Functions of the DRY motif and intracellular loop 2 of human melanocortin 3 receptor

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Abstract

The melanocortin 3 receptor (MC3R) regulates several physiological functions, including feed efficiency, nutrient partitioning, fasting response, natriuresis, and immune reactions. Naturally occurring mutations in the *MC3R* gene have been shown to be associated with increased adiposity and lung diseases such as tuberculosis and cystic fibrosis. The DRY motif at the cytoplasmic end of transmembrane domain 3 (TM3) and the second intracellular loop 2 (ICL2) are known to be important for receptor function in several G protein-coupled receptors (GPCRs). To gain a better understanding of the functions of this domain in MC3R, we performed alanine-scanning mutagenesis on 18 residues. We showed that alanine mutation of 11 residues reduced the maximal binding and maximal cAMP production stimulated by agonists. Mutation of two residues did not change maximal binding but resulted in impaired signaling in the G_s -cAMP pathway. Mutation of five residues impaired signaling in the ERK1/2 pathway. We have also shown that alanine mutants of seven residues that were defective in the cAMP pathway were not defective in the ERK1/2 pathway, demonstrating biased signaling. In summary, we demonstrated that the cytoplasmic end of TM3 and the ICL2 were critical for MC3R function. We also reported for the first time biased signaling in MC3R.

Key Words

- ▶ melanocortin 3 receptor
- ▶ intracellular loop 2
- ▶ DRY motif
- ▶ MAP kinase
- ▶ biased signaling

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Introduction

The melanocortin 3 receptor (MC3R) was identified in 1993 as a receptor for α - and γ -melanocyte-stimulating hormones (MSHs) (Gantz *et al.* 1993, Roselli-Rehfuß *et al.* 1993). It is highly expressed in the hypothalamus, especially in the ventromedial nucleus and arcuate nucleus, as well as in several peripheral tissues, such as the heart, liver, lung, kidney, and macrophages (Gantz *et al.* 1993, Roselli-Rehfuß *et al.* 1993, Ni *et al.* 2003, Getting *et al.* 2006). Co-expressed with proopiomelanocortin (POMC) in the arcuate nucleus, MC3R acts as an

inhibitory autoreceptor, providing a short-loop feedback to regulate POMC neuronal activity (Cowley *et al.* 2001).

MC3R plays vital roles in regulating various physiological functions. It regulates feed efficiency and nutrient partitioning; *Mc3r* knockout mice have elevated fat mass and reduced lean mass (Butler *et al.* 2000, Chen *et al.* 2000). More recently, several studies have shown that MC3R is required for normal nutrient anticipatory activities and fasting responses (Sutton *et al.* 2008, 2010, Renquist *et al.* 2012). Deletion of *Mc3r* results in

Cushing-like phenotypes with increased basal corticosterone levels and decreased bone density (Renquist *et al.* 2012). Consistent with an important role of MC3R in regulating energy homeostasis, mutations in MC3R have been identified to be associated with human obesity or adiposity (Tao 2010) as well as lung diseases including tuberculosis and cystic fibrosis (Cooke *et al.* 2008, Wright *et al.* 2011). In addition to the metabolism-related functions, MC3R is also involved in the regulation of natriuresis (Ni *et al.* 2003) and immune functions (Getting *et al.* 2001, 2006).

MC3R is a member of rhodopsin-like family A G protein-coupled receptors (GPCRs). Activation of MC3R results in GDP/GTP exchange in the stimulatory G protein (G_s), which then activates adenylyl cyclase to increase the production of cAMP. Although two studies have reported that MC3R activation stimulates ERK1/2 phosphorylation (Chai *et al.* 2007, Begriche *et al.* 2012), an earlier study has reported that while MC4R activation leads to ERK1/2 phosphorylation, MC3R activation does not (Daniels *et al.* 2003). The existence of biased signaling has been reported in multiple GPCRs with important therapeutic implications (for reviews, see Violin & Lefkowitz (2007), Rajagopal *et al.* (2010) and Reiter *et al.* (2012)). In addition to biased ligands, biased signaling may also be induced by mutations in the GPCRs (Rajagopal *et al.* 2010). We and others have previously identified both biased ligands and receptors in MC4R (Patten *et al.* 2007, Büch *et al.* 2009, Huang & Tao 2012, Mo *et al.* 2012, Mo & Tao 2013; reviewed by Breit *et al.* (2011) and Tao (2014)). However, until now, no biased signaling of MC3R has been reported. Therefore, it is important to determine whether MC3R mediates ERK1/2 phosphorylation and whether biased signaling also exists in MC3R.

The highly conserved DRYxxI motif at the cytoplasmic end of transmembrane domain 3 (TM3) and the intracellular loop 2 (ICL2) are known to be critical for receptor function in several GPCRs. DRY motif forms salt bridges with surrounding residues and with TM6, which forms an ionic lock constraining the receptor in an inactive conformation (Palczewski *et al.* 2000, Rosenbaum *et al.* 2009). Upon ligand binding, the ionic lock is broken and DRY forms new interactions with TM5, stabilizing the receptor in an active conformation (Palczewski *et al.* 2000, Rosenbaum *et al.* 2009). ICL2 forms a L-shaped structure or a short α -helix parallel to the cell membrane in different GPCRs (Palczewski *et al.* 2000, Rosenbaum *et al.* 2009, Chien *et al.* 2010). ICL2 directly interacts with $G\alpha$ and β -arrestin and therefore is important for G-protein coupling and receptor desensitization (Han *et al.* 2001,

Rasmussen *et al.* 2011). Until now, two residues of MC3R in this region, D178 and I183, have been previously reported to be important for MC3R function (Tao & Segaloff 2004, Wang *et al.* 2008). Hence, our knowledge about the function of the DRYxxI motif and ICL2 of MC3R is still very limited. In this study, we investigated the importance of each residue in the DRYxxI motif and ICL2 in regulating receptor cell surface expression, ligand binding, and their different contributions to the two signaling pathways of MC3R.

Materials and methods

Materials

[125 I]-[Nle⁴,D-Phe⁷]- α -MSH (NDP-MSH) was purchased from the American Radiolabeled Chemicals (St Louis, MO, USA), NDP-MSH from Peptides International (Louisville, KY, USA), and α -MSH from Pi Proteomics (Huntsville, AL, USA). Radiolabeled cAMP was iodinated using the chloramine T method (Tao *et al.* 2010). The N-terminal 3 $\times$ HA tagged WT human MC3R (hMC3R) cloned into pcDNA3.1 was obtained from Missouri S&T cDNA Resource Center (<http://www.cdna.org/>; Rolla, MO, USA).

Site-directed mutagenesis

Mutant hMC3Rs were generated from the WT receptor using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) as described in detail previously (Tao & Segaloff 2003) using primers listed in Table 1. The presence of desired mutations was verified by direct nucleotide sequencing by the DNA Sequencing Facility of University of Chicago Cancer Research Center (Chicago, IL, USA).

Cell culture and transfection

Human embryonic kidney 293T (HEK293T) cells and Neuro2a cells were purchased from American Type Culture Collection (Manassas, VA, USA). HEK293T cells were cultured in DMEM supplemented with 10% newborn calf serum, 10 mM HEPES, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin B. Neuro2a cells were cultured in the same media except that it was supplemented with 10% fetal bovine serum. Cells were seeded into gelatin-coated six-well clusters and were transfected with 1 μ g plasmid/well using the calcium phosphate precipitation method for HEK293T cells as described previously (Tao *et al.* 2010) or using jetPRIME

Table 1 Forward primer sequences that were used for site-directed mutagenesis studies. The mutated codons are underlined

Constructs	Primer sequences
D178A	GCCATCGCCGTCGCCAGGTACGTCACC
R179A	CCATCGCCGTCGACGCGTACGTCACCATC
Y180A	CATCGCCGTCGACAGGGCCGTCACCATCTTTAC
V181A	GTCGACAGGTACGCCACCATCTTTTAC
T182A	CGACAGGTACGTCGCCCATCTTTTACGC
I183A	CAGGTACGTCACCGCCTTTTACGCGCTC
F184A	CAGGTACGTCACCATCGCTTACGCGCTCCGCTAC
Y185A	GTACGTCACCATCTTTGCCGCGCTCCGTACCCAC
A186G	CACCATCTTTTACGGGCTCCGCTACCCAC
L187A	CACCATCTTTTACGGGCTCCGCTACCCACAGCATC
R188A	CATCTTTTACGCGCTCGCCTACCACAGCATCATG
Y189A	CTTTTACGCGCTCCGCGCCACAGCATCATGACC
H190A	CGCGCTCCGCTACGCCAGCATCATGACCG
S191A	GCGTCCGCTACCCAGCCATCATGACCGTGAG
I192A	GCTCCGCTACCCAGGCCATGACCGTGAGGAAG
M193A	CGTACCACAGCATCGCGACCGTGAGGAAGGC
T194A	CCACAGCATCATGGCCGTGAGGAAGGC
V195A	CACAGCATCATGACCGCGAGGAAGGCCCTCACC

transfection reagent (Polyplus-transfection, New York, NY, USA) for Neuro2a cells (Huang & Tao 2014, Tao & Huang 2014). Approximately 48 h later, cells were used for ligand binding, signaling, or flow cytometry studies. For western blot, HEK293T cells were plated into 100 mm dishes coated with 0.1% gelatin and were transfected using the calcium phosphate precipitation method.

Flow cytometry

HEK293T and Neuro2a cells were seeded and transfected as described above. On the day of experiment, cells in six-well clusters were placed on ice, washed once with ice-cold PBS for immunohistochemistry (PBS-IH; Tao & Segaloff 2003), detached, and then pelleted by centrifugation at 500 *g* for 5 min. To measure the total expression of receptors, cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 in PBS-IH for 15 min, and then blocked with 5% BSA in PBS-IH for 1 h. To measure the cell surface expression of receptors, cells were directly incubated with the primary antibody after centrifugation. The primary antibody anti-HA.11 (Covance, Princeton, NJ, USA) was added at a dilution of 1:50 in 0.5% BSA in PBS-IH. One hour later, cells were washed once with 0.5% BSA in PBS-IH and then were incubated with the secondary antibody Alexa Fluor 488-labeled goat anti-mouse IgG (Invitrogen) for 1 h. Cells were washed once with 0.5% BSA in PBS-IH and assayed using a C6 Accuri Cytometer

(Accuri Cytometers, Ann Arbor, MI, USA). Cells transfected with the empty vector pcDNA3.1 were used for background staining. Calculations were carried out as described previously (Wang *et al.* 2008).

Ligand binding assay

HEK293T cells were seeded and transfected as described above. On the day of experiment, cells were washed twice with warm Waymouth's MB752/1 media (Sigma-Aldrich) containing 1 mg/ml BSA (Waymouth/BSA). Then cells were incubated with 100 000 c.p.m. of ¹²⁵I-NDP-MSH and with or without different concentrations of unlabeled NDP- or α -MSH at 37 °C. One hour later, cells were washed twice with ice-cold Hank's balanced salt solution containing 1 mg/ml BSA to terminate the reaction and then were lysed by 100 μ l 0.5 M NaOH. Cell lysates were collected by cotton swabs and counted in a gamma counter. GraphPad Prism (San Diego, CA, USA) was used to calculate receptor occupancy (RO, as an estimate of maximal binding; Wang *et al.* 2008) and the concentration that results in 50% inhibition (IC₅₀).

cAMP assay

HEK293T cells were seeded and transfected as described above. On the day of experiment, cells were washed twice with warm Waymouth/BSA. Then cells were incubated with fresh Waymouth/BSA containing 0.5 mM isobutylmethylxanthine (Sigma-Aldrich) at 37 °C 15 min before the addition of different concentrations of NDP- or α -MSH. One hour after incubation with the ligand, cells were lysed with 0.5 M perchloric acid containing 180 μ g/ml theophylline (Sigma-Aldrich). The solution was neutralized with 0.72 M KOH/0.6 M KHCO₃. Intracellular cAMP levels were measured using RIA as described previously (Tao *et al.* 2010).

Protein preparation and western blot

The method for protein preparation and western blot was described previously in detail (Huang & Tao 2012, Mo *et al.* 2012). Briefly, HEK293T cells were seeded and transfected as described above. At 24 h after transfection, cells were starved in Waymouth/BSA for 24 h at 37 °C. On the day of experiment, cells were stimulated with or without 10⁻⁶ M NDP-MSH for 5 min at 37°C. Cells were lysed in 0.5% NP-40 lysis buffer and total protein concentrations of cell lysates were measured using the Bradford protein assay. Thirty microgram samples were subjected to 10%

SDS-PAGE and then transferred onto PVDF membranes. Membranes were incubated with 10% non-fat dry milk containing 0.2% Tween 20 for at least 3 h at room temperature, and then immunoblotted with a rabbit phosphorylated ERK1/2 (pERK1/2) antibody (Miyaji *et al.* 2009) (Cell Signaling, Billerica, MA, USA) at a dilution of 1:1000 and a mouse β -tubulin antibody (Chu & Klymkowsky 1989) (Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, IA, USA) at a dilution of 1:5000 in 5% BSA in Tris-buffered saline containing Tween 20 (TBST) overnight at 4 °C. After three washes in TBST, membranes were incubated with horseradish peroxidase-linked antibodies, an anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA, USA) at a dilution of 1:2000, and an anti-mouse antibody (Jackson ImmunoResearch) at a dilution of 1:5000 in 10% non-fat dry milk for 2 h at room temperature. Antibody binding was detected with ECL reagent (Thermo Scientific, Rockford, IL, USA), and staining intensity was determined using the ImageJ Software (NIH, Bethesda, MD, USA).

Statistical analysis

All data analyses were performed using the GraphPad Prism 4.0 Software. To compare cell surface expression

levels, pERK1/2 levels as well as the binding and signaling parameters between WT and mutant hMC3Rs, Student's *t*-test was carried out.

Results

Cell surface expression of the mutant hMC3Rs

To determine the function of each residue at the cytoplasmic end of TM3 and ICL2, we performed alanine-scanning mutagenesis by mutating each residue to alanine or mutating alanine to glycine (Table 1). We generated a total of 18 mutants (Fig. 1).

We first quantified the cell surface expression levels of the mutant hMC3Rs. HEK293T cells and Neuro2a cells transiently transfected with the 3×HA-tagged WT or mutant hMC3Rs were used for flow cytometry studies as described in 'Materials and methods' section. We found that no mutant had decreased cell surface expression levels in HEK293T cells (Fig. 2A). To confirm the results, we further performed flow cytometry studies using Neuro2a cells because MC3R is widely expressed in the CNS. Consistently, no mutant had significantly decreased total expression levels (data not shown) or cell surface expression levels (Fig. 2B) in Neuro2a cells. Compared

Figure 1

Schematic of hMC3R. Residues that were found to be important for the receptor function are indicated.

Figure 2

Cell surface expression of the WT and mutant hMC3Rs. HEK293T cells (A) or Neuro2a cells (B) transiently transfected with WT or mutant hMC3Rs were stained with Alexa Fluor 488-conjugated antibody and the immunostaining was measured using flow cytometry. Results are expressed as mean $\pm$ S.E.M. of at least three independent experiments. *Significantly different from the cell surface expression of WT hMC3R, $P < 0.05$.

with the WT hMC3R, two mutants (A186G and M193A) had a 40% reduction in cell surface expression levels that did not reach statistical significance in Neuro2a cells (Fig. 2B).

Ligand-binding properties of the mutant hMC3Rs

To study ligand binding and signaling properties of the mutant hMC3Rs, two ligands of MC3R, NDP-MSH, and α -MSH were used. NDP-MSH is a superpotent agonist and α -MSH is an endogenous agonist of the melanocortin receptors except for the MC2R. Both ligands are widely used in MC3R studies.

To perform the competitive binding assay, HEK293T cells transiently transfected with the WT or mutant hMC3Rs were incubated with the radiolabeled ^{125}I -NDP-MSH and different concentrations of unlabeled ligands. NDP-MSH was first studied as the unlabeled ligand. Our data showed that three mutants, D178A, M193A,

and T194A, had no detectable binding (Table 2 and Fig. 3). Four mutants, R179A, Y180A, I183A, and I192A, had significantly decreased IC_{50}s compared with the WT hMC3R and therefore increased affinities with the ligand (Table 2 and Fig. 3).

The competitive binding assay was then performed using α -MSH as the ligand. Consistently, three mutants, D178A, M193A, and T194A, had no measurable binding, and one mutant I192A had decreased IC_{50}s (Table 3 and Fig. 4). By contrast, five mutants, R179A, Y180A, T182A, F184A, and L187A, had increased IC_{50}s (Table 3 and Fig. 4).

RO values were also calculated from the binding assays. In addition to the three mutants with no detectable binding, eight mutants, R179A, Y180A, I183A, Y185A, A186G, R188A, Y189A, and I192A, had significantly decreased RO values compared with the WT hMC3R (Table 2). Two mutants, T182A and L187A, had increased RO by $\sim 50\%$ (Table 2).

Signaling properties of the mutant hMC3Rs in the G_s -cAMP signaling pathway

To study whether residues in the DRYxxI motif and ICL2 are required for MC3R signaling in the G_s -cAMP pathway, HEK293T cells transfected with WT or mutant hMC3Rs were stimulated with different concentrations of the ligand and intracellular cAMP accumulation was measured using RIA. We first studied the signaling properties in response to NDP-MSH. We showed that NDP-MSH increased intracellular cAMP production of WT hMC3R with an EC_{50} of 0.61 nM in a dose-dependent manner. Four mutants, R179A, I183A, M193A, and T194A, had no response to NDP-MSH stimulation (Table 2 and Fig. 5). Additionally, nine mutants, D178A, Y180A, V181A, T182A, Y185A, A186G, L187A, R188A, and I192A, had significantly decreased maximal cAMP production (R_{max}) compared with the WT hMC3R (Table 2 and Fig. 5). Although there was no measurable binding, D178A responded to NDP-MSH stimulation with a reduced R_{max} , 13% of the WT hMC3R. EC_{50} values of the mutants were also analyzed compared with the WT hMC3R. We showed that two mutants, T182A and L187A, had increased EC_{50}s (Table 2 and Fig. 5).

The cAMP assay was also carried out using α -MSH as the ligand. The EC_{50} of α -MSH was 3.3 nM for the WT hMC3R. Three mutants, I183A, M193A, and T194A, did not respond to α -MSH stimulation. Additionally, nine mutants, D178A, R179A, Y180A, Y185A, A186G, L187A, R188A, Y189A, and I192A, had significantly reduced R_{max}

Table 2 The ligand binding, basal, and NDP-MSH-stimulated signaling properties of WT and mutant hMC3Rs. Values are expressed as mean $\pm$ S.E.M. of at least three independent experiments for IC_{50} , EC_{50} , and R_{max} , and at least six independent experiments for RO and basal activities. The R_{max} of WT hMC3R was 2410.86 ± 341.73 pmol/ 10^6 cells with NDP-MSH stimulation

hMC3R construct	NDP-MSH binding		Basal activities (% WT)	NDP-MSH-stimulated cAMP		NDP-MSH-stimulated ERK1/2
	IC_{50} (nM)	RO (% WT)		EC_{50} (nM)	R_{max} (% WT)	
WT	3.08 ± 0.69	100 ± 0	100 ± 0	0.61 ± 0.05	100 ± 0	+
D178A	ND	ND	87.88 ± 16.26	1.95 ± 0.67	13.19 ± 1.28^a	+
R179A	0.98 ± 0.06^b	50.85 ± 4.69^a	91.25 ± 13.86	ND	ND	+
Y180A	0.72 ± 0.16^c	13.41 ± 3.53^a	66.23 ± 11.04^c	1.22 ± 0.34	10.06 ± 1.95^a	+
V181A	1.84 ± 0.32	103.20 ± 13.55	144.59 ± 16.81^c	0.49 ± 0.12	70.46 ± 10.82^c	+
T182A	3.07 ± 0.33	154.30 ± 21.92^c	122.14 ± 20.05	495.33 ± 166.65^c	61.59 ± 5.25^b	+
I183A	1.16 ± 0.11^c	45.90 ± 5.95^a	94.7 ± 17.92	ND	ND	+
F184A	2.00 ± 0.29	91.21 ± 9.50	79.67 ± 7.72^c	0.46 ± 0.10	96.43 ± 10.58	—
Y185A	2.62 ± 0.86	39.16 ± 5.54^a	111.96 ± 15.87	0.73 ± 0.22	59.22 ± 7.18^c	—
A186G	5.94 ± 2.25	77.43 ± 7.58^c	86.86 ± 14.43	1.26 ± 0.35	42.26 ± 15.82^c	—
L187A	3.81 ± 0.65	152.40 ± 16.56^c	47.51 ± 10.11^b	3.66 ± 1.24^c	8.65 ± 2.23^a	+
R188A	1.61 ± 0.26	50.28 ± 5.07^a	74.4 ± 10.57	0.95 ± 0.17	39.03 ± 7.44^b	—
Y189A	4.69 ± 1.50	53.34 ± 8.10^a	69.45 ± 14.37	1.05 ± 0.23	69.25 ± 18.45	+
H190A	3.34 ± 1.01	78.47 ± 13.53	94.97 ± 18.38	0.47 ± 0.16	77.13 ± 19.82	+
S191A	2.17 ± 0.34	97.93 ± 11.05	84.92 ± 12.02	0.43 ± 0.11	91.64 ± 13.86	—
I192A	1.17 ± 0.31^c	24.33 ± 5.32^a	78.43 ± 16.24	0.49 ± 0.05	26.30 ± 7.00^b	+
M193A	ND	ND	62.87 ± 16.74	ND	ND	—
T194A	ND	ND	67.25 ± 17.72	ND	ND	—
V195A	4.16 ± 1.36	88.99 ± 14.83	98.9 ± 14.74	0.55 ± 0.18	99.45 ± 34.83	+

ND, not detected; +, activated by 10^{-6} M NDP-MSH; —, not activated by 10^{-6} M NDP-MSH.

^aSignificantly different from WT hMC3R, $P < 0.001$.

^bSignificantly different from WT hMC3R, $P < 0.01$.

^cSignificantly different from WT hMC3R, $P < 0.05$.

(Table 3 and Fig. 6). The results were consistent with the results using NDP-MSH with three exceptions, R179A, Y189A, and S191A. Although they did not respond to NDP-MSH stimulation, R179A partially responded to α -MSH stimulation with an R_{max} 16% of the WT hMC3R. Y189A and S191A had similar R_{max} values with NDP-MSH, but a reduced R_{max} value with α -MSH. When EC_{50} s of the mutants were analyzed, six mutants, D178A, R179A, Y180A, T182A, A186G, and L187A, had a dramatic decrease in R_{max} (by more than 40%) and significantly increased EC_{50} s (Table 3 and Fig. 6).

The basal signaling of MC3R is low compared with another neural melanocortin receptor, the MC4R (Tao 2007, Tao *et al.* 2010). To study whether the mutants had altered basal signaling, intracellular cAMP concentrations were measured in the absence of any ligand. Our data showed that one mutant, V181A, had significantly increased basal cAMP levels, increased by $\sim 50\%$ compared with the WT hMC3R. Three mutants, Y180A, F184A, and L187A, had significantly reduced basal cAMP levels (Table 2). The other mutants had similar basal cAMP levels as the WT hMC3R (Table 2).

Figure 3

Ligand-binding properties of the WT and mutant hMC3Rs with NDP-MSH as the ligand. Intact cell surface binding was measured by competitive inhibition of 125 I-NDP-MSH with different concentrations of unlabeled NDP-MSH. Results are expressed as percentage of WT maximal binding. Each point is expressed as mean $\pm$ S.E.M. of duplicate measurements within one experiment. All curves are representative of at least three independent experiments.

Table 3 The ligand binding and signaling properties of WT and mutant hMC3Rs in response to α -MSH stimulation. Values are expressed as mean $\pm$ S.E.M. of at least three independent experiments. The $R_{\max}$ of WT hMC3R was 2851.50 ± 466.51 pmol/ 10^6 cells with α -MSH stimulation

hMC3R construct	α -MSH binding	α -MSH-stimulated cAMP	
	IC ₅₀ (nM)	EC ₅₀ (nM)	$R_{\max}$ (% WT)
WT	66.88 $\pm$ 4.57	3.30 $\pm$ 1.04	100 $\pm$ 0
D178A	ND	42.08 $\pm$ 13.49 ^b	9.04 $\pm$ 4.16 ^c
R179A	280.50 $\pm$ 57.87 ^a	63.04 $\pm$ 10.17 ^b	16.07 $\pm$ 2.35 ^c
Y180A	84.56 $\pm$ 3.99 ^a	62.38 $\pm$ 9.81 ^b	10.19 $\pm$ 3.30 ^c
V181A	45.67 $\pm$ 9.66	2.54 $\pm$ 0.85	89.33 $\pm$ 6.80
T182A	182.36 $\pm$ 31.63 ^a	17.44 $\pm$ 6.39 ^b	62.89 $\pm$ 14.48
I183A	62.42 $\pm$ 9.63	ND	ND
F184A	107.99 $\pm$ 7.56 ^b	4.37 $\pm$ 2.17	111.96 $\pm$ 16.62
Y185A	45.46 $\pm$ 8.09	7.12 $\pm$ 2.94	44.80 $\pm$ 10.91 ^b
A186G	73.55 $\pm$ 6.67	64.98 $\pm$ 17.28 ^a	36.40 $\pm$ 1.58 ^c
L187A	110.61 $\pm$ 13.32 ^a	65.96 $\pm$ 11.55 ^b	10.79 $\pm$ 1.18 ^c
R188A	46.48 $\pm$ 6.13	4.43 $\pm$ 0.63	30.67 $\pm$ 7.67 ^c
Y189A	83.14 $\pm$ 13.83	5.47 $\pm$ 0.79	46.41 $\pm$ 7.63 ^b
H190A	57.31 $\pm$ 2.67	1.28 $\pm$ 0.29	113.66 $\pm$ 15.73
S191A	56.82 $\pm$ 3.87	1.29 $\pm$ 0.36	82.82 $\pm$ 7.00
I192A	30.89 $\pm$ 2.55 ^a	2.92 $\pm$ 0.49	9.38 $\pm$ 2.39 ^c
M193A	ND	ND	ND
T194A	ND	ND	ND
V195A	69.28 $\pm$ 11.74	1.50 $\pm$ 0.48	136.25 $\pm$ 21.23

ND, not detected.

^aSignificantly different from WT hMC3R, $P < 0.05$.

^bSignificantly different from WT hMC3R, $P < 0.01$.

^cSignificantly different from WT hMC3R, $P < 0.001$.

Signaling properties of the mutant hMC3Rs in the ERK1/2 signaling pathway

To study the ERK1/2 signaling pathway of MC3R, HEK293T cells starved for 24 h were stimulated with or without 10^{-6} M NDP-MSH for 5 min. We showed that stimulation of WT hMC3R with NDP-MSH in turn stimulated ERK1/2 phosphorylation (Fig. 7). Compared with the basal pERK1/2 level, the pERK1/2 level of WT hMC3R was increased by more than twofold in the presence of NDP-MSH. When the 18 mutants were studied, our data indicated that five mutants (Y185A, A186G, R188A, M193A, and T194A) did not respond to NDP-MSH stimulation (Table 2 and Fig. 7). NDP-MSH stimulation increased ERK1/2 phosphorylation in two mutants, F184A and S191A, although not statistically significant, probably due to larger variation and increased basal ERK1/2 activation. The other 11 mutants had significantly increased ERK1/2 phosphorylation when stimulated by NDP-MSH (Table 2 and Fig. 7).

Discussion

In this study, we systematically studied the function of 18 residues in the DRYxxI motif and the ICL2 of hMC3R.

We showed that i) 11 residues, D178, R179, Y180, I183, Y185, A186, R188, Y189, I192, M193, and T194, were critical for ligand binding and signaling; ii) two residues, T182 and L187, were critical for signaling in the G_s -cAMP

Figure 4

Ligand-binding properties of the WT and mutant hMC3Rs with α -MSH as the ligand. Intact cell surface binding was measured by competitive inhibition of 125 I-NDP-MSH with different concentrations of unlabeled α -MSH. Results are expressed as percentage of WT maximal binding. Each point is expressed as mean $\pm$ S.E.M. of duplicate measurements within one experiment. All curves are representative of at least three independent experiments.

Figure 5

Signaling properties of the WT and mutant hMC3Rs with NDP-MSH as the ligand. HEK293T cells were treated with or without different concentrations of NDP-MSH, and intracellular cAMP accumulations were measured using RIA. Each point is expressed as mean $\pm$ s.e.m. of triplicate measurements within one experiment. All curves are representative of at least three independent experiments.

pathway; iii) five residues, Y185, A186, R188, M193, and T194, were critical for signaling in the ERK1/2 pathway; and iv) seven residues, D178, R179, Y180, T182, I183, L187, and I192, were critical for conferring biased signaling in MC3R.

Alanine or glycine mutations of 11 residues, D178, R179, Y180, I183, Y185, A186, R188, Y189, I192, M193, and T194, did not interfere with the receptor cell surface expression but resulted in decreased RO (Fig. 2, Tables 2 and 3). These mutations might interfere with the ligand binding with a consequent defect in signaling. We have reported previously that D178E and D178Q are expressed well on the cell surface but have either no detectable (D178Q) or reduced RO (D178E) (Wang *et al.* 2008). These 11 residues are located in the cytoplasmic side of MC3R and, therefore, are not expected to directly interact with the ligand. Instead, these residues may indirectly modulate the ligand-receptor interaction through a conformational change. Although it is not a common feature of residues that are important for ligand binding located remotely from the binding sites, observations have been reported previously in MC3R (Wang *et al.* 2008) as well as other GPCRs, such as gonadotropin-releasing hormone receptor (Lu *et al.* 2005) and V2 vasopressin receptor (Erlenbach *et al.* 2001).

It has been suggested that D^{3.49} forms hydrogen bonds with R^{3.50}, which is critical for the conformational switch between inactive and active states of GPCRs

(the superscript numbers refer to the Ballesteros & Weinstein (1995) numbering scheme; Palczewski *et al.* 2000, Rosenbaum *et al.* 2007, Wacker *et al.* 2013). In addition to be involved in multiple intramolecular interactions constraining the receptor in an inactive status (Flanagan 2005), R^{3.50} has also been indicated to directly contact G α stabilizing the active status of the receptor-G protein complex upon ligand binding (Scheerer *et al.* 2008, Rasmussen *et al.* 2011). Compared with D^{3.49} and R^{3.50}, Y^{3.51} is the least conserved in the DRY motif. The side chain of Y^{3.51} has been indicated to direct toward the inside of the receptor and form hydrophobic interaction with P^{3.57} in rhodopsin (Okada *et al.* 2001). Consistent with these well-known findings, we observed that the DRY motif of MC3R also played a vital role in MC3R binding and signaling (Fig. 2, Tables 2 and 3).

Alanine mutant of I183^{3.54} (I183^{3.54}A) with partially reduced RO did not respond to NDP- or α -MSH stimulation (Tables 2 and 3). We reported that mutations of I183^{3.54} to polar residues result in a profound loss of function, whereas valine mutation (I183^{3.54}V) does not alter receptor activity (Tao & Segaloff 2004), implying that I183^{3.54} forms hydrophobic interactions required for MC3R activation. Phosphorylation of T157^{3.65} has been suggested to regulate the forward trafficking of MC1R (Sanchez-Laorden *et al.* 2009). However, in MC3R,

Figure 6

Signaling properties of the WT and mutant hMC3Rs with α -MSH as the ligand. HEK293T cells were treated with or without different concentrations of α -MSH, and intracellular cAMP accumulations were measured using RIA. Each point is expressed as mean $\pm$ s.e.m. of triplicate measurements within one experiment. All curves are representative of at least three independent experiments.

Figure 7

ERK1/2 signaling properties of the WT and mutant hMC3Rs. In (A), HEK293T cells were transiently transfected with WT or mutant hMC3Rs. Cells were starved for 24 h and then stimulated with or without 10^{-6} M NDP-MSH for 5 min. Lysates were analyzed by western blot using antibodies against pERK1/2 or antibodies against β-tubulin as a control. In (B), results are

expressed as mean $\pm$ S.E.M. of at least four independent experiments.

*Significantly different from the basal signaling of WT or mutant hMC3Rs, $P < 0.05$. Images had not been modified and are representative of at least four independent experiments. The β-tubulin and pERK1/2 bands were developed from the same blot.

T194^{3.65} did not affect MC3R trafficking but abolished MC3R binding and signaling.

Although T182^{3.53}A and L187^{3.58}A bound similarly with the ligands as the WT hMC3R, they had impaired cAMP production upon NDP- or α -MSH stimulation (Tables 2 and 3). These two positions are highly conserved in rhodopsin-like GPCRs, with 79% being A/S/T at position 3.53 and 75% I/L/V/F at position 3.58 respectively (Marion *et al.* 2006). The position 3.58 was reported to be critical for G protein coupling, and mutations result in impaired receptor activation in β_2 -AR (F139^{3.58}A; Rasmussen *et al.* 2011) and GPR54 (L148^{3.58}S; Wacker *et al.* 2008). Consistently, our data indicated that L187^{3.58} was also a key residue in MC3R activation.

The basal activity of MC3R is relatively low compared with the closely related MC4R. Until now, there is only one artificial mutation of MC3R, F347^{7.64}A, that has been reported to be constitutively active (Tao *et al.* 2010). In this study, we found that only one mutant (T182^{3.53}A) had a less than twofold increase in basal activity. It has been suggested that the hydrogen bonding interaction between D^{3.49} and Y^{3.60} in β_1 -AR and A_{2A}-adenosine receptor accounts for their low basal activities, whereas the interaction between D^{3.49} and S^{3.62} in β_2 -AR accounts for its high basal activity (Jaakola *et al.* 2008, Rosenbaum *et al.* 2009). In the MC4R, mutation of D146^{3.49}N identified from obese patients pronouncedly increases the basal activity (Wang & Tao 2011). However, in this study, alanine mutation of D178^{3.49}A and Y189^{3.60}A had no effect on the basal activity of MC3R, implying that such hydrogen bonding interactions do not exist or do not affect the basal activity of MC3R. There is usually a correlation between the basal activity of the WT receptor and the propensity of mutations to be constitutively active (Tao *et al.* 2002, Tao 2008). As MC3R basal activity is low, the propensity of MC3R mutations to be constitutively active is probably low.

To delineate the ERK1/2 signaling pathway of MC3R, we also studied the pERK1/2 levels of WT and mutant hMC3Rs stimulated with NDP-MSH. We showed that activation of MC3R by NDP-MSH activated ERK1/2. Five mutants, Y185A, A186G, R188A, M193A, and T194A, did not activate ERK1/2 when stimulated with NDP-MSH; these mutants also had decreased maximal cAMP production. Interestingly, some of the mutants with decreased RO, especially D178A that had almost no detectable binding, still functioned well in ERK1/2 signaling. It is possible that the mutation D178A increased the disassociation rate of the ligand from MC3R, resulting in decreased RO; however, as ERK1/2 activation is rapid and transient, ERK1/2 was still activated. ERK1/2 are

known to be involved in various processes, such as cell proliferation and cell migration (Roskoski 2012). However, the physiological role of ERK1/2 mediated by MC3R is still not clear. Further studies need to be conducted to determine and differentiate the different functions of the cAMP and ERK1/2 pathways of MC3R.

Seven mutants (D178A, R179A, Y180A, T182A, I183A, L187A, and I192A) that had very low or even no cAMP production had similar ERK1/2 signaling as the WT MC3R, suggesting that these seven mutants had ERK1/2 biased signaling (Table 2 and Fig. 7). A previous study had reported that MC3R induces ERK1/2 phosphorylation not through cAMP-PKA but through phospho-inositol-3 kinase (Chai *et al.* 2007). To the best of our knowledge, this is the first report describing biased signaling in MC3R. Biased signaling induced either by mutation or by biased ligand has been reported in the MC4R (Patten *et al.* 2007, Büch *et al.* 2009, Huang & Tao 2012, Mo *et al.* 2012, Mo & Tao 2013; reviewed by Breit *et al.* (2011) and Tao (2014)) and several other GPCRs (reviewed by Violin & Lefkowitz (2007) and Lefkowitz (2013)). It has been suggested that biased ligands interact with different residues of the receptor and, therefore, induce different conformational changes in β_1 -AR (Warne *et al.* 2012) and serotonin receptors (Wacker *et al.* 2013). The biased mutants of MC3R might also have different structures from the WT receptor and, consequently, have different signaling capacities when stimulated by NDP-MSH.

In summary, our present data demonstrated that the cytoplasmic end of TM3 and ICL2 were critical for MC3R function. We identified residues that were critical for ligand binding, signaling in the G_s-cAMP pathway, and signaling in the ERK1/2 pathway. We also reported biased signaling of MC3R that advances our understanding of the complexity of the MC3R signaling cascades and also might have therapeutic potential.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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