Molecular Mechanism of the Intracellular Segments of the Melanocortin-4 Receptor for NDP−MSH Signaling

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ABSTRACT: Mutations of the human melanocortin-4 receptor (hMC4R) have been previously identified to be the most common cause of monogenic human obesity. Specifically, mutations of the intracellular C terminus and the third intracellular loop of hMC4R have been reported to play an important role in human obesity. However, the molecular basis of these hMC4R intracellular segments in receptor function remains unclear. In this study, we utilized deletions and mutations of specific portions of the hMC4R to determine the molecular mechanism of both the C terminus and the third intracellular loop in receptor signaling. Our results indicate that deletions of the distal 25 (the entire C terminus), 22, 18, 17, 16, and 15 amino acids of the C terminus result in the complete loss of both [Nle^4-D-Phe^7]-α-melanocyte stimulating hormone (NDP−MSH) binding and NDP−MSH-mediated cAMP production. Deletion of the distal 14 amino acids of the C terminus significantly decreases both NDP−MSH binding affinity and potency, but deletion of the distal 13 amino acids of the C terminus does not affect NDP−MSH activity. Further analysis revealed that the proximal 12 amino acids of the C terminus are not only important for receptor signaling but also important for ligand binding. Our results also indicate that the third intracellular loop of the hMC4R is important for receptor signaling but not ligand binding. In summary, our findings suggest that the proximal region of the melanocortin-4 receptor (MC4R) C terminus is crucial not only for receptor signaling but also for ligand binding, while the third intracellular loop is important mainly for receptor signaling.

Obesity has become one of the most significant public health problems facing the world today (*1*−*3*). Obese humans are facing an increased risk of mortality and morbidity because of obesity-associated diseases such as type-II diabetes mellitus, hypertension, stroke, coronary artery diseases, and cancer (*3*−*5*). In recent years, researchers have produced exciting new insights into the physiological systems of the hypothalamus governing metabolic, appetite, and energy expenditure signaling (*6*−*12*). The melanocortin-4 receptor (MC4R) has been identified to play a key role in the regulation of body weight (*13*−*18*). Mutations of MC4R have been identified as the most common monogenic cause of human obesity described to date, accounting for up to 6% of all cases of severe obesity (*19*−*21*). Known human melanocortin-4 receptor (hMC4R)[1] mutations associated with human obesity are located throughout the receptor, including the extracellular loops, the transmembrane domains (TMs), and the intracellular loops (*22*). A better understanding of the molecular basis of hMC4R function is therefore critical to understanding the development of human obesity and to guide the development of effective therapeutic strategies for its treatment.

Identification of receptor-specific determinants for G-protein coupled receptor (GPCR) signaling has been the subject of intense investigation, but the identity of a conserved GPCR-specific consensus motif responsible for receptor signaling has not been obtained. Numerous studies have indicated that the C-terminal tails of some GPCRs are involved in G-protein coupling but not important for ligand binding because deletion of their C termini abolished receptor signaling but maintained normal ligand binding (*23*−*26*). However, in other cases, the C termini of receptors are not necessary for agonist-dependent G-protein coupling and signaling (*27*). Therefore, to date, it would rather seem that multiple receptor domains contribute to GPCR-specific differences in receptor function. hMC4R is a member of the family of GPCRs, and it consists of a single polypeptide featuring seven α-helical TMs, an extracellular N terminus, three extracellular loops, three intracellular loops, and an intracellular C terminus (Figure 1). hMC4R transduces a signal by coupling to the heterotrimeric G_s protein, thereby activating adenylate cyclase. The C terminus of hMC4R contains a dihydrophobic sequence composed of leucine, isoleucine, proline, phenylalanine, arginine, lysine, threonine, and cysteine, preceded by an acidic glutamate or aspartate residues (*28*). This sequence is highly conserved within the melanocortin receptor (MCR) family. It has been reported that mutation of the conserved isoleucine (I) motif in the C terminus of hMC4R is found in select cases of human obesity (*29*). In addition, deletion of the entire C terminus and partial

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[1] Abbreviations: MCR, melanocortin receptor; hMC4R, human melanocortin-4 receptor; GPCR, G-protein coupled receptor; NDP−MSH, [Nle^4-D-Phe^7]-α-melanocyte stimulating hormone; TM, transmembrane domain; AGRP, agouti-related protein; IBMX, 3-isobutylmethylxanthine; PCR, polymerase chain reaction; FACs, fluorescence-activated cell sorting.

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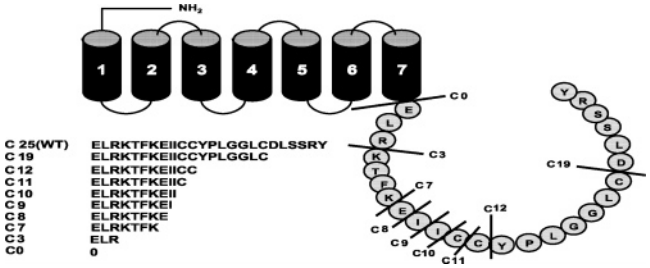

FIGURE 1: Schematic representation of the truncated hMC4R utilized in these studies depicts the seven transmembrane domain structures of the WT MC4R and sequences of the deletions of the C terminus.

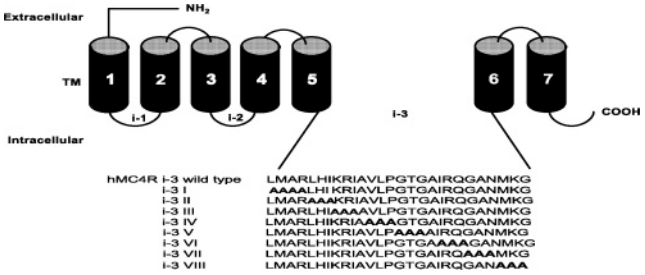

FIGURE 2: Schematic representation of the hMC4R third intracellular loop mutations utilized in these studies depicts the seven transmembrane domain structure of the WT MC4R and the sequence of the intracellular loop mutations of MC4R.

seventh TM has been reported to result in the loss of receptor expression and signaling (*30*). However, to date, the molecular determinants of the hMC4R intracellular loops involved in receptor signaling are limited. We hypothesized that the C terminus and the third intracellular loop of MC4R would be important for receptor signaling. In the present study, we examined a series of hMC4R C-terminal and third intracellular loop deletions and mutations and demonstrate that the C terminus is crucial not only for receptor signaling but surprisingly also important for ligand binding, while the third intracellular loop is involved only in receptor signaling.

MATERIALS AND METHODS

Materials. [Nle^4-D-Phe^7]-α-melanocyte stimulating hormone (NDP−MSH) was purchased from Peninsula Laboratories (Belmont, CA). 3-Isobutyl-methylxanthine (IBMX) was purchased from Sigma, and [^{125}I]NDP−MSH was purchased from Amersham Biosciences (Piscataway, NJ). The HEK-293 cell line was purchased from ATCC (Manassas, VA), and DMEM and lipofectamine were purchased from Life Technologies (Rockville, MD).

Construction of Truncated hMC4R. The C-deletion constructs of hMC4R utilized in these studies are schematically depicted in Figure 1. They were constructed using the polymerase chain reaction (PCR) (*31*), with PCR primer oligonucleotides that were antisense to the desired C-terminal truncated domains. For protein expression studies, we also utilized PCR to insert a FLAG tag onto its NH_2 terminus of hMC4R 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 (*32*, *33*). The 5′ (forward) primer is a 78 base pair sequence that contains an *Eco*R V restriction site (shown in bold), a signal sequence, and the FLAG-coding sequence. The sequence of the sense primer is 5′-CAC **GAT ATC** CCC ACC ATG GAC TAC AAG GAC GAC GAT GAC AAG ATG GTG AAC TCC ACC CAC CGT GGG ATG CAC ACT TCT. The 3′ (reverse) primer is a 27 base pair sequence that contains an *Xba* I restriction site (shown in bold). The sequence of the antisense primer is 5′-CAC **TCT AGA** TTA ATA TCT GCT AGA CAA. After amplification, the truncated receptor DNA fragments were separated by agarose gel electrophoresis and cut with the restriction enzymes *Eco*R V and *Xba* I. The DNA fragments were then subcloned into the eukaryotic expression vector pcDNA 3.1/CT-GFP (Invitrogen, Carlsbad, CA). In addition, we performed forward sequencing on the mutated receptors to confirm that the desired truncated sequences were present and that no sequence errors had been introduced.

Site-Directed Mutagenesis of the Third Intracellular Loop. The third intracellular loop mutations of hMC4R utilized in these studies are schematically depicted in Figure 2. Receptors with multiple mutations were constructed using multiphase PCR techniques. During the initial round of PCR, partial-length receptor fragments were generated (*31*). The sequence of the first PCR primer oligonucleotides consisted of the third intracellular loop amino acids of interest coupled to a portion of the sixth TM required to form a chimeric receptor. The second oligonucleotide primer consisted of either the 5′ or 3′ end of the hMC4R. Receptor fragments were separated by agarose gel electrophoresis and used in a second round of PCR in which full-length chimeric receptor constructs were assembled by cycling the appropriate fragments together for 10 cycles prior to adding both 5′ and 3′ receptor primers. The constructs were then subcloned into the eukaryotic expression vector pcDNA 3.1/CT-GFP. A single mutation was constructed using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The presence of the desired mutations was confirmed by sequencing performed at the University of Alabama at Birmingham Sequence Core.

Cell Culture and Transfection. The HEK-293 cell line was purchased from ATCC (Manassas, VA) and cultured in DMEM medium containing 10% bovine fetal serum and HEPES. Cells at 80% confluence were washed twice, 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 (*34*).

Binding Assays. Binding experiments were performed using the conditions previously described (*35*). Briefly, after removal of the media, cells were incubated with nonradioligand NDP−MSH from 10^{-10} to 10^{-6} M in 0.5 mL of DMEM containing 0.2% BSA and 2×10^5 cpm of ^{125}I-NDP−MSH for 1 h. The binding reactions were terminated by removing the media and washing the cells twice with DMEM containing 0.2% BSA. The cells were then lysed with 0.2 N NaOH, and the radioactivity in the lysate was quantified in an analytical γ counter (Perkin−Elmer, Shelton, CT). Nonspecific binding was determined by measuring the amount of ^{125}I label bound on the cells in the presence of excess 10^{-6} M unlabeled ligand. Specific binding was calculated by subtracting nonspecifically bound radioactivity from total bound radioactivity. Binding data are reported as B_{max} and IC_{50}.

cAMP Assay. Cellular cAMP generation was measured using a competitive binding assay kit (TRK 432, Amersham, Arlington Heights, IL). Briefly, cell culture media was removed, and cells were incubated with 0.5 mL of Earle's balanced salt solution (EBSS), containing the melanocortin agonist NDP−MSH (10^{-10}−10^{-6} M), for 1 h at 37 °C in the presence of 10^{-3} M IBMX. The reaction was stopped by adding ice-cold 100% ethanol (500 μL/well). The cells in each well were scraped, transferred to a 1.5 mL tube, and centrifuged for 10 min at 1900*g*, and the supernatant was evaporated in a 55 °C water bath with prepurified nitrogen gas. cAMP content was measured as previously described, according to instructions accompanying the assay kit (*36*).

Receptor Expression by Using FACs (37). hMC4R transfected cells were harvested using 0.2% EDTA and washed twice with phosphate-buffered saline (PBS). Aliquots of 3 $\times$ 10^6 cells were centrifuged and fixed with 3% paraformaldehyde in PBS (pH 7.4). The cells were incubated with 50 μL of 10 μg/mL murine anti-FLAG M1 monoclonal antibody (Sigma, catalog number 316) in incubation buffer for 45 min. Under this condition, the primary antibody binds only to receptors located at the cell surface. The cells were collected by centrifugation and washed 3 times with incubation buffer. The cell pellets were suspended in 100 μL of incubation buffer containing CY three-conjugated affinity pure donkey anti-mouse Ig G (ImmunoResearch Lab, Inc., West Grove, PA) and incubated at room temperature for 30 min. Flow cytometry was performed on a fluorescence-activated cell sorter (Becton Dickinson FACStar plus six parameter cytometer/sorter with a dual Argon ion laser, San Jose, CA). The results were analyzed using the software CellQuest (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Statistical Analysis. Each experiment was performed in duplicate three separate times. 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). Data are expressed as mean ± SEM. The significant difference was assessed by one-way ANOVA, with $p < 0.05$ considered to be statistically significant.

RESULTS

Contribution of the C Terminus of hMC4R to Ligand Binding and Agonist-Induced Receptor Signaling. All hMC4R wild-type (WT) and truncated receptors analyzed in this study were expressed in HEK-293 cells and assayed for their ^{125}I-NDP−MSH binding ability and for NDP−MSH-induced cAMP generation. Consistent with our previously reported hMC4R functional profile (*35*), cells expressing hMC4R WT demonstrated high NDP−MSH binding and agonist-mediated cAMP production. To determine the contribution of the C terminus of hMC4R in receptor function, we created a series of the truncated receptors, in which the C-terminal amino acids of hMC4R were progressively deleted. These are schematically depicted in Figure 1 and designated as C19, C12, C7, C3, and C0, representing deletion of the distal 6, 13, 18, 22, and 25 amino acid residues, respectively. Our results indicate that the truncated receptors C19 and C12 possess high NDP−MSH binding, similar to that of the hMC4R WT (C25), while the truncated receptors C7, C3, and C0 completely lost specific NDP−MSH binding activity (Figure 3A). Further characterization of receptors C19 and C12 indicates that both receptor-binding profiles are nearly identical to that of the hMC4R WT (Figure 3C).

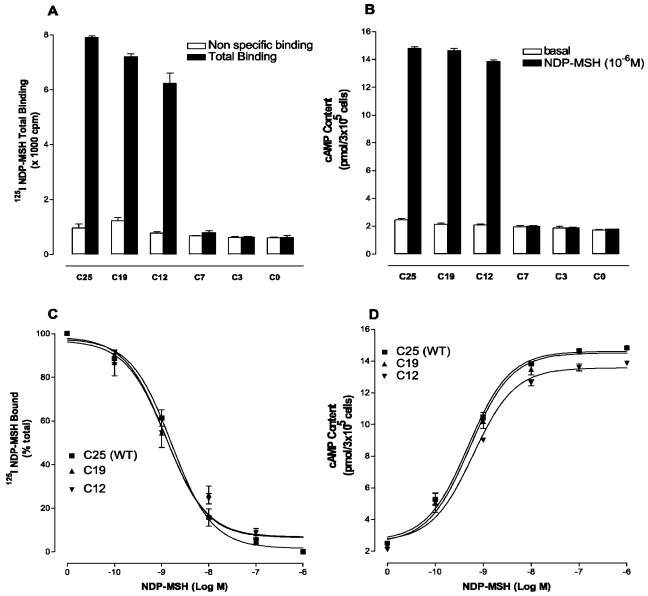

FIGURE 3: (A) Binding of the radioligand ^{125}I-NDP−MSH at these C-truncated receptors. (B) Effect of NDP−MSH on cAMP generation at these C-truncated receptors. (C) Displacement of the radioligand ^{125}I-NDP−MSH at receptors C19 and C12 with unlabeled NDP−MSH. (D) Dose-dependent response of NDP−MSH stimulation of cAMP production at the receptors C19 and C12. Transfection into HEK-293 cells and ligand-binding studies were performed as described under the Experimental Procedures. Each point is the mean of duplicate values obtained from three independent experiments. Competitive binding curves were fit as described under the Experimental Procedures. Parameters of receptor function are detailed in Tables 1 and 2.

To determine the effect of successive deletions of the hMC4R C terminus on agonist-mediated receptor signaling, cells expressing the truncated receptors were treated with NDP−MSH (10^{-6} M) and cAMP production was measured. Our results indicate that NDP−MSH significantly induces cAMP production at the receptors C19 and C12 but it loses its ability to stimulate cAMP production at the receptors C7, C3, and C0 (Figure 3B). To further determine whether the truncated receptors C19 and C12 alter NDP−MSH potency, dose−response experiments were performed at these two receptors. Our results indicate that the NDP−MSH dose dependently increases cAMP production at receptors C19 or C12 in a manner similar to that of the hMC4R WT (Figure 3D). Kinetic parameters of receptor binding and signaling are shown in Tables 1 and 2.

The findings that partial or total deletion of the hMC4R C terminus (receptors C0, C3, and C7) resulted in a complete loss of receptor function suggested that the proximal region of the C terminus is crucial not only for receptor signaling but also for ligand binding. We subsequently designed a second series of C deletion constructs to determine the exact number of amino acids in the proximal C terminus required for ligand binding and receptor signaling. These are schematically depicted in Figure 1 and are designated as the truncated receptors C11, C10, C9, and C8. Our results show that the truncated receptor C11 maintains substantial NDP−

Table 1: Effect of NDP−MSH on ^{125}I-NDP−MSH Binding on HEK Cells Transfected with the C-Terminal Deletions of hMC4R

	^{125}I-NDP−MSH	
	B_{max} (fmol/mg of pro)	NDP−MSH IC_5 (nM ± SEM)
C25(WT)	325 ± 5.8	3.8 ± 0.4
C19	314 ± 16.3	3.9 ± 0.1
C12	284 ± 26.4	4.3 ± 0.2
C11	257 ± 14.9*	27.6 ± 3.1*
C10	NB[a]	NB
C9	NB	NB
C8	NB	NB
C7	NB	NB
C3	NB	NB
C0	NB	NB

[a] NB = no binding.

Table 2: Effect of NDP−MSH on cAMP Formation on HEK Cells Transfected with the C-Terminal Deletions of hMC4R

	NDP−MSH		
	EC_{50} (nm ± SEM)	E_{max} (pm ± SEM)	percent of WT
C25(WT)	0.6 ± 0.2	14.8 ± 3.3	100
C19	0.6 ± 0.1	14.7 ± 0.7	98.6
C12	0.9 ± 0.1	13.4 ± 0.7	89.6
C11	9.2 ± 0.1*	9.4 ± 1.1*	63.1
C10	NA[a]	NA	
C9	NA	NA	
C8	NA	NA	
C7	NA	NA	
C3	NA	NA	
C0	NA	NA	

[a] NA = no activity.

MSH binding and NDP−MSH-stimulated cAMP generation. However, cells expressing the truncated receptors C10, C9, and C8 resulted in a complete loss of NDP−MSH binding and NDP−MSH-induced cAMP production (parts A and B of Figure 4).

To further characterize the kinetics of the receptor C12 and C11 binding affinity, we incubated HEK cells expressing the receptor C12 or C11 with NDP−MSH from 10^{-10}−10^{-6} M and radioactive ligand ^{125}I-NDP−MSH for 1 h and NDP−MSH binding kinetics were analyzed. Our results indicate that the receptor C12 retains high NDP−MSH binding affinity. However, cells expressing the truncated receptor C11 show a decrease in NDP−MSH binding affinity when compared to that of hMC4R WT (Figure 4C). We then examined the effect of NDP−MSH-stimulated cAMP production at these two deleted receptors with the dose of NDP−MSH from 10^{-10} to 10^{-6} M for 1 h and measuring cAMP production. Our results indicate that NDP−MSH dose dependently increases cAMP production at both the receptors C12 and C11; however, the potency of NDP−MSH-stimulated cAMP production is reduced at the latter (Figure 4D). The maximal response of C11 to stimulation was only 63% of hMC4R WT. Kinetics parameters of receptor function are shown in Tables 1 and 2.

Effect of C-Terminal Deletions on Cell-Surface Expression of hMC4R Evaluated by FACs. In above the experiments, we have identified that truncation in receptors C0, C3, C7, C8, C9, and C10 results in the complete loss of NDP−MSH binding and signaling. To ensure that the loss of receptor function from these C-terminal deletions was not due to the

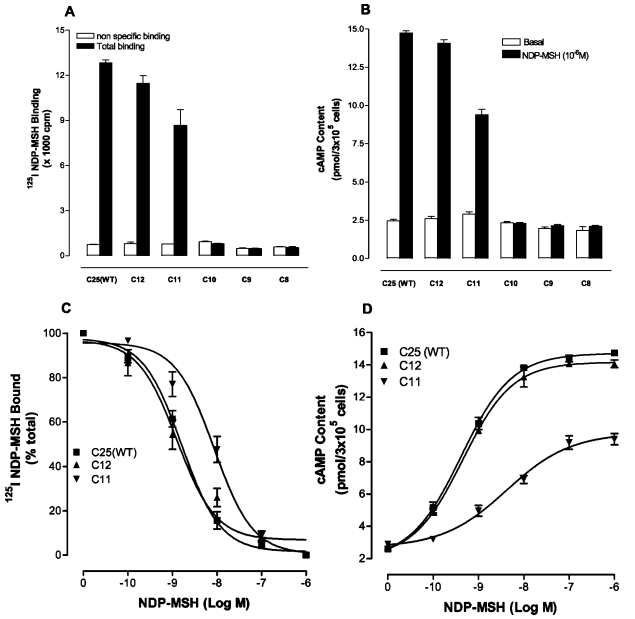

FIGURE 4: (A) Binding of the radioligand ^{125}I-NDP−MSH at the C-truncated receptors. (B) Effect of NDP−MSH stimulation on cAMP production at the C-truncated receptors. (C) Displacement of the radioligand ^{125}I-NDP−MSH at receptors C12 and C11 with unlabeled NDP−MSH. (D) Complete dose−response curves of NDP−MSH stimulation at receptors C12 and C11. Parameters of receptor function are detailed in Tables 1 and 2.

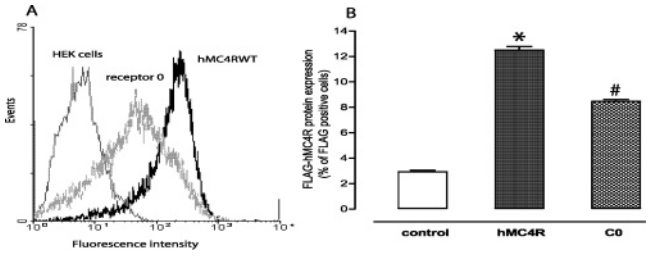

FIGURE 5: FACs of HEK-293 cells transfected with the N-terminal FLAG-tagged wild type or the single clone of the whole C-terminal deletion of MC4R (C0). Receptor expression analysis by FACs is described under the Experimental Procedures. (A) Fluorescence intensity of HEK cells (negative control), FLAG-hMC4R, and the mixed cloned receptor C0. (B) Histograph of the results. Values of the bar represent the percentage of 1×10^5 cells expressing N-terminal FLAG tagged that have FLAG-fluorescent intensity signals.

failure of receptor expression on the cell surface, we utilized the aforementioned FLAG sequence to detect receptor cell-surface expression. FLAG-tagged hMC4R WT or truncated hMC4Rs were transfected into HEK-293 cells, and the expression of FLAG-tagged WT or truncated receptor was detected by FACs. A total of 10 000 cells from each transfection were sorted, and the percentage of cells having a fluorescence intensity signal was determined via a basal cutoff value. We detected a strong FLAG signal in hMC4R WT receptors, as we expected. Because of the low expression of mutant receptor, we selected the high expressed mixed receptor C0 by G418. A strong FLAG signal was also observed in truncated receptor C0. Figure 5 shows the protein expressions of hMC4R WT and the receptor C0. These results demonstrate that the truncated receptor C0 is in fact expressed on the cell surface, verifying that the lack of cell-surface receptor expression is not the culprit for altered receptor ligand binding and signaling.

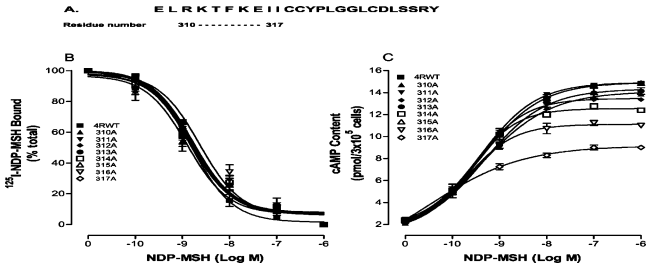

FIGURE 6: Effect of a single mutation of the proximal C terminus of MC4R on ligand binding and receptor signaling. (A) Amino acid sequence of hMC4R C terminus and mutation number. (B) Displacement of the radioligand ^{125}I-NDP−MSH with unlabeled NDP−MSH at the mutated receptors. (C) Effect of NDP−MSH stimulation on cAMP generation at the mutated receptors. Ligand binding and cAMP accumulation measurements were performed as described under the Experimental Procedures. Each point is the mean of duplicate values obtained from three independent experiments. Parameters of receptor function are detailed in Tables 3 and 4.

Table 3: Effect of NDP−MSH on ^{125}I-NDP−MSH Binding on HEK Cells Transfected with the Mutations of the C Terminus of hMC4R

	^{125}I-NDP−MSH	
	B_{max} (fmol/mg of pro)	NDP−MSH IC_{50} (nM)
hMC4R WT	325 ± 5.8	3.8 ± 0.4
R310A	284 ± 26.4	4.9 ± 0.6
K311A	293 ± 13.6	3.6 ± 0.1
T312A	272 ± 11.2	4.1 ± 0.1
F313A	258 ± 6.5	4.2 ± 0.2
K314A	298 ± 9.5	4.3 ± 0.1
E315A	319 ± 7.5	4.0 ± 0.2
I316A	287 ± 11.2	5.0 ± 0.7
I317A	271 ± 12.3	3.8 ± 0.1

Effects of Single-Point Mutation of C-Terminal Amino Acids of MC4R on Ligand Binding and Signaling. Having identified that the proximal 12 amino acids of the C terminus of hMC4R are important for maintaining high receptor ligand binding and signaling, we next attempted to determine whether a specific amino acid within the proximal 12 residues was crucial for these activities. We individually replaced each amino acid with alanine and assessed the impact of a single residue mutation on ligand binding and signaling (Figure 6A). Alanine was chosen because its small nonpolar side chain should only minimally alter the receptor tertiary structure. hMC4R WT or mutated hMC4R were transfected into HEK-293 cells and tested for ^{125}I-labeled NDP−MSH binding. In comparison to that of hMC4R WT, cells expressing the mutated receptors K314A, I316A, and I317A demonstrate decreased receptor expression and decreased maximal cAMP production induced by NDP−MSH however maintained high NDP−MSH binding affinity and potency (parts B and C of Figure 6 and Tables 3 and 4). Cells expressing other single mutations did not show significant reductions in receptor expression and NDP−MSH binding affinity and potency (parts B and C of Figure 6 and Tables 3 and 4).

Effect of Multiple Amino Acid Substitution within the MC4R C Terminus on NDP−MSH Binding and Signaling. With the above experiments, we demonstrated that the proximal 12 amino acids of the C terminus are important not only for receptor signaling but also for ligand binding. To gain insight into the molecular details of these amino acids on receptor signaling and ligand binding, we examined

Table 4: Effect of NDP−MSH on cAMP Formation HEK Cells Transfected with the Mutations of the C Terminus of hMC4R

	NDP−MSH		
	B_{max} (fmol/mg of pro)	NDP−MSH IC_{50} (nM)	percent of WT
hMC4R WT	0.6 ± 0.1	14.9 ± 3.3	100
R310A	2.2 ± 0.6	13.2 ± 1.1	88.7
K311A	0.9 ± 0.1	13.6 ± 2.3	91.4
T312A	0.7 ± 0.1	14.7 ± 3.2	98.3
F313A	1.2 ± 0.4	14.9 ± 1.9	99.7
K314A	0.8 ± 0.2	10.8 ± 2.3*	72.2*
E315A	0.7 ± 0.1	13.7 ± 2.2	89.2
I316A	0.8 ± 0.1	6.7 ± 3.3*	44.6*
I317A	0.7 ± 0.2	5.3 ± 2.1*	35.1*

a series of multiple substitutions of the proximal residues of the hMC4R on ligand binding and receptor signaling. We created three new C-terminal substitution constructs based on the previous finding that the truncated receptor C12 maintains high NDP−MSH binding and signaling. We utilized the C12 receptor as a blueprint and replaced the last 5, 9, and 11 amino acids of this receptor with alanine to maintain the C-terminal length. These truncated receptor constructs are schematically depicted in Figure 7A and are designated C7 + 5A, C3 + 9A, and C1 + 11A. Receptor function was analyzed by measuring NDP−MSH binding and NDP−MSH-mediated cAMP production at each of the constructs. We transfected HEK cells with the mutated receptors, and half of the cells were used for ligand binding and the other half of the cells, for NDP−MSH-induced cAMP production. Therefore, the mutated receptor expression should be identical for these two different experiments. Our results show that cells expressing receptor C7 + 5A, C3 + 9A, and C1 + 11A show high NDP−MSH binding compared to that of the receptor C7, C3, and C0. However, cells expressing receptor C3 + 9A and C1 + 11A have decreased NDP−MSH binding (Figure 7B). NDP−MSH was found to induce cAMP production at receptors C7 + 5A,

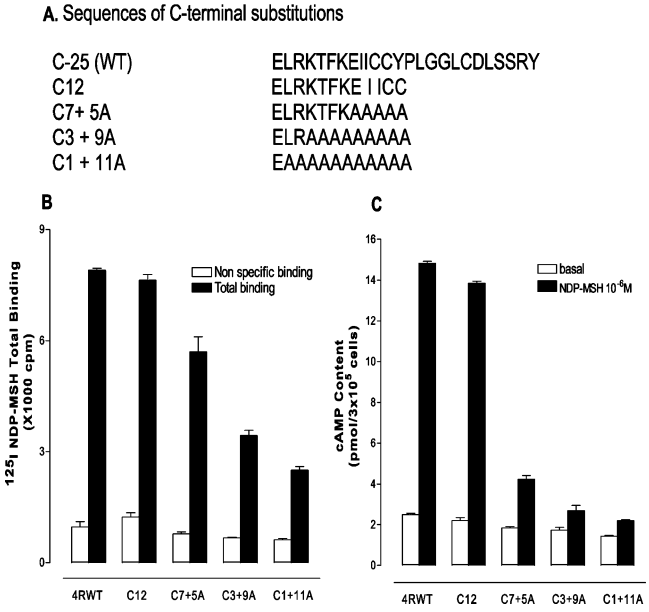

FIGURE 7: Effect of multiple substitutions of the hMC4R proximal C-terminus amino acids with alanine on the receptor function. (A) Sequences of the substituted receptors. (B) NDP−MSH binding at the cells expressing these constructs. (C) Effect of NDP−MSH on cAMP generation at these receptors.

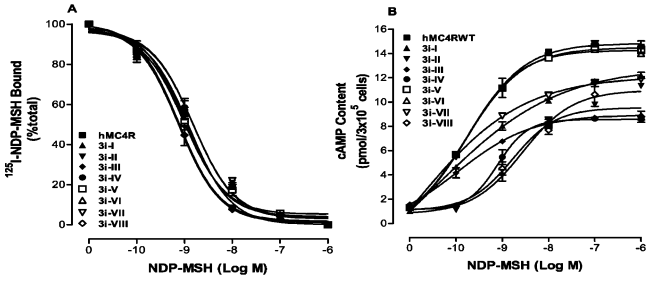

FIGURE 8: Effects of multiple substitutions of the third intracellular loop of MC4R on ligand and receptor signaling. (A) Displacement of ^{125}I-NDP−MSH by unlabeled NDP−MSH from the mutated receptors. (B) Effect of NDP−MSH agonism on cAMP generation at the mutated receptors. Measurement of ligand binding and cAMP accumulation was performed as described under the Experimental Procedures. Each point is the mean of duplicate values obtained from three independent experiments. Parameters of receptor function are detailed in Tables 5 and 6.

Table 5: Effect of NDP−MSH on ^{125}I-NDP−MSH Binding on HEK Cells Transfected with the Mutations of the Third Intracellular Loop of hMC4R

	^{125}I-NDP−MSH	
	B_{max} (fmol/mg of pro)	NDP−MSH IC_{50} (nM ± SEM)
hMC4R WT	325 ± 5.8	3.8 ± 0.4
3i-I	314 ± 12.5	4.3 ± 0.3
3i-II	298 ± 11.3	3.9 ± 0.7
3i-III	305 ± 14.6	3.7 ± 0.6
3i-IV	286 ± 17.2	4.7 ± 0.7
3i-V	312 ± 2.3	3.9 ± 0.1
3i-VI	312 ± 4.3	3.6 ± 0.1
3i-VII	312 ± 10.6	4.3 ± 0.2
3i-VIII	295 ± 7.6	4.9 ± 0.1

C3 + 9A, and C1 + 11A, but in comparison to C7 + 5A, potency was successively reduced. For the receptor C7 + 5A, total NDP−MSH binding was only reduced 18% compared to hMC4R WT, but cAMP production induced by NDP−MSH was significantly decreased by 72% compared to that of hMC4R WT (Figure 7C), suggesting that this portion of the receptor is important for receptor signaling.

The Role of the Third Intracellular Loop of hMC4R in Ligand Binding and NDP−MSH-Induced Signaling. We next sought to determine whether the third intracellular loop of hMC4R was important in receptor binding and agonist-mediated receptor signaling. We divided the third intracellular loop of hMC4R into eight subregions of three amino acids each and used alanine substitution to construct mutant receptors. These are schematically depicted in Figure 2 and are designated 3i-I, 3i-II, 3i-III, 3i-IV, 3i-V, 3i-VI, 3i-VII, and 3i-VIII. These designations represent substitution of the first three, second three, third three, fourth three, fifth three, sixth three, seventh three, and eighth three amino acids, respectively. We examined the effects of these residue substitutions on receptor ligand binding and signaling. We found that NDP−MSH dose dependently displaced ^{125}I-NDP−MSH binding at all of these mutated receptors in a manner similar to that of hMC4R WT (Figure 8A), indicating that these specific residues are not critical for ligand binding. IC_{50} values are presented in Table 5. Our results also indicate that NDP−MSH dose dependently increases cAMP formation at mutated receptor 3i-I, 3i-III, 3i-V, 3i-VI, and 3i-VII similar to that of hMC4R WT. However, the signaling potency of NDP−MSH was decreased at substituted receptors 3i-II, 3i-IV, and 3i-VIII, reflected by 10-, 9-, and 8-fold decreases in EC_{50}, with a markedly blunted maximal cAMP response (approximately 78, 56, and 76% of WT receptor levels, respectively) (Table 6).

Table 6: Effect of NDP−MSH on cAMP Formation on HEK Cells Transfected with the Mutations of the Third Intracellular Loop of hMC4R

	^{125}I-NDP−MSH		
	B_{max} (fmol/mg of pro)	NDP−MSH IC_{50} (nM ± SEM)	percent of WT
hMC4R WT	0.6 ± 0.1	14.9 ± 3.3	100
3i-I	0.9 ± 0.1	11.7 ± 0.1	781
3i-II	4.6 ± 0.2*	7.3 ± 0.2*	49.7
3i-III	0.9 ± 0.1	8.9 ± 0.1*	61.2
3i-IV	5.6 ± 0.8*	9.8 ± 1.2*	66.8
3i-V	0.6 ± 0.1	12.1 ± 0.2	85.6
3i-VI	0.6 ± 0.2	12.3 ± 0.3	86.3
3i-VII	0.7 ± 0.4	9.9 ± 0.4*	66.9
3i-VIII	3.8 ± 0.2*	8.4 ± 0.1*	56.4

DISCUSSION

In this study, we have identified that the proximal 12 amino acids of the hMC4R C terminus are important not only for receptor signaling but also critical for ligand binding, while the third intracellular loop of hMC4R is important for receptor signaling but not for ligand binding. To date, the localization of receptor−G-protein contact sites and the receptor motifs required for G-protein activation have been the subjects of intense investigation. In all GPCR-mediated signaling events, the cytoplasmic location of the G protein dictates that the interaction with the receptor must involve the intracellular loops or the intracellular C-terminal tail. Extensive mutational analysis and experiments with synthetic peptides have elicited the involvement of intracellular loops 3 and the C-terminal cytoplasmic domain in G-protein interactions in the GPCR family (*38*). Many studies indicate that the C terminus of selected GPCRs plays an important role in most GPCR signaling (*39*, *40*). An entire C-terminal deletion of many GPCRs abolishes receptor signaling while still maintaining ligand binding affinity (*23*, *26*). For example, deletion of the angiotensin-2 receptor C terminus causes reduction of cGMP levels, while the receptor retains its ability to bind ^{125}I-Ang II (*23*). However, in other studies, the third intracellular loop of GPCR has been reported to be important for receptor signaling (*41*). All findings to date indicate that there is no single sequence motif shared by all GPCRs with similar G-protein specificity and that multiple receptor domains contribute to GPCR-specific differences in individual receptor signaling. This assessment highlights the complex diversity in receptor−G-protein interaction upon agonist-mediated receptor signaling (*39*, *42*). Our findings that the C terminus and IC3 of hMC4R play an important role in receptor signaling is consistent with other GPCRs; however, our finding that C terminus plays an important role in ligand binding is unique.

All members of the MCR family are coupled to adenylate cyclase (AC) activity. However, the structural sites for interactions between the receptor and its G proteins have not been thoroughly investigated. The C terminus of all melanocortin receptors (MC1R, 2, 3, 4, and 5) has high

sequence conservation in the proximal residues (*28*, *43*). The dihydrophobic motif, at C-terminal residues 12 and 13, is completely conserved in this receptor family. Our first set of experiments indicates that deletion of the entire C terminus of MC4R results in the complete loss of not only receptor signaling but also ligand binding, suggesting that the C terminus is not only crucial for receptor signaling but also important for ligand binding. Further experiments show that deletion of more than 14 amino acids of the MC4R C terminus results in the loss of NDP−MSH binding and intracellular cAMP signaling, while the deletion of the distal 13 amino acids does not significantly affect receptor signaling. These combined results clearly suggest that G-protein coupling with the MC4R must require conservation of the proximal 12 amino acids and that the C terminus is important not only for receptor signaling but also for ligand binding, which is different from the role of the C terminus in other GPCRs (*23*, *26*).

To determine whether the inserted mutations affected proper expression of the receptor on the cell surface, we utilized FACs to examine receptor expression. Because of the low transfection efficiency of the mutant receptor, we selected highly expressed mixed mutant receptor C0 by G418. Cell-surface expressions of the mixed receptor C0 were confirmed using a single cloned antibody directed against the FLAG sequence. These immunolocalizing studies confirm that the receptor protein is expressed on the cell surface, eliminating the possibility that reduced cell expression might account for reduced ligand binding and receptor signaling. Therefore, the proximal 12 amino acids of the C terminus of MC4R are not only crucial for maintaining the receptor's tertiary structure for receptor signaling but also important for ligand binding.

It has been reported by van Leeuwen and his colleagues that the dileucine motif in the C terminus of hMC4R is important for receptor cell-surface expression (*44*). Our results also indicate that single-point mutations of I316A or I317A decrease mutant receptor expression on the cell surface, although the mutations did not affect ligand binding affinity and potency. Because of the fact that transfection efficiency and expression is low with the mutant receptors, we selected highly expressed single clone mutant receptor C0, C7 + 5A, C3 + 9A, and C1 + 11A to perform the NDP−MSH binding and NDP−MSH-stimulated cAMP production experiment.

The MCRs are characterized by a large number of cysteine residues. However, MCRs lack the conserved disulfide bond between TM3 and extracellular loop 2, which participates in structural stabilization in numerous other GPCRs (*45*). Therefore, in MCRs, other extracellular disulfides may exist to maintain a correct receptor conformation. Recently, it was suggested that conserved cysteines from the N terminus and EL3 of MC4R could form structurally and functionally important disulfide bonds (*46*−*48*). Many GPCRs have a conserved Cys residue in the C-terminal tail that serves as a site for palmitoylation, thereby forming a putative "fourth intracellular loop". Our results indicate that the receptor function of the C12 truncation (two Cys present) is normal; however, the C11 truncation is impaired (one Cys), and the C10 truncation (no Cys) is nonfunctional. This suggests that these two cysteines may be involved in stabilizing receptor conformation to allow ligand binding. Further experiments are required to determine whether the MC4R is palmitoylated on either of these Cys residues. In addition, cysteines 318, 319, and 325 in the C terminus have been reported to play a major role in agonist-mediated receptor phosphorylation and downregulation (*49*). Our data indicates that deletion of the distal 13 amino acids of the C terminus, including cysteine 325, has no effect on NDP−MSH binding and agonist-induced receptor signaling; however, deletion of cysteine 318 or 319 greatly affects ligand binding and signaling. These combined results suggest that cysteine 325 may be important for ligand-mediated receptor downregulation but not for receptor signaling, while cysteines 318 and 319 are crucial not only for ligand binding, receptor signaling but also perhaps for ligand-mediated receptor downregulation as well.

Molecular determinants of MC4R responsible for ligand binding have been studied by extensive site-directed mutagenesis and creation of receptor chimeras. It has been found that the amino acid residues in the TMs and the extracellular loops of MC4R are involved in α-MSH and AGRP binding and activity, highlighting a set of residues forming the ligand-binding pockets for α-MSH and AGRP (*50*−*52*). Computer modeling of MCR has suggested that the D-Phe-Arg-Trp triplet of α-MSH might interact with a cluster of negatively charged residues in TM3 and aromatic residues in TM3, 5, and 6 of MC4R (*35*, *53*). It is interesting that deletion of the entire C terminus of MC4R results in the loss of NDP−MSH binding and intracellular cAMP signaling, while single-point mutations of the proximal region of the MC4R C terminus do not affect ligand binding affinity. This information leads us to believe that multiple amino acids in the proximal region of the C terminus may be required for maintaining normal receptor structure for ligand binding and receptor signaling. To prove this hypothesis, we used multiple amino acid substitution with alanine in the proximal region of the MC4R C terminus to test their effects on receptor function. Our results indicate that, as more amino acids of the proximal region of the MC4R C terminus were substituted, ligand binding becomes weaker and less receptor signaling occurs. The tolerance for multiple amino acid substitutions in the MC4R C terminus indicates that G-protein activation by interaction between NDP−MSH and MC4R involves multiple intramolecular interactions between the C terminus of MC4R and the G protein that do not strongly depend on a particular amino acid element. The certain length of the C terminus appears to be more critical than the presence of specific residues because introduction of multiple alanine residues into the C terminus leads to a reduced but not entirely eliminated level of signaling. Defects in signaling caused by substitution of amino acids are not caused by changes in the abundance of receptors at the cell surface, which we confirmed using FACs. When our results are taken together with point-mutation findings, they confirm that a certain number of amino acids in the proximal region of the MC4R C terminus are required for maintaining receptor conformation and for ligand binding and receptor signaling.

The third intracellular loop of the receptor has also been reported to play an important role in some GPCR activation (*39*, *40*). Mutation of the third intracellular loop has been shown to result in the loss of agonist-induced receptor activation (*54*). For instance, deletions within the i-3 loop

of the thyrotropin receptor results in only constitutive activity. Trimming the i-3 loops in the glucagon receptor, the glucagon-like peptide-1 receptor, and in the muscarinic acetylcholine receptor has been shown to cause both severely impaired and unaltered signaling abilities (*55*, *56*). The sequence analysis of the i-3 loop portion of the MC4R shows that this region is particularly rich in charged residues. Our results indicate that NDP−MSH has a high binding affinity and potency at mutant receptor 3i-V and 3i-VI, suggesting that this region is not involved in receptor ligand binding and signaling, whereas the mutant receptors 3i-II, 3i-IV, and 3i-VIII have diminished NDP−MSH-mediated cAMP production with little or no effect on ligand binding. These results suggest that these regions may be important for hMC4R interaction with G-protein and receptor activation but have little importance in receptor ligand binding.

In conclusion, we have identified that the proximal 12 residues of the C terminus of the hMC4R are crucial not only for NDP−MSH-mediated cAMP production but also important for ligand binding. The proximal 12 amino acids of the hMC4R C terminus are critical for maintaining receptor function in both receptor G-protein coupling and ligand binding. Further experiments indicate that a certain number of amino acids in the proximal region of the C terminus are required for intact MC4R ligand binding and signaling. In addition, the third intracellular loop of hMC4R appears only important for receptor signaling. This study has provided information extending our current knowledge of the molecular basis of the C terminus and intracellular loop 3 of hMC4R responsible for receptor activation. These results may eventually provide specific insight into the pathogenesis of human obesity and its treatment.

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