Molecular Identification of the Human Melanocortin-2 Receptor Responsible for Ligand Binding and Signaling†

Min Chen,‡ Charles J. Aprahamian,§ Robert A. Kesterson,|| Carroll M. Harmon,§ and Yingkui Yang*,§

Departments of Nutrition, Surgery, and Genetics, University of Alabama at Birmingham, Birmingham, Alabama 35233

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ABSTRACT: The melanocortin-2 receptor (MC2R), also known as the adrenocorticotropic hormone (ACTH) receptor, plays an important role in regulating and maintaining adrenocortical function, specifically steroidogenesis. Mutations of the human MC2R (hMC2R) gene have also been identified in humans with familial glucocorticoid deficiency; however, the molecular basis responsible for hMC2R ligand binding and signaling remains unclear. In this study, both truncated ACTH peptides and site-directed mutagenesis studies were used to determine molecular mechanisms of hMC2R binding ACTH and signaling. Our results indicate that ACTH1−16 is the minimal peptide required for hMC2R binding and signaling. Mutations of common melanocortin receptor family amino acid residues E80 in transmembrane domain 2 (TM2), D107 in TM3, F178 in TM4, F235 and H238 in TM6, and F258 in TM7 significantly reduced ACTH-binding affinity and signaling. Furthermore, mutations of unique amino acids D104 and F108 in TM3 and F168 and F178 in TM4 significantly decreased ACTH binding and signaling. In conclusion, our results suggest that the residues in TM2, TM3, and TM6 of hMC2R share similar binding sites with other MCRs but the residues identified in TM4 and TM7 of hMC2R are unique and required for ACTH selectivity. Our study suggests that hMC2R may have a broad binding pocket in which both conserved and unique amino acid residues are required, which may be the reason why α-MSH was not able to bind hMC2R.

The melanocortin-2 receptor (MC2R),[1] also known as the adrenocorticotropic hormone (ACTH) receptor, plays an important role in the regulation of adrenal cortisol secretion (*9*). Mutations of the hMC2R have been identified in a potentially fatal disease, called familial glucocorticoid deficiency (FGD), in which affected individuals are deficient in cortisol and prone to succumb to hypoglycemia or overwhelming infection in infancy or childhood if not treated (*8*, *10*, *16*, *35*, *39*, *40*, *44*, *49*, *50*, *52*, *58*−*60*). Overexpression of this receptor is also found in adrenal adenomas or hyperplasia associated with glucocorticoid overproduction (Cushing's syndrome) (*1*, *22*, *37*, *38*).

Extensive studies have been performed to determine the molecular basis of the ACTH interaction with cognate melanocortin receptors (MCRs) in the last 2 decades, but inconsistent results have failed to produce a clear picture of mechanistic function. In early years, ACTH1−14, ACTH11−19, and ACTH11−24 were reported to increase corticosterone levels in the isolated adrenal cell system, with ACTH11−24 playing an important role in adipocyte function (*14*, *24*, *42*, *45*); however, ACTH1−14 was reportedly not able to bind MC2R (*48*). A potential explanation for these variations is that different methods of adrenal cell preparation were used and/or separate receptors may be involved in these peptide activities (*53*). Further studies using some ACTH peptide truncations indicated that ACTH1−17 is the minimal peptide required for ligand binding and signaling (*14*, *24*, *42*, *45*). However, detailed structure−function analysis of ACTH residues responsible for MC2R binding and signaling is still unclear.

Cloning of MCRs, structure−function studies, and mutagenesis studies of the receptor have provided great insights into the ligand−receptor interaction (*26*, *61*, *63*, *64*). During the past several years, many studies have indicated that the transmembrane domains (TMs) 2, 3, and 6 of melanocortin 1, 3, and 4 receptors are important for α-MSH binding and signaling (*15*, *26*, *30*, *46*, *62*, *64*). However, the molecular determinants of the hMC2R responsible for binding the ACTH agonist selectively remain unknown.

MC2R is one of five known MCRs belonging to the seven transmembrane G-protein-coupled receptor family (GPCR) (*41*). MC2R shares nearly 50% homology with other MCR subtypes, but it is unique among MCRs because of its ligand selectivity. ACTH is the only known endogenous agonist at MC2R, whereas ACTH as well as α-, *β*-, and *γ*-MSH bind to other MCRs (*2*, *4*, *11*−*13*, *48*, *54*, *55*, *57*). All melanocortins have a shared core amino acid sequence, His-Phe-

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* To whom correspondence should be addressed: Division of Pediatric Surgery, University of Alabama at Birmingham, 300 ACC, 1600 7th Avenue South, Birmingham, AL 35233. Telephone: (205) 939-9688. Fax: (205) 975-4972. E-mail: ying-kui.yang@ccc.uab.edu.

‡ Department of Nutrition.

§ Department of Surgery.

|| Department of Genetics.

[1] Abbreviations: MCR, melanocortin receptor; hMC2R, human melanocortin-2 receptor; GPCR, G-protein-coupled receptor; ACTH, adrenocorticotropic hormone; α-MSH, α-melanocyte stimulating hormone; ASIP, agouti-signaling protein; TM, transmembrane domain; IBMX, 3-isobutyl-methylxanthine; PCR, polymerase chain reaction; FACS, flow cytometry.

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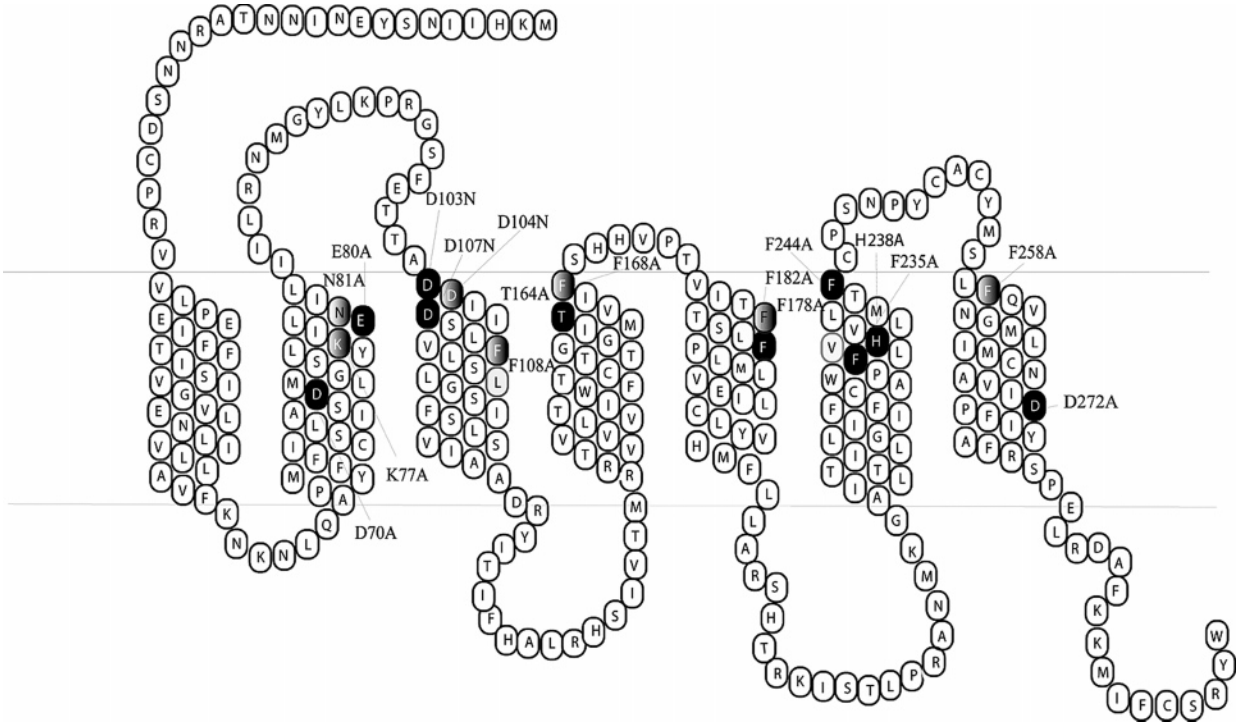

FIGURE 1: Two-dimensional representation of the seven TM structure of the hMC2R. The conserved TM residues mutated in these experiments are denoted by black highlighting, and the unique TM residues mutated in these experiments are denoted by gray highlighting.

Arg-Trp (HFRW), which is critical for melanocortin binding and signaling (*27*, *28*, *47*). In this study, both truncated ACTH peptides and site-directed mutagenesis studies were used to determine the molecular basis of hMC2R-selective ACTH binding and signaling. Our results indicate that ACTH1−16 is the minimal peptide for hMC2R binding and both conserved as well as unique amino acid residues in TMs of hMC2R are involved in ACTH binding and signaling. Not only are TM2, TM3, and TM6 domains of hMC2R important for ACTH recognition, but TM4, TM5, and TM7 are also required for ACTH binding and signaling.

MATERIALS AND METHODS

Peptides. ACTH1−39 and the truncated ACTH peptides were purchased from Peninsula Laboratories, Inc. (Belmont, CA). ACTH1−15 was purchased from Genscript Corporation (Piscataway, NJ).

Site-Directed Mutagenesis. Single-residue mutations were constructed using the quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA). The entire coding region of each mutant receptor was sequenced by the University of Alabama at Birmingham Sequence Core to confirm that the desired mutation sequences were present and that no sequence errors had been introduced. The mutated receptors are shown in Figure 1. The mutant receptors were subcloned into the eukaryotic expression vector pCDNA 3.1 (Invitrogen, Carlsbad, CA).

Cell Culture and Transfection. The OS3 adrenal cell line was cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% bovine fetal serum, 2% CBS, and 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES). Cells at 80% confluence were washed twice with DMEM, and the receptor constructs were transfected into cells using lipofectamine (Life Technologies, Rockville, MD). The permanently transfected clonal cell lines were selected by resistance to the neomycin analogue G418 (*62*).

Binding Assays. After removal of media, OS3 cells expressing hMC2R wild type (WT) or mutants were incubated with various nonradioligands in 0.5 mL of MEM (Fisher Scientific, Pittsburgh, PA) containing 0.2% bovine serum albumin (BSA) and radioligand. Binding experiments were performed using conditions previously described (*62*, *65*). Briefly, 2×10^5 cpm of ^{125}I-ACTH (Amersham, Arlington Heights, IL) was used in combination with nonradiolabeled ligand ACTH. Binding reactions were terminated by removing the media and washing the cells twice with MEM containing 0.2% BSA. The cells were lysed with 0.2 N NaOH, and the radioactivity in the lysate was quantified in an analytical γ counter. Nonspecific binding was determined by measuring the amount of ^{125}I-label bound in the presence of 10^{-6} M unlabeled ligand. Specific binding was calculated by subtracting nonspecifically bound radioactivity from the total bound radioactivity.

Cyclic Adenosine Monophosphate (cAMP) Assay. cAMP generation was measured using a competitive binding assay (TRK 432, Amersham, Arlington Heights, IL). Briefly, OS3 cells stably expressing hMC2R were used in these assays (*6*, *65*). Cell-culture media was removed, and cells were incubated with 0.5 mL of Earle's balanced salt solution (EBSS), containing ACTH (10^{-10}−10^{-6} M), for 1 h at 37 °C in the presence of 10^{-3} M isobutylmethylxanthine. The reaction was stopped by adding ice-cold 100% ethanol (500 μL/well). The cells in each well were scraped, transferred to a 1.5 mL tube, and centrifuged for 10 min at 1900*g*, and the supernatant was evaporated in a 55 °C water bath with purified nitrogen gas. cAMP content was measured according to instructions accompanying the assay kit. Each

Table 1: Effect of ACTH Truncations on ^{125}I-ACTH Binding and cAMP Generation[a]

		K_i (nM)	EC_{50} (nM)
Ac-Ser-Tyr-Ser-Nle-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-AA14−39	(ACTH1−39)	9.8 ± 2.1	8.59 ± 0.65
Ac-Ser-Tyr-Ser-Nle-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-AA14−24	(ACTH1−24)	5.4 ± 1.5[b]	2.8 ± 0.5[b]
Ac-Ser-Tyr-Ser-Nle-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg	(ACTH1−17)	78 ± 3.7[b]	46.8 ± 8.5[b]
Ac-Ser-Tyr-Ser-Nle-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg	(ACTH1−16)	92 ± 12.3[b]	567 ± 34[b]
Ac-Ser-Tyr-Ser-Nle-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys	(ACTH1−15)	no	NR
Ac-Ser-Tyr-Ser-Nle-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly	(ACTH1−14)	no	NR
Ac-Ser-Tyr-Ser-Nle-Glu-His-Phe-Arg-Trp-Gly	(ACTH1−10)	no	NR
His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro	(ACTH6−24)	839 ± 13.5[b]	NR
Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu	(ACTH7−38)	no	NR
Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe	(ACTH18−30)	no	NR

[a] no = no binding. NR = no response. $n > 3$, with mean ± SEM. [b] $p < 0.01$ compared with ACTH1−39.

experiment was performed a minimum of 3 times with duplicate wells.

Receptor Expression. For receptor protein expression studies, an eight amino acid FLAG tag (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) was inserted [via polymerase chain reaction (PCR)] onto the NH_2 terminus of hMC2R to characterize receptor protein cell-surface expression by flow cytometry using fluorescence-activated cell sorting (FACS) (*25*, *57*). hMC2R or mutant receptor transfected cells were harvested using 0.2% ethylenediaminetetraacetic acid (EDTA) and washed twice with phosphate-buffered saline (PBS). Aliquots of 3×10^6 cells were centrifuged and fixed with 3% paraformaldehyde in PBS (pH 7.4). The cells were incubated with 50 μL of 10 μg/mL murine anti-FLAG M1 monoclonal antibody (Sigma, catalog number 316, St. Louis, MO) in incubation buffer for 45 min. Under these conditions, the primary antibody binds only to receptors located at the cell surface. The cells were collected by centrifugation and washed 3 times with incubation buffer. The cell pellets were suspended in 100 μL of incubation buffer containing CY3-conjugated affinity pure donkey anti-mouse Ig G (ImmunoResearch Lab, Inc., West Grove, PA) and incubated at room temperature for 30 min. Flow cytometry was performed on a fluorescence-activated cell sorter (Becton−Dickinson FACStar plus six parameter cytometer/sorter with a dual argon ion laser, San Jose, CA). The results were analyzed using the software CellQuest (Beckton−Dickinson Immunocytometry Systems, San Jose, CA).

Statistical Analysis. Each experiment was performed at 3 separate times with duplicate wells. Data are expressed as mean ± standard error of the mean (SEM). The mean value of the dose−response data of binding and cAMP production was fit to a sigmoid curve with a variable slope factor using nonlinear squares regression analysis (Graphpad Prism, Graphpad Software, San Diego, CA). Significant differences were assessed by one-way analysis of variation (ANOVA), with $p < 0.05$ considered to be statistically significant.

RESULTS

Structure−Function Study of ACTH Peptides at hMC2R Wild Type. To determine which region of ACTH is essential for high-affinity binding and potency at hMC2R, we performed structure−activity studies using truncated ACTH peptides (primary sequences are shown in Table 1). Our results indicate that ACTH1−39, ACTH1−24, ACTH1−17, and ACTH1−16 possess full agonist activities. ACTH1−24 is the most potent, and ACTH1−16 is the least potent among the ACTH peptides tested (Figure 2 and Table 1). ACTH peptides with carboxyl-terminus truncations of less than 16 amino acids lost both binding affinity and biological activities. Further studies indicate that truncation of the amino-terminus residues of ACTH similarly leads to a loss of activity, with the retention of the first six amino acids absolutely required for full binding and agonist activity. Interestingly, ACTH6−24 displayed high-affinity binding but no agonist activity (Figure 3 and Table 1).

Substitutions of the Conserved Amino Acid Residues of hMC2R on ACTH Binding and Potency. The hMC2R shares 67% conserved amino acid residues with other MCRs in the transmembrane domains (*17*−*19*, *41*). However, the pharmacological profile of hMC2R is substantially different from other MCRs. The rationale for selecting amino acids of hMC2R in the mutagenesis experiments is based on our previous studies of other MCRs. Our previous results indicate that the conserved amino acid residues in the TMs of the hMC1R, hMC3R, and hMC4R are important for ligand binding and signaling (*6*, *7*, *62*, *64*). Moreover, our recent studies of chimeric receptors with hMC4R and hMC2R indicate that the conserved amino acid residues of hMC2R are important for ligand binding and signaling. The substitutions of the TMs of the hMC4R with the corresponding regions of the hMC2R did not significantly alter or abolish NDP−MSH-specific activity, suggesting that conserved amino acid residue in TMs of the hMC2R may be crucial for ACTH binding and activity (*7*). A sequence alignment of hMC2R with other MCRs identified 10 amino acid residues residing in transmembrane upper regions of TM2, TM3, TM4, TM5, and TM6 as being conserved. We hypothesized that these conserved amino acid residues of hMC2R may be involved in ACTH binding and receptor activation in a similar fashion to that of other MCRs. With this rationale, the conserved ionic and aromatic amino acid residues in TMs of the hMC2R were individually mutated with alanine (Figure 1) and the ACTH binding affinity and potency were evaluated. Alanine is the generally accepted amino acid of choice for mutagenesis substitution in this type of analysis because its small neutral nature theoretically makes it unlikely to disturb the receptor tertiary structure. Asparagine, instead of alanine, is used for some mutations when alanine results in the complete loss of the receptor function (*64*). Our results indicate that mutant receptors K77A, E80A, D103N, D107N, F178A, F235A, H238A, and

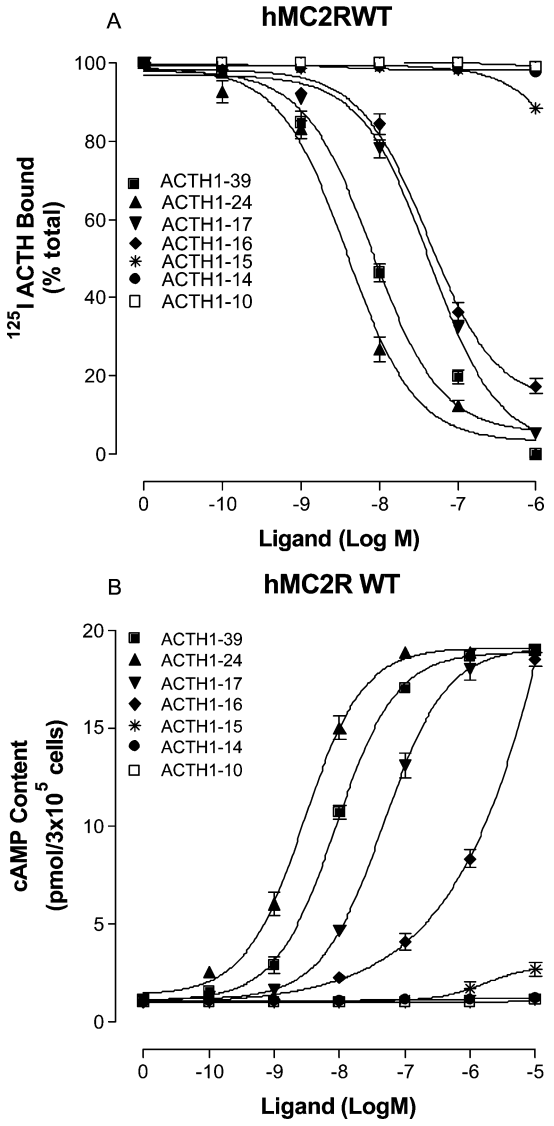

FIGURE 2: Binding affinity and potency of truncated ACTH peptides in OS3 cells stably transfected with the wild-type hMC2R. A shows that OS3 cells transfected with hMC2R were incubated with ^{125}I-ACTH in the presence of the indicated amounts of unlabeled ligands, and total ^{125}I-ACTH binding was determined. B shows that the cells were incubated with the indicated amounts of peptides, and total cAMP accumulation was determined ($n = 3$; see Table 1 for K_i and EC_{50} values).

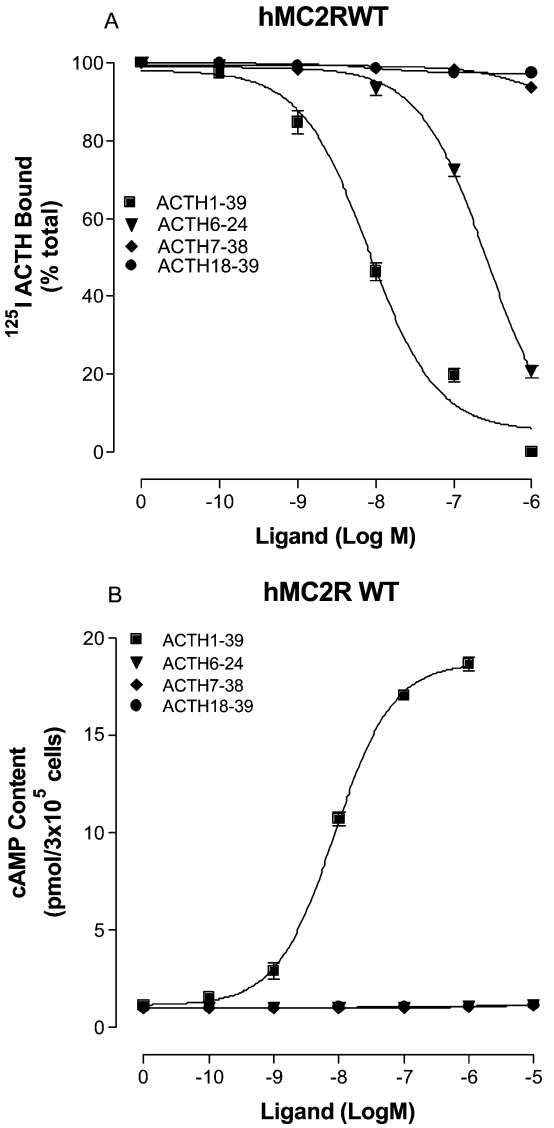

FIGURE 3: Binding affinity and potency of truncated ACTH peptides in OS3 cells stably transfected with the wild-type hMC2R. A shows that OS3 cells transfected with hMC2R were incubated with ^{125}I-ACTH in the presence of the indicated amounts of unlabeled ligands, and total ^{125}I-ACTH binding was determined. B shows that the cells were incubated with the indicated amounts of peptides, and total cAMP accumulation was determined ($n = 3$; see Table 1 for K_i and EC_{50} values).

F258A were expressed and found at the cell surface (Table 2), albeit at lower levels than that of the wild-type receptor. Receptor function analyses indicate that ACTH dose-dependently displaced ^{125}I-ACTH binding at the mutations K77A, E80A, D103N, D107N, F178A, F235A, H238A, and F258A but their binding affinities for ACTH were significantly reduced compared to that of the hMC2R WT (Figures 4A and 5A and Table 2). Consistent with the binding results, the mutations K77A, E80A, D103N, D107N, F178A, F235A, H238A, and F258A significantly reduced ACTH-mediated cAMP production (Figures 4B and 5B; K_i and EC_{50} are shown in Table 2).

It has been reported that mutations of aspartic acid residues in TM2 and TM7 of hMC3R and hMC4R resulted in not only altered ligand binding but also altered receptor signaling (*6*, *64*). To determine whether the corresponding residues of hMC2R are also involved in agonist-mediated receptor activation, we mutated residues D70 in TM2 and D272 in TM7 of hMC2R with alanine and tested their function. Substitutions D70A and D272A resulted in a complete loss of receptor−ligand binding and activation (Table 2). To examine the possibility that these mutations may not be properly expressed at the cell surface, we used FACS analysis to determine whether the alanine substitution alters receptor expression. Our results show that a strong signal was detected at FLAG-tagged MC2R WT but no signal was detected in FLAG-tagged mutants D70A and D272A, suggesting that these two mutants were not properly expressed at the cell surface.

Substitutions of the Unique Amino Acids in the TMs of hMC2R: ACTH Binding and Activation. ACTH is the only endogenous ligand for hMC2R, and three regions of ACTH are proposed for receptor ligand binding and signaling (*3*, *36*, *48*). Our results indicate that the residues 15−17 Lys15−Lys16−Arg17 are important for hMC2R binding and signaling because the loss of this region (ACTH1−14) impairs

Table 2: Effect of the Substitutions of the Conserved Amino Acid Residues of hMC2R on ^{125}I-ACTH Binding and cAMP Production

	receptor expression (percent of WT)	^{125}I-ACTH binding K_i (nM)	cAMP production EC_{50} (nM)
hMC2R WT	100	5.6 ± 1.0	0.8 ± 0.2
D70A	no	NB	NR
K77A	73 ± 9.5	25.2 ± 4.1[a]	6.2 ± 0.9[a]
E80A	75 ± 12.3	65.2 ± 7.1[a]	7.8 ± 1.1[a]
D103N	68 ± 14	15.7 ± 0.7[a]	5.8 ± 1.2[a]
D107N	66 ± 11	>10^3 [a]	>10^3 [a]
F178A	67 ± 8.7	>10^3 [a]	>10^3 [a]
F235A	84 ± 6.8	98 ± 6.9[a]	216 ± 18[a]
H238A	69 ± 11.7	>10^3 [a]	>10^3 [a]
F258A	78 ± 12.7	78 ± 4.4[a]	187 ± 10[a]
D272A	no	NB	NR

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

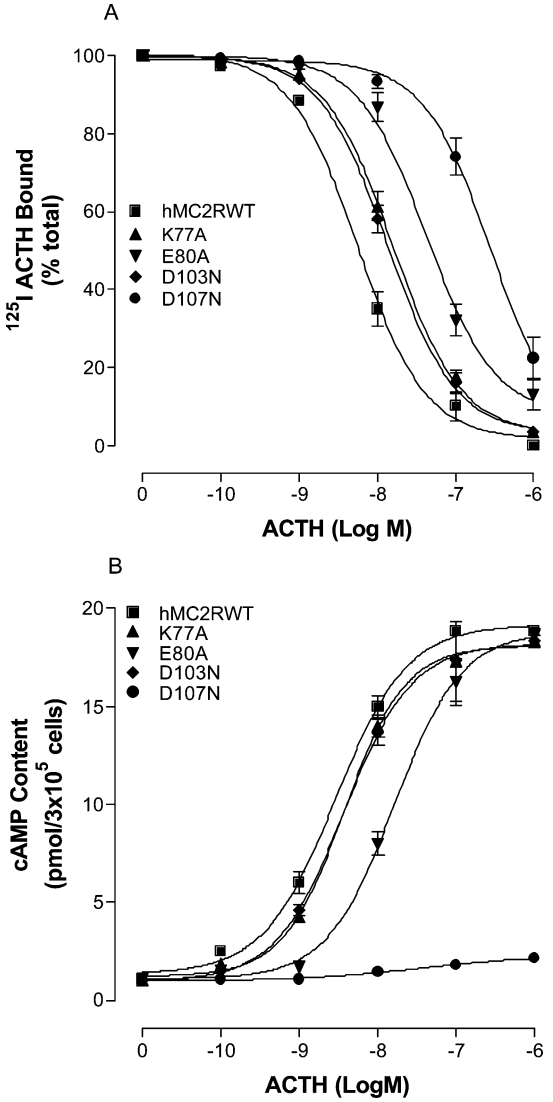

FIGURE 4: Effects of the mutations of the conserved, charged amino acid hMC2R TM residues with alanine on the ACTH-binding affinity and receptor activity. A shows the ACTH-binding affinity of these mutants. B shows the ability of ACTH-stimulated cAMP production at these mutants ($n = 3$; see Tables 2 for actual K_i and EC_{50} values).

receptor activation. This highly positively charged domain suggests that additional binding sites at hMC2R may be required for ACTH15−17 binding, with amino acid residues unique to hMC2R potentially responsible. The sequence of

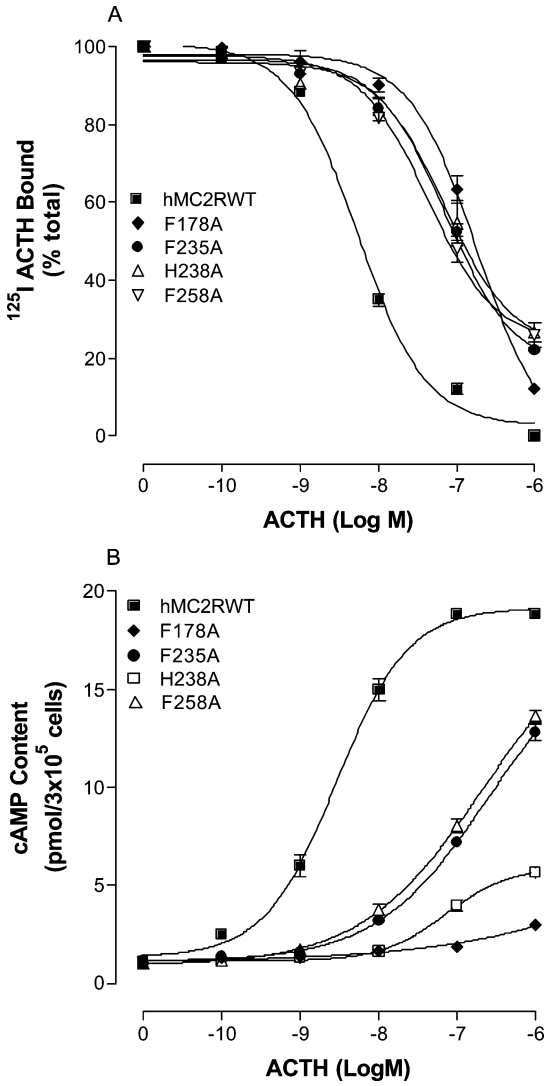

FIGURE 5: Effect of the mutations of various conserved, aromatic amino acids in the hMC2R on the ACTH-binding affinity and receptor activity. A shows the ACTH-binding affinity of these mutant receptors. B shows the ability of ACTH to stimulate cAMP production upon binding to the mutant receptors ($n = 3$; see Tables 2 for actual K_i and EC_{50} values).

amino acids in TMs of hMC2R was compared with other MCRs, and six amino acid residues are revealed to be unique, including N81 in TM2, D104 and F108 in TM3, T164 and F168 in TM4, and F244 in TM6. To assess their role in determining ACTH-specific binding and activity, each residue was individually mutated with alanine and evaluated as above. Mutant receptors were expressed at the cell surface, but expression levels of the mutations N81A, D104N, F108A, and F168A were found lower than that of the hMC2R WT (Table 3). ACTH dose-dependently displaced ^{125}I-ACTH binding at these mutations, but the binding affinity of ACTH at mutations N81, D104, F108, and F168 was significantly reduced (Figures 6A and 7A and Table 3). Consistent with the binding results, the mutations N81A, D104A, F108A, and F168A significantly reduced ACTH-mediated cAMP production (Figures 6B and 7B and Table 3).

DISCUSSION

The melanocortin peptides α-MSH and ACTH belong to a group of neuropeptides derived from the pro-opiomelano-

Table 3: Effect of the Substitutions of Unique Amino Acid Residues of hMC2R on ^{125}I-ACTH Binding and cAMP Production

	receptor expression (percent of WT)	^{125}I-ACTH binding K_i (nM)	cAMP production EC_{50} (nM)
hMC2R WT	100	8.6 ± 1.0	4.8 ± 0.1
N81A	89 ± 12.4	15.9 ± 0.6[a]	11.6 ± 1.2[a]
D104N	78 ± 13.4	87.3 ± 9.8[a]	87.7 ± 9.3[a]
F108A	65 ± 10.2	>10^3 [a]	>10^3 [a]
T164A	93 ± 14.2	6.4 ± 0.7	3.9 ± 0.1
F168A	56 ± 9.5	217.8 ± 13[a]	327.6 ± 30.3[a]
F244A	95 ± 11.2	9.9 ± 0.9	4.9 ± 0.9

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

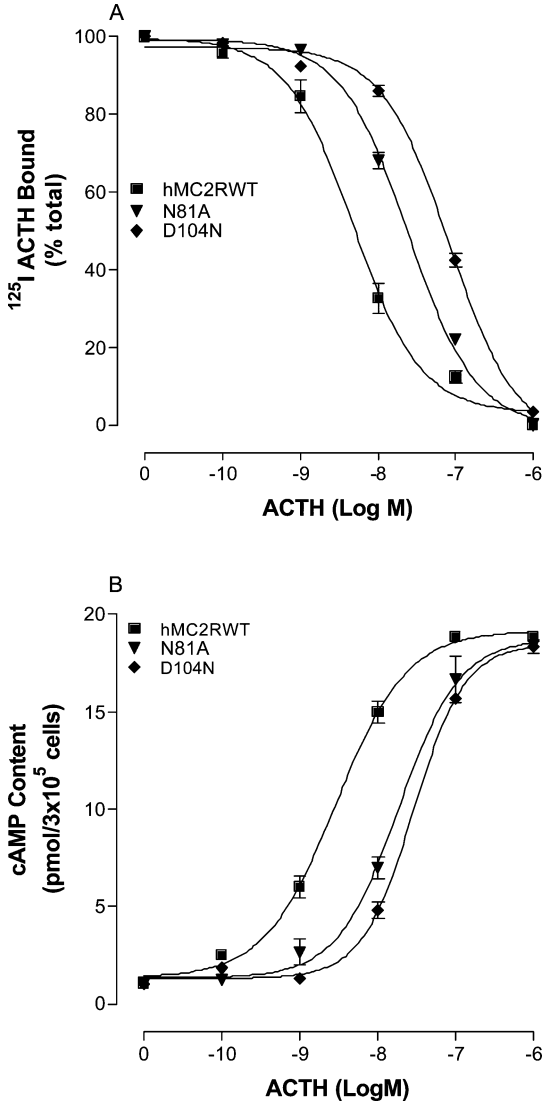

FIGURE 6: Effects of the mutations of unique, charged amino acid hMC2R TM residues on the ACTH-binding affinity and receptor activity. A shows the ACTH-binding affinity of these mutants. B shows the ability of ACTH-stimulated cAMP production at these mutants ($n = 3$; see Tables 3 for actual K_i and EC_{50} values).

cortin prohormone and contain the common amino acid sequence, His-Phe-Arg-Trp (*23*, *27*, *51*). These residues in MSH and ACTH were identified as important residues for ligand binding and biological activities at hMC1R, hMC3R, and hMC4R (*31*−*34*, *56*). However, this tetrapeptide was not able to bind and activate MC2R. Facilitated by the cloning of MC2R, studies with cells expressing transfected hMC2R indicated that α-MSH was not able to bind to MC2R

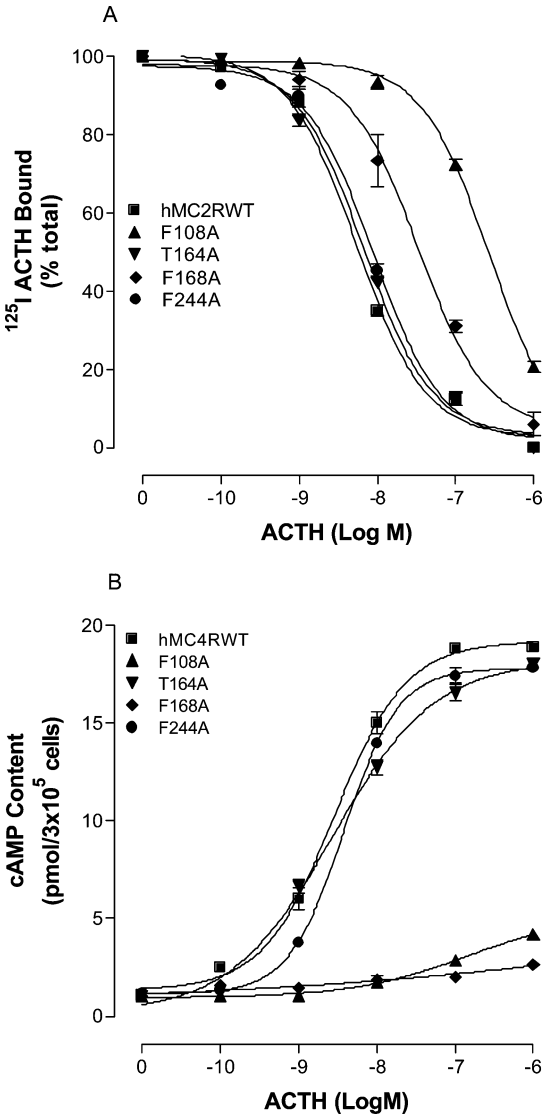

FIGURE 7: Effect of the mutations of various unique, aromatic amino acids in the hMC2R on the ACTH-binding affinity and receptor activity. A shows the ACTH-binding affinity of these mutant receptors. B shows the ability of ACTH to stimulate cAMP production upon binding to the mutant receptors ($n = 3$; see Tables 3 for actual K_i and EC_{50} values).

and ACTH1−17 is the minimal peptide for receptor binding and activation (*3*, *36*, *48*). In our study, we transfected hMC2R into OS3 cells, which are an adrenal cell line lacking endogenous MC2R, and determined which region of ACTH is important for hMC2R binding and activation. Our results indicate that ACTH1−24 is the most potent truncated peptide among those tested. ACTH loses its activity at MC2R if the length of the peptide is less than 16 amino acids in the N terminus, which is one amino acid less than the length of the peptide reported before (*36*). Our results also indicate that the N terminus of ACTH is also important for ligand binding and receptor activation because peptide ACTH6−24 completely loses its agonist activity, which are consistent with the previous reports (*5*, *24*). Our results therefore support the model proposed that three key regions of ACTH are important for ACTH binding and signaling at hMC2R (*36*). One region is located in ACTH1−5, which is important for receptor activation but not for ligand binding. The second region is located in ACTH 6−9 (HFRW), which is important for binding and activation. The third one is located between

residues 14 and 16 of ACTH that is critical for hMC2R binding and activation, which is different from that of other MCRs. The results of truncated ACTH peptides also suggest that the binding pockets of MC2R may differ structurally from that of other MCRs.

Extensive studies have been performed to examine the molecular basis of MC1R, MC3R, and MC4R responsible for ligand binding and signaling (*6*, *62*, *64*), with conserved residues in TMs of MCRs emerging as key elements that use proposed electrostatic and hydrophobic forces to facilitate both MSH binding and receptor activation (*29*). MCR subtypes are believed to have the same basic molecular architecture, and not surprisingly, hMC2R shares nearly 50% identity with other MCRs in the putative TM regions (*18*−*20*, *41*). To determine the molecular basis of hMC2R responsible for ACTH binding and activity, we first examined the roles of the highly conserved residues across the MC1−4 receptor subtypes, which have been previously identified to have functional significance. This included 10 conserved amino acid residues of hMC2R that had previously been shown to modulate ligand binding and activity in MC1R, MC3R, and MC4R (*6*, *7*, *15*, *26*, *30*, *62*, *64*). Our findings support the hypothesis that conserved residues E80 in TM2, D107 in TM3, F178 in TM4, and F235 and H238 in TM6 are involved in ACTH binding; therefore, hMC2R uses similar structural elements to that seen in other MCRs (*6*, *15*, *26*, *62*, *64*). However, significant differences between MC2R and other MCRs were also identified in this study. hMC2R has three aspartic acids (D103, D104, and D107) in TM3, but other MCRs have only two (D117 and D121 in hMC1R, D154 and D158 in hMC3R, and D122 and D126 in hMC4R). Residue D104 in TM3 of hMC2R is unique among the MCRs. Our observation that D103 did not dramatically alter ACTH-binding affinity and potency indicate that, unlike MC1R, MC3R, and MC4R, the residue D103 in hMC2R may not be functionally important for ligand binding. Instead, residue D104 in TM3 is important in ACTH binding and activity because mutation of this residue significantly decreased both ACTH-binding affinity and potency. Our analysis of D107N is also consistent with the results identified in FGD, in which mutation of D107N resulted in receptor malfunction (*43*). Our results suggest that hMC2R shares similar ionic-binding sites with hMC1R, hMC3R, and hMC4R. Altogether, these data suggest a model for the ACTH and hMC2R interaction (Figure 8), in which an ionic pocket formed by amino acid residues E80 in TM2 and D104 and D107 in TM3 of hMC2R along with a second hydrophobic binding pocket consisting of F235 and H238 in TM6 of hMC2R. In addition, two unique amino acid residues, F178 in TM5 and in TM7 of hMC2R, may be involved in the Lys15−Lys16 interaction.

MC2R is unique among MCRs because of the fact that ACTH is its only endogenous ligand. We speculate that the binding pockets of MC2R may be substantially different from those of other MCRs and D104 may play an important role in differential recognition of ACTH or MSH at MC2R. We examined the effect of α-MSH at mutation D104N, but this mutation did not increase MSH binding, hereby suggesting that this residue is not crucial for MSH binding (data not shown). By integrating mutagenesis and truncated ligand studies, we present data indicating that several similarities exist between the hMC2R and other hMCRs. First, all MCR

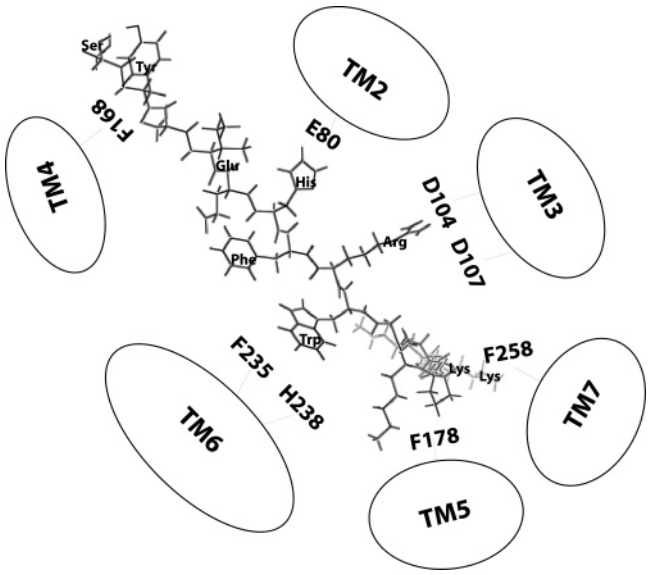

FIGURE 8: Two-dimensional representation of a proposed three-dimensional model illustrating ACTH1−16 docked inside the hMC2R. On the basis of our results, three main receptor-binding pockets are proposed. The first is a predominantly ionic pocket formed by E80, D104, and D107. The second binding pocket is formed by aromatic residues F235 and H238 in TM6. The third binding pocket is formed by F178 in TM5 and F258 in TM7.

subtypes are fully activated by ACTH1−16. Second, the mutation of homologous residues in TM2, TM3, and TM6 of hMC1R, hMC2R, hMC3R, and hMC4R are found to affect agonist-binding affinity (hMC1R residues E94, D117, D121, and H260, hMC3R residues E131, D154, D158, and H258, and hMC4R residues E100, D122, D126, and H264 are homologous to hMC2R residues E80, D104, D107, and H238) (*6*, *62*, *64*). However, the present studies did identify a potentially important subtype-specific difference between the hMC2R and other hMCRs. TM4, TM5, and TM7 of hMC2R are important for ACTH binding and signaling because mutation of residue F168 (Y182 in MC1R, Y219 in MC3R, Y180 in MC5R, and Y187 in MC4R) in TM4, F178 (L192 in MC1R, L229 in MC3R, and L190 in MC5R) in TM5, and F258 in TM7 affects both ACTH-binding affinity and potency. This observation suggests that hMC2R may have a broad binding pocket in which conserved amino acid residues are involved in ACTH binding, which are similar to that of other MCRs, but some unique amino acid residues are perhaps crucial for ACTH14−16 binding and activity. This may explain why α-MSH is not able to bind hMC2R, although α-MSH shares the first 13 amino acids with ACTH1−39.

Activation of GPCRs has been proposed to involve the rotation of TMs with the outward movement of their cytoplasmic ends (*21*). This theoretically would enable G proteins to interact with some of the intracellular loops as well as the C-terminal tail of GPCRs. Some previous MCR studies support this theory because mutations of some TM residues of MCRs can result in the inactivation of MCRs (*6*, *64*). Our current results indicate that mutations of the homologous residues of hMC2R (D70A and D272A) abolish both ACTH binding and receptor signaling because these two mutated receptors are not fully expressed at the cell surface. Altered receptor function may reflect the loss of cell-surface receptor expression. This suggests that D70 and D272 in hMC2R play important roles in receptor expression on

the plasma membrane, and their role in hMC2R is likely similar to that of MC3R and MC4R.

In summary, our results indicate that ACTH1−16 is the minimal peptide required for hMC2R binding and signaling. Both conserved and unique residues of hMC2R are required to form a broad binding pocket for ACTH binding and signaling, which includes TM2, TM3, TM4, TM6, and TM7 of hMC2R. These results may provide important information about the molecular determinants of hMC2R responsible for ACTH binding and receptor signaling.

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