Substituted NDP-MSH Peptides Paired with Mutant Melanocortin-4 Receptors Demonstrate the Role of Transmembrane 6 in Receptor Activation

Beth A. Fleck,*,‡ Nicholas Ling,§ and Chen Chen§

Departments of Pharmacology and Medicinal Chemistry, Neurocrine Biosciences Inc., 12790 El Camino Real, San Diego, California 92130

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ABSTRACT: The melanocortin-4 receptor (MC4R) is involved in regulating energy homeostasis and is a potential therapeutic target for obesity and cachexia. Molecular interactions between peptide ligands and MC4R have been studied in detail. Less is known regarding the role of these interactions in the mechanism of MC4R activation. The aim of this study was to investigate the molecular mechanism of human MC4R activation by [Nle^4, D-Phe^7]α-melanocyte-stimulating hormone (NDP-MSH), by first defining the role of the His^6-D-Phe^7-Arg^8-Trp^9 residues in receptor activation (E_{max} for stimulation of cAMP accumulation) using modified peptides, then understanding how their interaction with the receptor modulates activation using site-directed mutagenesis and a molecular model of NDP-MSH bound to the active state of the receptor. Alanine substitution indicated that the D-Phe^7, Arg^8, and Trp^9 side chains contribute binding energy but are not essential for the receptor activation event. Conversely, His^6 to Ala^6 substitution reduced receptor activation but did not affect affinity. Chlorine substitutions on the D-Phe^7 side chain also inhibited receptor activation. F261$^{(6.51)}$A and F284$^{(7.35)}$A receptor mutations acted as gain-of-function mutations, restoring efficacy to the His^6 and D-Phe^7 substituted peptides that had lost efficacy at the wild-type receptor. Based on a model of NDP-MSH and MC4R interaction, the antagonist behavior of these peptides is consistent with the prevention of transmembrane 6 (TM6) rotation. This data supports the hypothesis that increasing the size of D-Phe^7 directly interferes with TM6 rotation, preventing receptor activation. We further propose that removing the interaction with the His^6 side chain reorients the peptide within the binding pocket, indirectly impeding TM6 rotation by strengthening peptide interaction with F261$^{(6.51)}$ and F284$^{(7.35)}$. These findings refine the molecular basis for the mechanism of ligand-stimulated hMC4R activation and will be useful for the development of hMC4R agonists and antagonists.

The melanocortin-4 receptor (MC4R[1]) is responsible for mediating energy homeostasis and is a major target for drug design in the treatment of obesity and cachexia (*1*−*3*). It is one of five melanocortin receptors that have been cloned and sequenced and belongs to the class A superfamily of rhodopsin-like G-protein-coupled receptors (GPCRs), characterized by having seven transmembrane α-helices (TM1−TM7) linked by three extracellular and three intracellular loops (*4*−*6*). Several endogenous agonist ligands for the MC4 and other melanocortin receptors have been identified, including α-, *β*-, *γ*-melanocyte-stimulating hormone (MSH) and adrenocorticotropin hormone, all of which are derived from the precursor peptide proopiomelanocortin (*7*, *8*).

The molecular interactions between the melanocortin receptors and their peptide ligands have been elucidated in detail. (For extensive reviews see (*9*, *10*).) With respect to MC4R, extensive structure−activity relationship (SAR) studies of the endogenous ligands have identified amino acids involved in receptor interaction. The His^6-Phe^7-Arg^8-Trp^9 core sequence, conserved within the endogenous agonist peptides, is important for MC4 receptor interaction. α-MSH truncation studies identified the His^6-Phe^7-Arg^8-Trp^9 fragment as the minimal sequence required for detectable melanocortin receptor interaction (*11*−*14*). The identification of this small binding determinant within the peptides formed the basis for the development of a large array of cyclic peptides, which have provided highly useful templates for the design of agonist and antagonist peptides selective for individual melanocortin receptor subtypes (*9*, *10*, *15*). Some of these templates form more rigid structures that have allowed the determination of potential bioactive conformations, which have been used to optimize nonpeptide leads identified from high-throughput screening (*15*−*17*).

Substitution of the individual amino acids of the His^6-Phe^7-Arg^8-Trp^9 motif modifies receptor−ligand interaction (*9*, *10*, *15*, *18*). The effects of the substitutions can be dependent on the context of the peptide backbone, particularly in comparing linear and cyclic peptides (*9*). Within the endogenous agonist α-MSH, the substitution of each of the four core residues has demonstrated each to be involved in MC4R interaction (*19*−*23*), (reviewed in ref (*9*)). For

* Corresponding author. Phone: (858) 617-7600. Fax: (858) 617-7925. E-mail: bfleck@neurocrine.com.

‡ Department of Pharmacology.

§ Department of Medicinal Chemistry.

[1] Abbreviations: NDP-MSH, [Nle^4, D-Phe^7]α-melanocyte-stimulating hormone; hMC4R, human melanocortin-4 receptor; TM, transmembrane domain; WT, wild-type; SAR, structure−activity relationship; GPCR, G-protein-coupled receptor; cAMP, cyclic adenosine monophosphate; Tic, *R*-1,2,3,4-tetrahydroisoquinolin-3-ylcarbonyl; DPBS, Dulbecco's phosphate-buffered saline.

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example, the replacement of Phe^7 with D-Phe^7 substantially improves peptide potency (a substitution combined with Nle^4 to produce the potent and metabolically stable NDP-MSH (*19*, *24*)). Peptide SAR has been combined with site-directed mutagenesis of MC4R and molecular modeling to provide high-resolution molecular models of ligand orientation within the binding pocket (*9*, *13*, *25*, *26*). Two regions of MC4R have been identified as critical for peptide binding: (1) an acidic pocket formed by negatively charged residues in transmembrane domains (TM) 2 and 3; (2) a hydrophobic pocket or cage formed by aromatic or hydrophobic residues in TM4, TM6, and TM7 (*13*, *27*−*30*).

A recently published model describes NDP-MSH interaction with MC4R (*25*). In this model, D-Phe^7 and Trp^9 bind within the putative hydrophobic cage of the receptor and are in close proximity to W258$^{(6.48)}$, F261$^{(6.51)}$, H264$^{(6.54)}$, and F284$^{(7.35)}$ receptor residues, among others. His^6 and Arg^8 of the peptide face a different direction and interact with residues in TM2 and TM3. This model suggests that His^6 may interact with receptor residues E100$^{(2.60)}$ and D122$^{(3.25)}$, while Arg^8 may interact with D122$^{(3.25)}$ and D126$^{(3.29)}$.

While the molecular interactions involved in MC4R binding have been identified in detail, less is known regarding the mechanism of MC4R activation. Understanding the mechanisms by which some ligands activate the receptor while others do not is important for the development of agonists as potential treatments for obesity and antagonists for cachexia. Substitution of Phe^7 in α-MSH or cyclic analogues with halogenated or bulkier aromatic residues reduces MC4R activation, yielding antagonists (e.g., D-(4-Cl)Phe in the linear peptide SHU9005 (*30*, *31*), halogen substituents in the *R*-1,2,3,4-tetrahydroisoquinolin-3-ylcarbonyl (Tic)-D-Phe-Arg-Trp tetrapeptide (*32*), D-(4-I)Phe (SHU8914) and D-2′Nal (SHU9119) in the cyclic peptide MTII (*33*), and D-2′Nal in the small cyclic peptide MBP10 (*34*)). It has been proposed that this effect involves modified interaction with F254$^{(6.52)}$ and F259$^{(6.57)}$ residues within TM6 (mouse receptor) (*30*).

Beyond these findings, the role of binding interactions in MC4R activation has not been extensively investigated. Loss of bioactivity upon amino-acid substitution has been used to infer the role of a residue in receptor−ligand interaction, and in peptide SAR and receptor mutagenesis studies, this is typically quantified as an increase in EC_{50} in functional assays or an increase in IC_{50} or K_i in binding assays. By contrast, measuring the maximal signaling response (E_{max}) is necessary to investigate the role of ligand and receptor residues in receptor activation (*30*). The aim of this study was to further define the mechanism of MC4R activation by the linear peptide NDP-MSH. This peptide was used because it closely resembles the endogenous agonist α-MSH but binds more strongly to the receptor (*19*), allowing large reduction-of-function substitutions to be quantified accurately without the use of impractically high concentrations of ligand. This peptide was also chosen because it was utilized in a molecular model of the ligand-bound active state of MC4R (*25*), allowing us to experimentally test the inferences of the model. The experimental approach used was to first systematically evaluate the effect of His^6-D-Phe^7-Arg^8-Trp^9 substitutions on hMC4R activation, separating effects on binding affinity (K_i, EC_{50}) from those on activation (E_{max}). The substituted peptides were then evaluated on mutant receptors, and the findings were interpreted in the context of a molecular model to further define the role of specific receptor−ligand interactions in hMC4R activation.

EXPERIMENTAL PROCEDURES

Materials. [^{125}I]NDP-MSH was from Perkin-Elmer Life Sciences (Boston, MA) (specific activity of 2200 Ci/mmol). G418 (Geneticin), Dulbecco's phosphate-buffered saline (DPBS), and cell culture supplies were from Invitrogen (Carlsbad, CA). Fetal bovine serum was from HyClone (Logan, UT).

Peptide Synthesis. All peptides were synthesized by solid-phase methodology using the 4-methylbenzhydrylamine resin (Bachem California, Torrance, CA) on a model CS 536 peptide synthesizer (CS Bio Corp. San Carlos, CA). The synthetic method employed the use of *t*-butyloxycarbonyl protection, trifluoroacetic acid deprotection, and hydrogen fluoride cleavage of the finished peptide from the resin anchor. The derivatized amino acids were obtained from either Bachem California or Novabiochem (EMD Biosciences, Inc., San Diego, CA). The crude peptide product recovered from the cleavage reaction was purified on a KP 100 preparative high performance liquid chromatography (HPLC) system (Biotage, Charlottesville, VA) on a C18 cartridge, using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. The purity of the synthetic product was verified by analytical HPLC and its structure confirmed by mass spectrometric analysis on a SCIEX AP1 LC/MS system equipped with an electrospray ion source (Perkin-Elmer Corp. Norwalk, CT).

Construction of Mutant Receptors and Expression in HEK293 Cells. The hMC4R cDNA in pcDNA3.1 was used as the template for site-directed mutagenesis, using the QuikChange kit (Stratagene, La Jolla, CA) as previously described (*35*). Clones were sequenced using an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA), and clones containing the desired mutation were subcloned into the *Eco*RI/*Xho*I site of a fresh pcDNA3.1 vector. Complete receptor sequences were confirmed by DNA sequencing. Wild-type or mutant hMC4R plasmid cDNA was transfected into HEK293 cells using LipofectAMINE (Invitrogen) according to the manufacturer's protocol. Stable single cell clones were isolated after selection using 1 mg/mL G418 in Dulbecco's modified Eagle's medium, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 50 IU/mL penicillin, and 50 μg/mL streptomycin. Stable cell lines were maintained in medium containing 250 μg/mL G418.

Preparation of Cell Membranes. Cell membranes were prepared using a high-pressure nitrogen cell and differential centrifugation as previously described (*35*). The lysis buffer was DPBS, and the final membrane pellet was resuspended in binding assay buffer (25 mM HEPES, 1.5 mM $CaCl_2$, 1 mM $MgSO_4$, and 100 mM NaCl at pH 7.0). The protein concentration in the membrane pellet was determined using the Coomassie method (Pierce, Rockford, IL), using bovine serum albumin as the standard. Membranes were stored at −80 °C before use.

Radioligand Binding Assays. The binding affinity of unlabeled ligands for wild-type and mutant MC4 receptors

was measured by displacement of [^{125}I]NDP-MSH binding by 12 individual concentrations of ligand to generate dose−response curves. The following were added sequentially to low-binding 96-well plates (Corning, Palo Alto, CA) in binding assay buffer (see Preparation of Cell Membranes for the recipe): 50 μL of unlabeled ligand, 75 μL of [^{125}I]-NDP-MSH (0.2 nM, final concentration), and 75 μL of membrane suspension. The amount of membrane protein added per well was 6 μg for WT, 9 μg for F261$^{(6.51)}$A, and 20 μg for F284$^{(7.35)}$A. In each assay, total [^{125}I]NDP-MSH binding (obtained in the absence of unlabeled ligand) was less than 20% of the total radioligand added. The assay was incubated for 90 min at room temperature on a plate shaker (Titer Plate Shaker, setting 4, Lab-Line Instruments, Melrose Park, IL). Bound and free radioligands were then separated by rapid filtration, using UniFilter GF/C filters (Packard, Meriden, CT) pretreated with 0.1% polyethylenimine in DPBS, on a UniFilter-96 vacuum manifold (Packard). The filter was then washed three times with 0.2 mL/well 0.01% Triton X-100 in DPBS, then dried under electric fans for 40 min−1 h. Following the addition of scintillation fluid (50 μL per filter disc; Microscint 20, Packard), scintillation counts were measured in a Packard Topcount NXT. The CPM resulting from the emission of Auger electrons from ^{125}I was converted to DPM, using the predetermined counting efficiency of 30%. The total amount of radioligand added was measured using a Packard Cobra II gamma counter (78% efficiency).

Measurement of cAMP Accumulation. Cells were dislodged from tissue culture flasks and dissociated using enzyme-free cell dissociation buffer (Invitrogen, Carlsbad, CA). Cells were then isolated by centrifugation and resuspended in cAMP assay buffer (137 mM NaCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 0. 6 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 1 mM IBMX). Cells were then plated into low-binding white polystyrene 384-well plates (#3652, Corning, Palo Alto, CA) in a volume of 15 μL, using the following numbers of cells per well: 5,000 for the wild-type MC4 receptor and F261$^{(6.51)}$A receptor, and 4,000 for the F284$^{(7.35)}$A receptor. Peptide ligands diluted in cAMP assay buffer were then added in a volume of 5 μL, and the assay was incubated at room temperature for 30 min. For Schild analysis, NDP-MSH was diluted in buffer containing various concentrations of substituted peptides for simultaneous addition to cells. The accumulated cAMP was then measured with the LANCE cAMP kit (Perkin-Elmer Life and Analytical Sciences, Wellesley, MA), using the following volume of reagents per well: 10 μL of antibody (1:100 dilution) in lysis buffer with 0.1% (w/v) BSA; 10 μL of detection mix (detection buffer with 1:2,250 dilution of Eu-streptavadin and 1:750 dilution of biotin cAMP). Fluorescence was measured per the manufacturer's instructions using a ViewLux 1430 Ultra Microplate Imager (Perkin-Elmer Life and Analytical Sciences). All EC_{50} and E_{max} data were obtained using 12-point dose−response curves.

Data Analysis. Inhibition of [^{125}I]NDP-MSH binding by unlabeled peptides was fitted to a four parameter logistic equation to determine K_i using XL*fit* (ID Business Solutions Ltd., Emeryville, CA). In this analysis, K_i was determined from IC_{50} using the Cheng−Prusoff equation (*36*). The Hill slope factor in the analysis was allowed to float and fell between 0.8 and 1.2. Ligand-stimulated cAMP accumulation

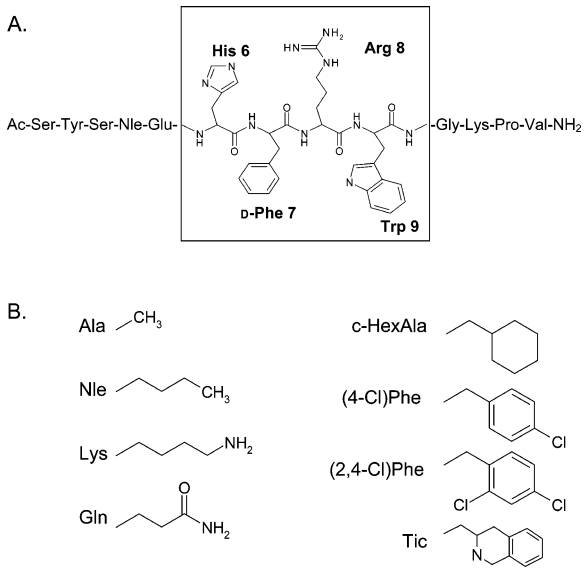

FIGURE 1: (A) Amino acid sequence/structure of NDP-MSH. Residues substituted in this study (His^6-D-Phe^7-Arg^8-Trp^9) are indicated inside the box. (B) Structures of various amino acid side chains used in this study. Tic is technically not a side chain since its nitrogen makes up part of the peptide backbone.

was analyzed using a four parameter logistic equation using GraphPad Prism 4.01 to provide an estimate of EC_{50}. The maximal amount of cAMP produced by NDP-MSH, represented by the top (plateau) of its dose−response curve, was determined for each experiment. This was defined as full stimulation (E_{max} = 100%) for that experiment, and the E_{max} values for other peptides (also defined as the top (plateau) of their dose−response curves) were calculated by dividing their specific cAMP accumulation by that for NDP-MSH. Statistical analysis of differences between values for all data was performed by analysis of variance (ANOVA) using GraphPad Prism 4.01, except for the difference between NDP-MSH and substituted peptide E_{max} on the WT receptor, which was determined using the one-sample *t*-test. For Schild analysis, the concentration of substituted peptide required to shift the EC_{50} of NDP-MSH by 2-fold was determined by plotting log((EC_{50}'/EC_{50})-1), where EC_{50}' is NDP-MSH EC_{50} in the presence of substituted peptide, on the *y*-axis versus log concentration of substituted peptide on the *x*-axis. The *x*-intercept determined by linear regression is an empirical estimate of the antagonist potency of the substituted peptide. The slopes of the Schild analysis linear regressions did not vary significantly from unity.

hMC4 Receptor Model. The hMC4R model with NDP-MSH bound has been previously described and was downloaded from the University of Michigan Peptide Synthesis and Molecular Recognition Lab website at http://mosberglab.phar.umich.edu/resources/index.php (*25*).

RESULTS

Peptides on Wild-Type Receptor. To investigate the molecular basis of hMC4R activation, we first examined the role of His^6, D-Phe^7, Arg^8, and Trp^9 residues of NDP-MSH in activation and binding assays (peptide **1**, NDP-MSH structure in Figure 1A). The residues were individually substituted to produce 16 analogue peptides (peptides **2**−**17**, substitution moieties in Figure 1B). These peptides were tested on the wild-type (WT) receptor using competition

Table 1: Peptide Affinity and Efficacy on Wild-Type hMC4 Receptor[a]

no.	peptide substitution				wild−type receptor		
					$K_i \pm$ SEM (nM)	$EC_{50} \pm$ SEM (nM)	$E_{max} \pm$ SEM (%)
1	NDP-MSH						
	His^6	D-Phe^7	Arg^8	Trp^9	0.79 ± 0.2	13 ± 2	100
2	Ala^6	—	—	—	1.4 ± 0.1	20 ± 6	41 ± 3 *
3	Phe^6	—	—	—	1.5 ± 0.2	25 ± 4	50 ± 2 *
4	Tic^6	—	—	—	0.28 ± 0.08	3.6 ± 0.3	60 ± 4 *
5	—	D-Ala^7	—	—	13,000 ± 2,000 *	10,000 ± 2,000 *	88 ± 10
6	—	D-(4-Cl)Phe^7	—	—	0.24 ± 0.03 *	3.4 ± 0.3 *	32 ± 2 *
7	—	D-(2,4-Cl)Phe^7	—	—	0.22 ± 0.03 *	6.4 ± 2	9 ± 3 *
8	—	—	Ala^8	—	110 ± 20 *	410 ± 20 *	88 ± 1 *
9	—	—	Nle^8	—	47 ± 1 *	500 ± 100 *	71 ± 5
10	—	—	Gln^8	—	29 ± 2 *	210 ± 20 *	98 ± 7
11	—	—	His^8	—	1.9 ± 0.1	11 ± 0.8	102 ± 1
12	—	—	Lys^8	—	2.7 ± 0.6	7.7 ± 0.3	88 ± 1 *
13	—	—	—	Ala^9	>30,000 *	>100,000 *	NP
14	—	—	—	Gln^9	>30,000 *	>100,000 *	NP
15	—	—	—	c-$HexAla^9$	120 ± 40 *	440 ± 70 *	88 ± 10
16	—	—	—	Phe^9	3.4 ± 0.2 *	18 ± 4	105 ± 5
17	—	—	—	(4-Cl)Phe^9	0.48 ± 0.04	1.9 ± 0.3 *	92 ± 9

[a] The K_i values were determined from competition binding against [^{125}I]-NDP-MSH on wild-type hMC4 receptor; data are the mean ± standard error from three independent experiments. The EC_{50} and E_{max} values were determined by agonist response in a cAMP assay; data are the mean ± standard error from three to four independent experiments. The maximal cAMP produced by NDP-MSH is defined as 100%, and the E_{max} values for all other peptides on that receptor are determined by comparison to that standard. E_{max} values could not be determined for curves that did not reach a distinct plateau and are indicated by NP. K_i, EC_{50}, and E_{max} values for substituted peptides that are significantly different from those for NDP-MSH ($p < 0.01$) are marked with an asterisk.

binding assays measuring the displacement of [^{125}I]NDP-MSH to determine affinity (K_i) and functional assays measuring the stimulation of cAMP accumulation to evaluate potency (EC_{50}) and efficacy (E_{max}) (Table 1). None of the peptides stimulated cAMP accumulation in untransfected HEK cells (data not shown), indicating that functional efficacy was mediated solely through the hMC4R.

His^6 has been shown to be important for MC4R interaction. Substitution with alanine in the His^6-D-Phe^7-Arg^8-Trp^9 tetrapeptide and other linear templates reduces the functional potency (EC_{50}) of MC4R activation (*21*, *37*). Substitutions in MTII and other cyclic peptide templates also reduce binding affinity (K_i) and functional potency (EC_{50}) (*34*, *38*−*41*). The role of His^6 in MC4R activation (E_{max}) is less well defined. His^6 may play a role in receptor activation in the context of cyclic templates (*40*, *41*). For example, substitution with proline or proline derivatives in the cyclic agonist MTII reduces MC4R activation (*39*, *42*). In this study on NDP-MSH, replacing the imidazole moiety with a methyl side chain by substituting His^6 of NDP-MSH with Ala^6 (**2,** Table 1) did not change the affinity (K_i) or functional potency (EC_{50}) of the peptide on the WT receptor, consistent with previous results (*43*), but did cause a large decrease in efficacy (E_{max} = 41%). This finding suggests that the imidazole side chain of His^6 is not essential for the binding of NDP-MSH to the hMC4R but is important for receptor activation. Peptide **2** was further tested for antagonist potency against NDP-MSH using Schild analysis and was found to have an empirical pA_2 (antagonist potency estimate) of 77 nM (Figure 2A). Increasing the concentration of peptide **2** causes a rightward shift of the NDP-MSH curve, suggesting that it competes with NDP-MSH, but further investigation would be needed to elucidate the mechanism causing the decrease in NDP-MSH E_{max}.

We also examined Phe (**3**) and Tic (**4**) substitutions at the 6-position because in nonpeptide ligands that bind in a similar orientation to NDP-MSH, these groups are thought to mimic His^6 (*16*, *25*, *44*). Similar to the Ala substitution, these substitutions had no effect on affinity or potency but caused a decrease in efficacy (40−50%, Table 1). The fact that neither an aromatic substituent (Phe) nor a substituent containing a restricted aromatic group (Tic) was sufficient to retain full efficacy suggests that the imidazole ring contains properties distinct from simply aromaticity that are important in NDP-MSH interaction with hMC4R. The nature of the distinction is presently unclear but may involve the basic nitrogen or hydrogen-bonding capacity.

Phe^7 is well known as a major contributor to the NDP-MSH/MC4R binding interaction, with L-Phe to D-Phe substitution increasing potency (*45*) and alanine substitution substantially reducing potency for MC4R (*13*, *20*), but the effect of alanine substitution in NDP-MSH on MC4R efficacy has not been reported. In this study, the D-Ala^7 substitution in NDP-MSH reduced potency and binding affinity (**5**, Table 1) but had minimal effect on the efficacy of the peptide (E_{max} = 88%, Table 1). (E_{max} was reliably determined using higher peptide concentrations (up to 100 μM) than those used in previous studies.) This finding indicates that the phenyl side chain of D-Phe^7 is essential for high-affinity binding but not for MC4R activation by NDP-MSH. While the phenyl side chain of D-Phe^7 is not required for NDP-MSH efficacy, modifying the benzene ring of the side chain by halogen substitution (*31*−*33*) or by increasing aromatic bulk (*33*, *34*) has been shown to decrease efficacy, demonstrating that modifications of the aromatic side chain at position 7 can affect receptor activation. In the present study, D-Phe^7 of NDP-MSH was substituted with D-(4-Cl)Phe^7 (**6**) and D-(2,4-Cl)Phe^7 (**7**) based on previous peptide and nonpeptide results showing that these substituents can reduce efficacy (*44*). 4-Chloro substitution of D-Phe^7 decreased efficacy (E_{max} = 32% for **6**, Table 1), and additional substitution at the 2-position further reduced

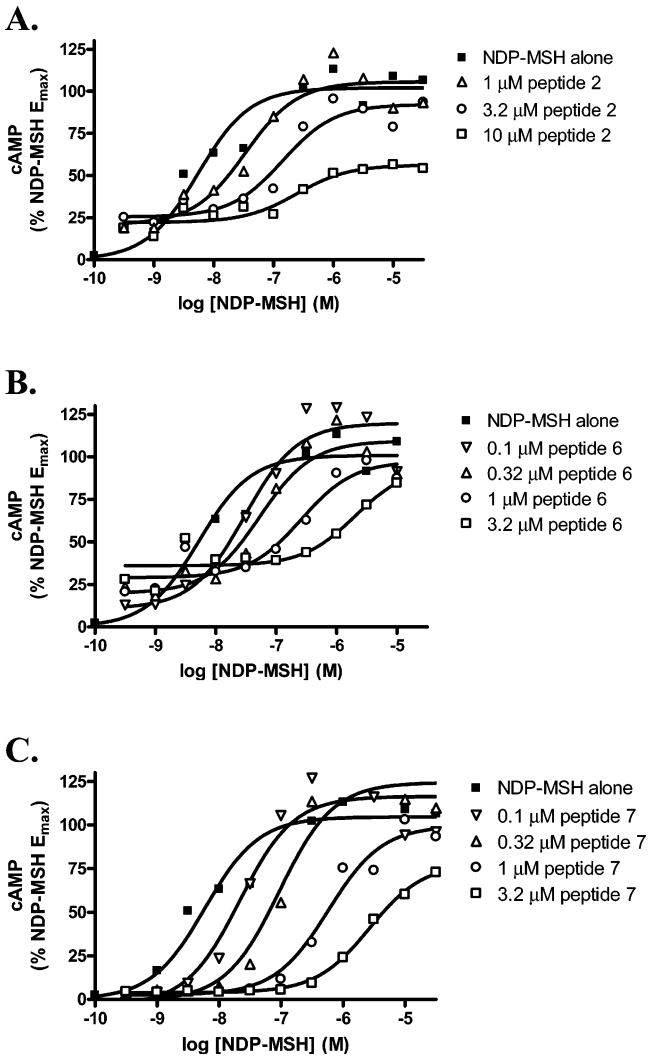

FIGURE 2: Schild analysis for partial agonist peptide antagonism of cAMP stimulated by NDP-MSH. Dose−response curves of NDP-MSH in the absence and presence of various concentrations of (A) peptide 2, Ala^6-NDP-MSH, (B) peptide 6, D-(4-Cl)Phe^7-NDP-MSH, and (C) peptide 7, D-(2,4-Cl)Phe^7-NDP-MSH. Increases in baseline are consistent with partial agonist activity (Table 1). Data are from representative experiments performed three times with similar results. Schild plots of the data were used to calculate an approximation of the log peptide concentration required to shift the EC_{50} of NDP-MSH by 2-fold: -7.11 ± 0.45 (SEM) for peptide 2, -7.57 ± 0.11 for peptide 6, and -7.57 ± 0.13 for peptide 7.

efficacy (9%, for **7**, Table 1), demonstrating that these chloro-substituted phenyl rings do not permit full receptor activation. These substitutions also slightly increased binding affinity (Table 1), consistent with the known role of Phe^7 as a major determinant of binding affinity (*9*, *19*). Schild analysis shows that the substituted peptides antagonize NDP-MSH stimulation of cAMP production, resulting in empirical pA_2 estimations (antagonist potency) of 27 nM for both peptide **6** and **7** (Figure 2B and C).

The Arg^8 residue of NDP-MSH is thought to contribute binding energy to the interaction with the TM2 and TM3 regions of the hMC4R (*13*), but Ala^8 substitutions in different peptide templates have not been shown to decrease E_{max} values (other than shifting the EC_{50} curve to the right so that a true E_{max} cannot be determined), suggesting that Arg^8 may not play a specific role in the receptor activation event (*23*, *37*, *41*, *46*). Consistent with this hypothesis, Arg^8 substitution with alanine did not appreciably affect the efficacy of NDP-MSH (**8**, E_{max} of 88%, Table 1), suggesting that the side chain of Arg^8 is not essential for MC4R activation. The substitution did reduce binding affinity (140-fold; Table 1). Several hypotheses have been proposed to explain this affinity-reducing effect: (1) the positive charge contributes to an ionic interaction with $D122^{(3.25)}$ and $D126^{(3.29)}$ (*13*); (2) the guanidine moiety is involved in a hydrogen-bonding interaction (*37*); and (3) the spatial orientation of the hydrophobic side chains is more important than the presence of nitrogen groups since many potent nonpeptide ligands lack a basic residue capable of mimicking Arg (*9*). Further Arg^8 substitutions in the present study were selected to elucidate the important characteristics of arginine in the binding affinity of NDP-MSH interaction with hMC4R. The aliphatic substitution, Nle^8 (**9**), produced a considerable decrease in affinity (60-fold), suggesting that the spatial orientation of the Nle side chain may not be sufficient to replace the binding energy of Arg^8 as Nle fills a space similar to the lipophilic portion of the Arg side chain without the potential for ionic interaction or hydrogen bonding. Substitution with glutamine (**10**), which allows for hydrogen bonding but not ionic interactions with the receptor, resulted in a 37-fold decrease in affinity. The substitutions retaining a basic group, His^8 (**11**) and Lys^8 (**12**), had minimal effect on both affinity and efficacy. These results establish that the basicity of Arg^8 of NDP-MSH is an important determinant of the affinity of NDP-MSH interaction, potentially through an ionic interaction. None of these additional substitutions appreciably affected efficacy (Table 1), consistent with the hypothesis that the Arg^8 side chain is not a major determinant of the MC4R activation event.

The Trp^9 residue has been shown to be crucial for peptide binding to hMC1R, as substitution with alanine in the α-MSH peptide results in a 2000-fold decrease in affinity and over 100-fold decrease in potency (*18*). Data from γ-MSH and MTII, a cyclic peptide derived from the 4−10 fragment of NDP-MSH, on MC3, MC4, and MC5 receptors suggest that Trp^9 may also be involved in receptor activation, though the observed lack of efficacy in these studies may have resulted from testing compound concentrations that were inadequate to elicit a full response (*46*, *47*). Additionally, it is not clear whether aromatic interactions, lipophilicity, or hydrogen-bonding plays an important role in the interaction. In the present study, [Trp^9]NDP-MSH substitutions were chosen to investigate the nature of its interaction with the receptor and whether it is involved in receptor activation. The Ala^9 (**13**) substitution resulted in a loss of binding affinity greater than 4 orders of magnitude (Table 1), confirming that the side chain of Trp^9 is crucial for high affinity binding (*13*). The Gln^9 (**14**) substitution has the potential for hydrogen bonding but showed a loss of affinity similar to that of the alanine substitution (Table 1). With both the Ala^9 and Gln^9 substitutions, it was not possible to determine efficacy because curves did not reach a plateau at the highest concentrations tested. The bulky lipophilic amino acid derivative c-$HexAla^9$ (**15**) produced a large decrease in affinity (150-fold) but still retained efficacy, while the phenyl analogues (**16** and **17**) were similar to the parent peptide in both affinity and efficacy. This implies that the aromatic nature of Trp^9 is critical for binding, while

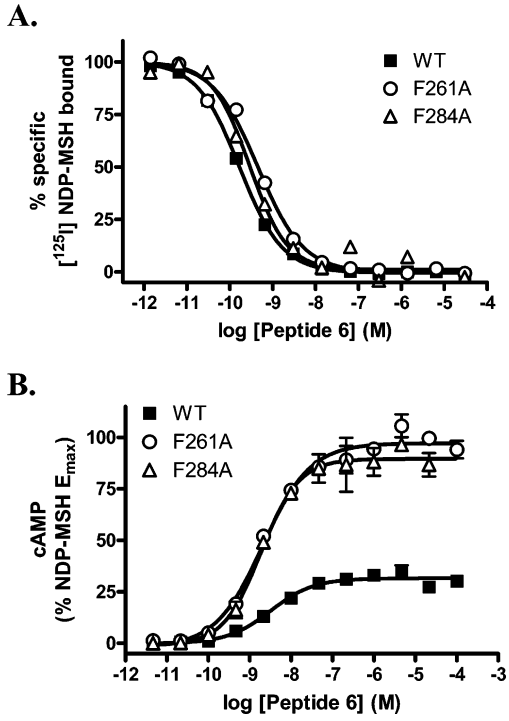

FIGURE 3: Effect of F261$^{(6.51)}$A and F284$^{(7.35)}$A hMC4 receptor mutations on the pharmacology of peptide 6 (D-(4-Cl)Phe^7-NDP-MSH). (A) Binding was measured by displacement of [^{125}I]NDP-MSH binding to membranes from HEK293 cells transfected with the WT, F261$^{(6.51)}$A, or F284$^{(7.35)}$A mutant hMC4 receptors. (B) Accumulation of cAMP production in the same cells was measured and analyzed as described in Experimental Procedures. Data are from representative experiments performed 3−6 times with similar results. F261$^{(6.51)}$A and F284$^{(7.35)}$A receptor mutations do not affect the affinity of peptide 6 but increase its efficacy.

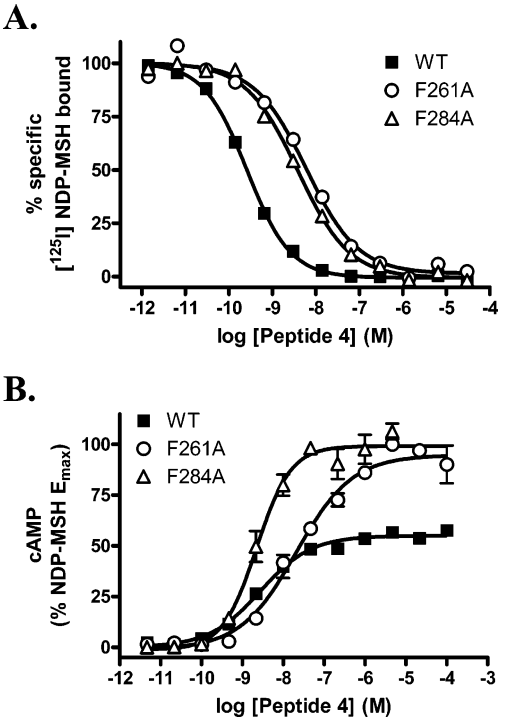

FIGURE 4: Effect of F261$^{(6.51)}$A and F284$^{(7.35)}$A hMC4 receptor mutations on the pharmacology of peptide 4 (Tic^6-NDP-MSH). (A) Binding was measured by the displacement of [^{125}I]NDP-MSH binding to membranes from HEK293 cells transfected with the WT, F261$^{(6.51)}$A, or F284$^{(7.35)}$A mutant hMC4 receptors. (B) Accumulation of cAMP production in the same cells was measured and analyzed as described in Experimental Procedures. Data are from representative experiments performed 3−6 times with similar results. Receptor mutations decrease the affinity of peptide 4 but increase its efficacy.

lipophilicity also plays a role. For the compounds where an E_{max} could be determined, there were no significant decreases in efficacy, suggesting that the Trp^9 interaction is minimally involved in activation.

Peptides on Mutant Receptors. Exploring the effects of the peptide substitutions on the WT hMC4R provided new insights into the nature of NDP-MSH binding and receptor activation. We were especially interested in the His^6 and D-Phe^7 peptide substitutions that were found to decrease efficacy and aimed to further define their interaction with the receptor and the mechanism by which they modulate efficacy. Previous studies have shown that the F261$^{(6.51)}$ and F284$^{(7.35)}$ receptor residues are involved in receptor−ligand interaction and receptor activation for both peptide and nonpeptide ligands (*13*, *25*, *29*, *30*, *35*). The mutation of these residues in the putative hydrophobic cage to alanine can increase the efficacy of both partial agonist and antagonist nonpeptide ligands (*35*). However, these mutations do not appear to be global enhancers of efficacy because some small molecule hMC4R antagonists do not gain any agonist activity on the mutant receptors despite high affinity binding (benzamidine-1 (*35*), from the benzamidine chemical series (*48*), and 12i (*49*)).

To investigate whether these receptor residues also play a role in NDP-MSH efficacy, the His^6 and D-Phe^7 substituted peptides causing a decrease in efficacy on the WT receptor were tested on the F261$^{(6.51)}$ and F284$^{(7.35)}$ mutant receptors (Figure 3, Figure 4, Table 2, and Table 3). These mutant receptors have been previously characterized, establishing

Table 2: Changes in Functional Efficacy Due to Receptor Mutations[a]

peptide		F261A receptor		F284A receptor	
no.	substitution	E_{max} ± SEM (%)	$E_{max(mut)}$ − $E_{max\ (WT)}$	E_{max} ± SEM (%)	$E_{max(mut)}$ − $E_{max\ (WT)}$
2	Ala^6	76 ± 15 *	(+35)	71 ± 4 *	(+30)
3	Phe^6	70 ± 6 *	(+20)	75 ± 4 *	(+25)
4	Tic^6	88 ± 10 *	(+28)	92 ± 2 *	(+32)
6	D-(4-Cl)Phe^7	88 ± 9 *	(+56)	82 ± 2 *	(+50)
7	D-(2,4-Cl)Phe^7	97 ± 9 *	(+88)	30 ± 9 *	(+21)

[a] Maximal cAMP produced by NDP-MSH (peptide **1**) is defined as 100% for each receptor, and the E_{max} values for all other peptides on that receptor are determined by comparison to that standard. The E_{max} values on the mutant receptors that are significantly larger ($p < 0.05$) than E_{max} on the wild-type receptor are marked with an asterisk. The difference between E_{max} on WT receptor and E_{max} on mutant receptor is indicated in parentheses. Data are the mean ± standard error from three to six independent experiments.

that their functional receptor expression is similar to that of WT (B_{max} = 0.72−2.0 pmol/mg) and that the affinity, potency, and efficacy of NDP-MSH are not significantly different between WT and mutant receptors, similar to previously published F261$^{(6.51)}$A data (*13*), thus demonstrating that the mutations do not dramatically alter receptor structure (*35*).

When the D-Phe^7 chlorophenyl and dichlorophenyl substituted peptides (**6** and **7**) were tested on the F261$^{(6.51)}$A and F284$^{(7.35)}$A receptors, we observed no change in their affinities compared to that of the WT receptor (Figure 3A

Table 3: Changes in Peptide Binding Affinity Due to Receptor Mutations[a]

peptide		F261A receptor		F284A receptor	
no.	substitution	$K_i \pm$ SEM (nM)	$K_{i(mut)}/K_{i\,(WT)}$	$K_i \pm$ SEM (nM)	$K_{i(mut)}/K_{i\,(WT)}$
1	NDP-MSH	1.2 ± 0.5	1.5	1.1 ± 0.2	1.4
2	Ala^6	50 ± 20 *	36	100 ± 9 *	71
3	Phe^6	190 ± 30 *	127	120 ± 10 *	80
4	Tic^6	3.5 ± 1 *	13	2.2 ± 0.4 *	8
6	D-(4-Cl)Phe^7	0.40 ± 0.08	1.7	0.28 ± 0.07	1.2
7	D-(2,4-Cl)Phe^7	0.31 ± 0.04	1.4	0.30 ± 0.04	1.4

[a] The K_i values were determined from competition binding against [^{125}I]NDP-MSH on WT and mutant hMC4 receptors and averaged from three to five independent experiments. The differences between K_i values on WT and mutant receptors and are calculated by (K_i, mut)/(K_i, WT). The K_i values on the mutant receptor that are significantly different from those on the WT receptor ($p < 0.01$) are marked with an asterisk.

and Table 3), indicating that neither receptor residue is crucial to peptide binding affinity. However, these two receptor mutations did result in significant increases in the efficacy of the two substituted peptides (Table 2). Peptide **6** displayed an E_{max} of 32% on the WT receptor (Table 1) but increased to near full efficacy on the F261$^{(6.51)}$A and F284$^{(7.35)}$A receptors (Figure 3B, Table 2). Similarly, peptide **7** had only 9% efficacy compared to that of NDP-MSH on the WT receptor (Table 1) but achieved nearly full efficacy with the F261$^{(6.51)}$A mutation and a lower but still statistically significant increase ($p < 0.05$) with the F284$^{(7.35)}$A mutation (Table 2). These results indicate that the hydrophobic cage residues F261$^{(6.51)}$ on TM6 and F284$^{(7.35)}$ on TM7 can play a role in modulating peptide efficacy.

This conclusion is also supported by functional assay results from the His^6-substituted peptides (**2**−**4**) on the F261$^{(6.51)}$A and F284$^{(7.35)}$A mutant receptors. The F261$^{(6.51)}$A and F284$^{(7.35)}$A receptor mutations increased the efficacies of the His^6-substituted peptides (Table 2) compared to that of the WT receptor. On the WT receptor, the Ala^6 and Phe^6 peptides both displayed nearly half-maximal efficacy levels, but on the F261$^{(6.51)}$A and F284$^{(7.35)}$A mutant receptors, the efficacy of these peptides increased to 70% or greater (Table 2). We also saw the same trend with the Tic^6 substitution, where efficacy levels were only 60% on the WT receptor but near full efficacy on F261$^{(6.51)}$A and F284$^{(7.35)}$A mutant receptors (Figure 4B and Table 2). While both receptor mutations caused increased efficacy, they also resulted in decreased affinity for the His^6-substituted peptides, shifting the K_i values approximately 10−100-fold (Figure 4A and Table 3). Therefore, both of these receptor residues contribute binding energy to the His^6-substituted peptide interactions with the receptor. However, neither of these receptor mutations changes the affinity of NDP-MSH, consistent with the His^6-substituted peptides having a slightly different orientation in the receptor binding pocket compared to that of NDP-MSH.

Model of NDP-MSH D*-Phe^7 Interaction with the hMC4 Receptor*. To rationalize how the His^6 and D-Phe^7 peptide residues and the F261$^{(6.51)}$ and F284$^{(7.35)}$ receptor residues interact to modulate receptor activation, we utilized a molecular model of NDP-MSH binding to the MC4 receptor (*25*). First, we examined D-Phe^7 interaction with the receptor. During hMC4R activation, TM6 undergoes counterclockwise

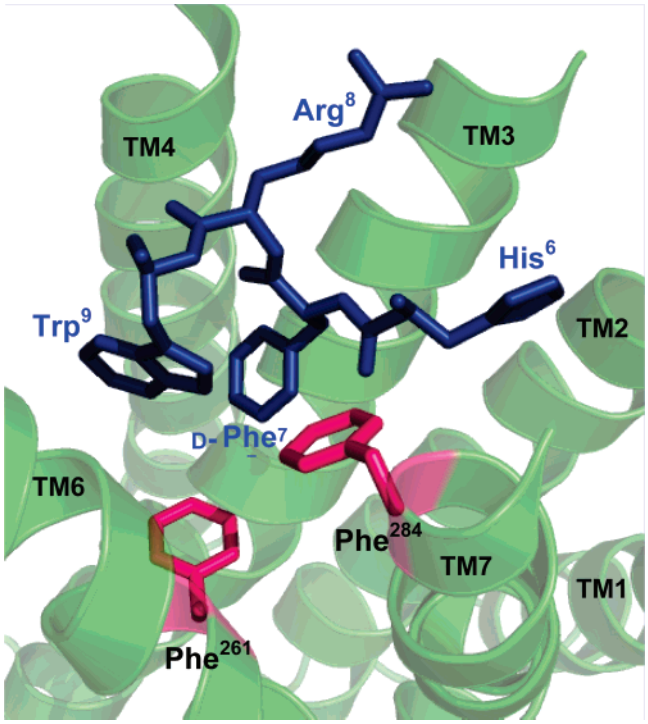

FIGURE 5: Computer-aided representation of NDP-MSH interaction with the active conformation of the hMC4 receptor (Pogozheva's model) (*25*) magnified to show only the His^6-D-Phe^7-Arg^8-Trp^9 motif of the peptide (blue). The remaining residues were removed for better visualization. Phe^{261} (TM6) and Phe^{284} (TM7) receptor residues are indicated in pink.

rotation, bringing H264 into the binding pocket and shifting the position of F261$^{(6.51)}$, F262$^{(6.52)}$, L265$^{(6.55)}$, and Y268$^{(6.58)}$ (*25*). According to NDP-MSH docking in the hMC4R model, D-Phe^7 is positioned within the hydrophobic cage of the receptor, formed by residues in TM6 and TM7 (Figure 5). D-Phe^7 allows for unrestricted rotation of TM6, resulting in full activation of the receptor upon NDP-MSH binding. Chlorophenyl and dichlorophenyl substitutions at position 7 (**6** and **7**) significantly reduce efficacy (Table 1), indicating that these substitutions interfere with receptor activation. Upon F261$^{(6.51)}$ or F284$^{(7.35)}$ mutation to alanine, however, we observe significant increases in the efficacies of these substituted peptides (Table 2). It is likely that the presence of the chloro groups on the phenyl ring of D-Phe^7 blocks the counterclockwise rotation of TM6 by means of steric hindrance through interaction with residues in the hydrophobic cage, resulting in the decreased efficacy seen on the wild-type receptor. When F261$^{(6.51)}$ or F284$^{(7.35)}$ is mutated to an alanine residue, TM6 rotation is no longer spatially constrained by the chloro groups and can freely rotate, allowing full receptor activation. This reversal of steric hindrance can result from the mutation of side chains in TM6 as well as TM7.

Neither the F261$^{(6.51)}$A nor the F284$^{(7.35)}$A receptor mutations affect the affinity of the D-Phe^7 substituted peptides (Table 3), indicating that neither receptor residue contributes significant binding energy to the interactions. This suggests that the modulation of receptor activation by relieving spatial constraints within the hydrophobic pocket does not require modifying an interaction that contributes to peptide affinity.

Model of NDP-MSH His^6 Interaction with the hMC4 Receptor. F261$^{(6.51)}$A and F284$^{(7.35)}$A receptor mutations can

Table 4: Affinity or Potency Changes Due to Alanine Substitution in Various Peptide Templates[a]

peptide template	fold shift in affinity or potency due to alanine substitution			
	His	Phe	Arg	Trp
NDP-MSH	1.8	16,000	140	>38,000
Ac-His-D-Phe-Arg-Trp	58[b]	>5,800[c]	1,300[d]	2,500[e]
Ac-Nle^4-c[Asp^5-His^6-D-Phe^7-Arg^8-Trp^9-Lys^{10}]-NH_2 (MTII)[f]	10	>100,000	3,700	>100,000
(O)C−$(CH_2)_2$-C(O)-c[His-D-Phe-Arg-Trp-Lys]-NH_2[g]	14	>6,300	>630	NR
Bu-His-D-Phe-Arg-Trp-Gly-NH_2	6[h]	NR	NR	NR

[a] The fold shift in affinity or potency due to alanine substitution is calculated by (K_i, substituted peptide)/(K_i, unsubstituted peptide) or (EC_{50}, substituted peptide)/(EC_{50}, unsubstituted peptide). [b] Ref *21*. [c] Ref *20*. [d] Ref *23*. [e] Ref *22*. [f] Ref *46*. [g] Ref *41*. [h] Ref *40*.

also recover the loss of efficacy caused by His^6 peptide substitutions (Figure 4B and Table 2). However, instead of interacting with F261$^{(6.51)}$ and F284$^{(7.35)}$ in the hydrophobic cage, His^6 is believed to face toward TM2 and TM3 (Figure 5) and interact with D122$^{(3.25)}$ and D126$^{(3.29)}$. We propose that the His^6-substituted peptides are oriented differently than NDP-MSH within the binding pocket of the receptor because of a modified or weakened interaction with D122$^{(3.25)}$ and D126$^{(3.29)}$. This modified orientation results in a stronger interaction with F261$^{(6.51)}$ and F284$^{(7.35)}$ in the hydrophobic pocket, thereby interfering with the rotation of TM6 and preventing receptor activation. When F261$^{(6.51)}$ or F284$^{(7.35)}$ is mutated to alanine, the interaction that prevents TM6 rotation is lost, and receptor activation can occur. This theory is supported by the affinity changes we have observed. F261$^{(6.51)}$A and F284$^{(7.35)}$A receptor mutations do not change the affinity of NDP-MSH but decrease the affinity of the His^6-substituted peptides, demonstrating that F261$^{(6.51)}$ and F284$^{(7.35)}$ have a stronger interaction with the His^6-substituted peptides than with NDP-MSH (Table 3).

DISCUSSION

There have been a large number of studies looking at the roles of melanocortin peptide residues interacting with the MC4R, and seemingly inconsistent data in the literature may result from the use of different peptide templates, receptor subtypes, and species (*9*, *43*). The first aim of this study was to further explore the role of the His^6, D-Phe^7, Arg^8, and Trp^9 residues, specifically within the NDP-MSH peptide, in hMC4 receptor binding and activation using modified peptides. We found that neither the Arg^8 nor the Trp^9 side chain is essential for the receptor activation event, but the basicity of Arg and the aromatic and lipophilic characteristics of Trp appear to contribute to binding affinity. Similarly, the aromatic side chain of D-Phe^7 contributes significant binding energy but is not essential for receptor activation. However, adding chlorine substituents to the aromatic side chain at this position interferes with receptor activation. The imidazole group of His^6 is the only side chain of the four residues that is not important for NDP-MSH binding affinity but is crucial in receptor activation.

The effects of the core tetrapeptide substitutions on the binding affinity of NDP-MSH are in reasonable agreement with those reported previously (*13*). Generally, alanine substitutions in NDP-MSH were better tolerated in terms of binding affinity than substitutions in the linear Ac-His-D-Phe-Arg-Trp-NH_2 tetrapeptide and Bu-His-D-Phe-Arg-Trp-Gly-NH_2 pentapeptide (Table 4 and refs therein). One potential explanation for this finding is that the additional amino acids of NDP-MSH form additional receptor interactions that stabilize the receptor−ligand interaction. Alanine substitutions were also generally better tolerated in NDP-MSH than in the cyclic templates (Table 4 and refs therein), potentially because of the greater flexibility of the linear peptide, which allows for compensating receptor interactions upon substitution (as suggested for the [His^6]NDP-MSH substitutions in this study). In terms of efficacy, template-dependent effects of the Phe^7 position are apparent. For example, D-(4-Cl)Phe^7 substitution in NDP-MSH substantially reduces E_{max} but does not appreciably affect MC4R activation by the cyclic MTII (*33*). These differences may be a result of a subtly different orientation of the cyclic peptides within the binding pocket.

The second aim of this study was to further explore the mechanism of receptor activation by examining the interaction of His^6 and D-Phe^7 with the receptor using site-directed mutagenesis and molecular modeling. The mechanism of GPCR activation has not been completely elucidated, but there is evidence that it is conserved across class A and B receptors (*50*). From numerous studies on rhodopsin (*51*−*53*), β_2-adrenergic (*54*), and PTH receptors (*55*), it appears that TM6, TM3, and perhaps TM7 play a critical role in the transition to the active state. Fluorescence spectroscopic techniques reveal that in both rhodopsin and β_2-adrenergic receptors (*51*, *53*), TM6 undergoes significant counterclockwise movement during transition (viewed from the extracellular side of the receptor) that is crucial to activation. Pogozheva and co-workers have modeled both the active and inactive states of hMC4R and propose that similar structural changes accompany MC4 receptor activation (*25*). TM6 shifts outward and undergoes counterclockwise movement, changing the position of F261$^{(6.51)}$, F262$^{(6.52)}$, L265$^{(6.55)}$, and Y268$^{(6.58)}$ and bringing H264$^{(6.52)}$ into the binding pocket.

Different groups have proposed that large aromatic side chain substitutions at the Phe^7 position can interfere with MC4R activation by interacting with receptor residues within TM6, physically hindering the conformational changes necessary to elicit full efficacy. The specific residues implicated were W258$^{(6.48)}$ in the human receptor and F254$^{(6.52)}$ and F259$^{(6.57)}$ in the mouse receptor (analogous to the human F262$^{(6.52)}$ and F267$^{(6.57)}$ residues) (*25*, *30*, *33*). In the mouse receptor studies, the antagonist peptide SHU9005, an NDP-MSH analogue where D-Phe^7 is replaced by D-(4-I)Phe^7, is converted to a partial agonist by F254S$^{(6.52)}$ and F259S$^{(6.57)}$ receptor mutations. This led to the hypothesis that these wild-type receptor residues may be interacting with the side chains at position 7 of the peptide and sterically hindering the rotation of TM6, resulting in decreased receptor activation (antagonism). When the receptor residues are mutated so that the steric hindrance is relieved, receptor

activation is restored. This hypothesis is strongly supported by the findings of the present study and positioning of the peptide within the molecular model and led us to conclude that the loss of efficacy we see with His^6 and D-Phe^7 substitutions is caused by steric hindrance to the rotation of TM6, without which the receptor cannot form the fully active state. The $F261^{(6.51)}A$ and $F284^{(7.35)}A$ receptor mutations relieve this steric hindrance and result in increased efficacy for these peptides.

In this study, we have uncovered unique receptor residues that can play a role in determining peptide efficacy. One residue, $F261^{(6.51)}$, is located in TM6 like the residues in the above-mentioned studies. We also discovered that $F284^{(7.35)}$ in TM7 can be involved in regulating receptor activation. This is the first demonstration that residues in TM7 may affect efficacy through steric hindrance. Since residues in either TM6 or TM7 can relieve steric hindrance within the hydrophobic pocket, allowing TM6 rotation and restoring efficacy, it suggests that no single interaction with a specific residue can be fully responsible for stabilization of the inactive state.

The Phe^7 residue has received considerable attention in peptide SAR focused on developing antagonists. The findings of this and other studies (*39*, *42*) indicate that His^6 could also be targeted to develop antagonist peptides. Substitution with proline in a modified MTII template reduces MC4R E_{max} (PG-975, (*39*)), substitution with the proline derivative 4-*R*-benzyloxy-L-proline in MTII generates a potent MC4R antagonist (PG103, (*42*)), and substitution with Ala^6, Phe^6, and Tic^6 in NDP-MSH reduces MC4R activation (Table 1). In the context of NDP-MSH, we propose that the reduction of MC4R activation results from an indirect effect, in which His^6 substitution weakens receptor interaction with the 6-position residue, which faces TM3, and strengthens the interaction of the peptide with residues within the hydrophobic pocket. This hypothesis is supported by the effect of $F261^{(6.51)}A$ and $F284^{(7.35)}A$ receptor mutations, which reduce the affinity of the Ala^6-, Phe^6-, and Tic^6-substituted analogues but do not affect the affinity of the His^6-bearing NDP-MSH (Table 3). Increasing the strength of peptide interaction with the hydrophobic cage could impair the rotation of TM6, hindering the MC4R activation event. Schild analysis results also show that [Ala^6]NDP-MSH is a functional antagonist of the NDP-MSH response. In short, His^6 substituents could indirectly utilize the same molecular mechanism directly exploited in Phe^7 SAR to develop useful antagonist ligands. The extent to which this mechanism could explain the antagonizing effect of proline substitutions in cyclic templates (*39*, *42*) remains to be determined.

The aim of this study focuses on receptor activation by the NDP-MSH peptide, but the conclusions may also be applicable to nonpeptides. The peptide data from the present study are supported by nonpeptide results (*35*) in which halogenated aromatic groups, D-(4-Cl)Phe, and D-(2,4-Cl)-Phe, mimic D-Phe^7 of NDP-MSH and are positioned similarly within the hMC4R binding pocket. On the WT receptor, both compounds have high affinity, but the D-(4-Cl)Phe compound has poor efficacy (E_{max} = 28%), and the D-(2,4-Cl)Phe compound does not stimulate the receptor at all. When the compounds interact with the $F261^{(6.51)}A$ mutant receptor, there is a significant increase in efficacy compared to that of the WT receptor, suggesting that both peptides and nonpeptides may stabilize an inactive conformation of the receptor by interacting with $F261^{(6.51)}$ and hindering the rotation of TM6. More experiments would be needed to establish whether other residues are also involved.

Furthermore, these nonpeptide studies have shown that the $F261^{(6.51)}A$ mutation does not affect the efficacy of all ligands. Benzamidine-1 (*35*), from the benzamidine chemical series (*48*), has no agonist activity on the wild-type, $F261^{(6.51)}A$, or $F284^{(7.35)}A$ MC4 receptors. Similarly the small molecule antagonist 12i (*49*) is unaffected by the mutations (data not shown). This suggests that the efficacy changes brought about by these receptor mutations involve specific receptor−ligand interactions and are not changes that globally affect receptor activation.

By applying an existing model to our results, we have tested a mechanism to explain the changes in efficacy resulting from both NDP-MSH peptide and hMC4 receptor mutations. Our data support a proposed theory that loss of agonist activity can be caused by sterically hindering TM6 rotation within the hydrophobic cage of the receptor. Closely related peptides can therefore have different efficacies depending on their interaction with residues in the hydrophobic cage. This information can be applied to the development of agonists for obesity and antagonists for cachexia. Antagonists can be specifically designed to obstruct the rotation of TM6, while agonists should avoid large substituents in the hydrophobic pocket. Furthermore, both the $F261^{(6.51)}A$ (TM6) and $F284^{(7.35)}A$ (TM7) receptor mutations relieve steric hindrance within the hydrophobic pocket, allowing TM6 rotation and restoring efficacy. This demonstrates that for the peptides tested in this study, no single interaction with a specific residue can be fully responsible for the stabilization of the inactive state. Additional experiments will need to be done to further test this hypothesis and determine its application to other receptor systems.

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