Med. Chem. 45*, 2801−2810.
(40) Grieco, P., Balse, P. M., Weinberg, D., MacNeil, T., and Hruby, V. J. (2000) D-Amino acid scan of γ-melanocyte-stimulating hormone: Importance of Trp^8 on human MC3 receptor selectivity. *J. Med. Chem. 43*, 4998−5002.
(41) Grieco, P., Balse-Srinivasan, P., Han, G., Weinberg, D., MacNeil, T., Van der Ploeg, L. H., and Hruby, V. J. (2002) Synthesis and biological evaluation on hMC3, hMC4 and hMC5 receptors of γ-MSH analogs substituted with L-alanine. *J. Pept. Res. 59*, 203−210.
(42) Joseph, C. G., Yao, H., Scott, J. W., Sorensen, N. B., Marnane, R. N., Mountjoy, K. G., and Haskell-Luevano, C. (2010) γ_2-Melanocyte stimulation hormone (γ_2-MSH) truncation studies results in the cautionary note that γ_2-MSH is not selective for the mouse MC3R over the mouse MC5R. *Peptides 31*, 2304−2313.
(43) Gantz, I., Miwa, H., Konda, Y., Shimoto, Y., Tashiro, T., Watson, S. J., DelValle, J., and Yamada, T. (1993) Molecular cloning, expression, and gene localization of a fourth melanocortin receptor. *J. Biol. Chem. 268*, 15174−15179.
(44) Gantz, I., Yamada, T., Tashiro, T., Konda, Y., Shimoto, Y., Miwa, H., and Trent, J. M. (1994) Mapping of the gene encoding the melanocortin-1 (α-melanocyte stimulating hormone) receptor (MC1R) to human chromosome 16q24.3 by fluorescence in situ hybridization. *Genomics 19*, 394−395.
(45) Mountjoy, K. G., Robbins, L. S., Mortrud, M. T., and Cone, R. D. (1992) The cloning of a family of genes that encode the melanocortin receptors. *Science 257*, 1248−1251.
(46) Chen, M., Cai, M., Aprahamian, C. J., Georgeson, K. E., Hruby, V., Harmon, C. M., and Yang, Y. (2007) Contribution of the conserved amino acids of the melanocortin-4 receptor in NDP-MSH binding and signaling. *J. Biol. Chem. 282*, 21712−21719.
(47) Hogan, K., Peluso, S., Gould, S., Parsons, I., Ryan, D., Wu, L., and Visiers, I. (2006) Mapping the binding site of melanocortin 4 receptor agonists: A hydrophobic pocket formed by $I3.28^{125}$, $I3.32^{129}$, and $I7.42^{291}$ is critical for receptor activation. *J. Med. Chem. 49*, 911−922.
(48) Fleck, B. A., Chen, C., Yang, W., Huntley, R., Markison, S., Nickolls, S. A., Foster, A. C., and Hoare, S. R. (2005) Molecular interactions of nonpeptide agonists and antagonists with the melanocortin-4 receptor. *Biochemistry 44*, 14494−14508.
(49) Pogozheva, I. D., Chai, B. X., Lomize, A. L., Fong, T. M., Weinberg, D. H., Nargund, R. P., Mulholland, M. W., Gantz, I., and Mosberg, H. I. (2005) Interactions of human melanocortin 4 receptor with nonpeptide and peptide agonists. *Biochemistry 44*, 11329−11341.
(50) Nickolls, S. A., Cismowski, M. I., Wang, X., Wolff, M., Conlon, P. J., and Maki, R. A. (2003) Molecular determinants of melanocortin 4 receptor ligand binding and MC4/MC3 receptor selectivity. *J. Pharmacol. Exp. Ther. 304*, 1217−1227.
(51) Sawyer, T. K., Castrucci, A. M., Staples, D. J., Affholter, J. A., De Vaux, A., Hruby, V. J., and Hadley, M. E. (1993) Structure-activity relationships of [Nle4,D-Phe7]-α-MSH. Discovery of a tripeptidyl agonist exhibiting sustained bioactivity. *Ann. N.Y. Acad. Sci. 680*, 597−599.