Functions of DPLIY motif and helix 8 of human melanocortin-3 receptor

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Abstract

The melanocortin-3 receptor (MC3R) is a member of the family A G protein-coupled receptors (GPCRs). The MC3R remains the most enigmatic of the melanocortin receptors with regard to its physiological functions, especially its role in energy homeostasis. The N/DPxxY motif and the eighth helix (helix 8) in the carboxyl terminus of GPCRs have been identified to be important for receptor expression, ligand binding, signal transduction and internalization. To gain a better understanding of the structure-function relationship of MC3R, we performed a systematic study of all 20 residues in this domain using alanine-scanning mutagenesis. We showed that although all mutants were expressed normally on the cell surface, eleven residues were important for ligand binding and one was indispensable for downstream cAMP generation. F347A showed constitutive activity in cAMP signaling while all the other mutants had normal basal activities. We studied the signaling capacity of nine mutants in the ERK1/2 signaling pathway. All of these mutants showed normal basal ERK1/2 phosphorylation levels. The pERK1/2 levels of six binding- or signaling-defective mutants were enhanced upon agonist stimulation. The unbalanced cAMP and pERK1/2 signaling pathways suggested the existence of biased signaling in MC3R mutants. In summary, we showed that the DPLIY motif and helix 8 was important for MC3R activation and signal transduction. Our data led to a better understanding of the structure-function relationship of MC3R.

Key Words

- ▶ melanocortin-3 receptor
- ▶ G protein-coupled receptor
- ▶ constitutively active mutant
- ▶ biased signaling
- ▶ energy homeostasis

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Introduction

The melanocortin-3 receptor (MC3R), a member of family A G protein-coupled receptors (GPCRs) (Gantz *et al.* 1993, Roselli-Rehfuß *et al.* 1993), has received increasing attention with regard to its multiple physiological functions (reviewed in Renquist *et al.* (2011)). The MC3R is primarily expressed in hypothalamus, especially in the arcuate nucleus, the ventromedial nucleus and the posterior hypothalamic region (Jegou *et al.* 2000). It is also expressed in several peripheral tissues, including the placenta, gut, heart, kidney, and peritoneal macrophages (Gantz *et al.* 1993, Chhajlani 1996, Getting *et al.* 2003,

Ni *et al.* 2006). Based on its wide distribution, the MC3R has been shown to be involved in regulating cardiovascular function (Versteeg *et al.* 1998, Mioni *et al.* 2003), natriuresis (Ni *et al.* 2006, Chandramohan *et al.* 2009), and inflammation (Catania *et al.* 2004, Getting *et al.* 2006, Getting *et al.* 2008).

The MC3R, together with MC4R, another member of melanocortin receptor family expressed in the central nervous system, has been considered as a potential regulator of energy homeostasis. But unlike the MC4R, which is a well-known mediator of leptin action

(Cone 1999) and is crucial for both food intake and energy expenditure regulation (Huszar *et al.* 1997, reviewed in Tao (2010a)), the MC3R is shown to be primarily involved in affecting feed efficiency rather than mediating food intake or energy expenditure (Butler *et al.* 2000, Chen *et al.* 2000). The MC4R plays an undisputed role in human obesity pathogenesis since mutations in *MC4R* have been characterized as the most common monogenic form of obesity in human (Farooqi *et al.* 2003, Tao 2009, Hinney *et al.* 2013). However, the role of MC3R in human obesity pathogenesis is more controversial (reviewed in Tao (2010b)), although some MC3R mutations (such as I183N and I335S) have been recognized as possible genetic contributors for morbid obesity (Lee *et al.* 2002, 2007, Rached *et al.* 2004, Tao & Segaloff 2004, Tao 2007, Mencarelli *et al.* 2008, Yang & Tao 2012, Yang *et al.* 2015).

The MC3R is a typical GPCR consisting of seven transmembrane helices (TMs) with an extracellular N-terminus and intracellular C-terminus. The currently known crystal structures of typical family A GPCRs reveal the existence of an eighth helix (helix 8) (Mustafi & Palczewski 2009, Rosenbaum *et al.* 2009), which initiates just after the highly conserved N/DPxxY motif (Asn/Asp-Pro-Xaa-Xaa-Tyr) in TM7 (DPLIY in the MC3R) and terminates either with anchorage into the plasma membrane by acylation of cysteine residues, or with kinks produced by proline residues. There are only a few GPCRs that do not have this helix in the crystal structures (Zhang *et al.* 2015). To date, the functional importance of the N/DPxxY motif and helix 8 has been emerging in GPCR expression, conformational switch upon GPCR activation, G protein coupling, and GPCR internalization (Wess *et al.* 1993, Barak *et al.* 1995, Prioleau *et al.* 2002, Fritze *et al.* 2003, Tetsuka *et al.* 2004, Delos Santos *et al.* 2006, Swift *et al.* 2006).

However, no systematic study of the DPLIY motif and helix 8 of MC3R has been reported. In order to gain a better understanding of the structure-function relationship of the human MC3R (hMC3R), we investigated the function of each residue in these two domains of the receptor using alanine-scanning mutagenesis. We generated 20 mutants and studied the cell surface expression, ligand binding and signaling properties of the mutant receptors. MC3R activation has also been reported to stimulate ERK1/2 phosphorylation (Chai *et al.* 2007, Begriche *et al.* 2012) (one report suggested that the MC3R does not activate ERK1/2 (Daniels *et al.* 2003)). However, we and others recently reported biased signaling in the MC3R (Huang & Tao 2014, Montero-Melendez *et al.* 2015, Yang *et al.* 2015), the ERK1/2 signaling pathway of the hMC3R was also investigated.

Materials and methods

Materials

[Nle⁴, D-Phe⁷]- α -melanocyte stimulation hormone (NDP-MSH) was purchased from Bachem (King of Prussia, PA, USA). ¹²⁵I-NDP-MSH was iodinated as previously described (Mo *et al.* 2012). Radiolabeled cAMP was iodinated in our lab with chloramine T method (Tao *et al.* 2010).

Site-directed mutagenesis

The WT hMC3R tagged at the N-terminus with 3×HA tag was obtained from Missouri S&T cDNA Resource Center (<http://www.cDNA.org>). Mutations were generated from the WT receptor by QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) using the primers listed in Table 1. Plasmid DNAs were purified by IsoPure Maxi Prep Kit (Denville Scientific, Metuchen, NJ, USA). DNA sequencing was performed by the DNA Sequencing Facility of University of Chicago Cancer Research Center (Chicago, IL, USA) to confirm the presence of intended mutations and nonexistence of unintended mutations.

Cell culture and DNA transfection

Human embryonic kidney (HEK) 293T cells, purchased from American Type Culture Collection (Manassas, VA,

Table 1 Forward primer sequences used for site-directed mutagenesis studies of hMC3R. The mutated codons are underlined

Constructs	Primer sequences
D332A	CAACTCCGTCATCGCCCCACTCATCTACG
P333A	CTCCGTCATCGACGCACTCATCTACGC
L334A	CTCCGTCATCGACCCAGCCATCTACGCTTCCGG
I335A	CATCGACCCACTCGCCTACGCTTCC
Y336A	CATCGACCCACTCATCGCCGCTTCCGGAGCCTG
A337G	CCACTCATCTACGGTTTCCGGAGCCTG
F338A	CCCACTCATCTACGCTGCCGGAGCCTGGAATTG
R339A	CTCATCTACGCTTCCGGAGCCTGGAATTGCG
S340A	CATCTACGCTTCCGGGCCCTGGAATTGCGCAAC
L341A	CTACGCTTCCGGAGCGCGGAATTGCGCAACACC
E342A	CGCTTCCGGAGCCTGGCAATTGCGCAACACCTTAG
L343A	CTTCCGGAGCCTGGGAAGCGCGCAACACCTTTAGGG
R344A	CGGAGCCTGGAATTGGCCAACACCTTTAGGGAG
N345A	GGAGCCTGGAATTGCGCGCCACCTTTAGGGAGATTG
T346A	CTGGAATTGCGCAACGCCCTTTAGGGAGATTG
F347A	GAATTGCGCAACACCGCTAGGGAGATTCTCTG
R348A	GCGCAACACCTTTGCGGAGATTCTCTGTG
E349A	CAACACCTTTAGGGCGATTCTCTGTGGC
I350A	CACCTTTAGGGAGGCTCTCTGTGGCTGC
L351A	CACCTTTAGGGAGATTGCTCTGTGGCTGCAACGGC

USA), were cultured in DMEM containing 10% newborn calf serum. The cells were plated into six-well clusters (or 100 mm dishes for western blot) pre-coated with 0.1% gelatin and transfected with purified plasmids at 50–70% confluency using calcium phosphate precipitation method (Chen & Okayama 1987). Flow cytometry assay, ligand binding, and signaling studies were performed approximately 48 h after transient transfection.

Quantification of MC3R cell surface expression by flow cytometry

Forty-eight hours after transfection, HEK 293T cells were washed with filtered PBS for immunohistochemistry (PBS-IH) (Tao & Segaloff 2003) and fixed by 4% paraformaldehyde in PBS-IH for 30 min. After blocking with PBS-IH containing 5% BSA for 1 h, the cells were then incubated with the primary antibody anti-HA.11 (Covance, Princeton, NJ, USA), which was diluted 1:100 in PBS-IH containing 5% BSA, for another 1 h. The cells were washed once with 0.5% BSA in PBS-IH and then incubated with the secondary antibody Alexa Fluor 488-labeled goat anti-mouse IgG (Invitrogen) for 1 h. The cell surface expression of WT and mutant hMC3Rs was analyzed by Accuri flow cytometer (Accuri Cytometers, Ann Arbor, MI, USA). The expression levels of the mutant hMC3Rs were calculated as the percentage of WT hMC3R expression using the formula: (mutant – pcDNA3.1)/(WT – pcDNA3.1) × 100%, where the pcDNA3.1 were used as control for background staining (Wang *et al.* 2008, Yang & Tao 2012).

Ligand binding assays

Forty-eight hours after transfection, HEK 293T cells were washed twice with warm Waymouth's MB752/1 media (Sigma–Aldrich) containing 1 mg/ml BSA (Waymouth/BSA). The cells were incubated with Waymouth/BSA containing 100 000 cpm of ¹²⁵I-NDP-MSH with or without different concentrations of unlabeled NDP-MSH (from 10^{−12} to 10^{−6} M) at 37 °C for 1 h. The reaction was terminated by washing twice with cold Hank's balanced salt solution on ice. The cells were lysed by 100 µl 0.5N NaOH, collected using cotton swabs, and detected in gamma counter.

Ligand stimulated cAMP production

Forty-eight hours after transfection, HEK 293T cells were washed twice with warm Waymouth/BSA and then incubated with fresh Waymouth/BSA containing 0.5 mM

isobutylmethylxanthine (Sigma–Aldrich) at 37 °C for 15 min. Then different concentrations of NDP-MSH were added into each well to make the final volume 1 ml and final concentration ranging from 10^{−12} to 10^{−6} M. After 1 h incubation at 37 °C, the reaction was terminated on ice and the intracellular cAMP was extracted by adding 0.5 N perchloric acid containing 180 µg/ml theophylline and 0.72 M KOH/0.6 M KHCO₃ into each well. Cyclic AMP concentrations were determined by radioimmunoassay as described in detail before (Tao *et al.* 2010, Yang & Tao 2012).

Protein preparation and western blot

Twenty-four hours after transfection, HEK 293T cells were starved in Waymouth/BSA at 37 °C for 24 h. After being treated with or without 1 µM NDP-MSH for 5 min at 37 °C on the second day, the cells were transferred directly to ice, washed twice using cold 0 G (150 mM NaCl and 20 mM Hepes, pH 7.4), and then scraped into lysis buffer (0G containing 0.5% NP-40, 2 mM EDTA, 1 mM Na₃VO₄, and 1 mM NaF). Total protein concentrations were determined by Bradford protein assay and 35 µg protein samples were separated on 10% SDS–PAGE gel, and then blotted onto PVDF membranes. After blocking in 10% nonfat dry milk (containing 0.2% Tween-20) for at least 4 h at room temperature, the membranes were then immunoblotted with rabbit anti-pERK1/2 antibody (Cell Signaling, Beverly, MA, USA) 1:2000 and mouse anti-β-tubulin antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA) 1:5000 diluted in Tris-buffered saline containing Tween 20 (TBST) with 5% BSA overnight at 4 °C. The membranes were then probed with HRP-conjugated secondary antibody, donkey anti-rabbit (Jackson ImmunoResearch, West Grove, PA, USA) 1:1500 and donkey anti-mouse IgG (Jackson ImmunoResearch) 1:5000 diluted in 10% nonfat dry milk for 2 h at room temperature. Specific bands were visualized using ECL reagent (Thermo Scientific, Rockford, IL, USA) and then analyzed using ImageJ Software (NIH, Bethesda, MD, USA).

Statistical analysis

GraphPad Prism 4.0 Software (San Diego, CA, USA) was used to calculate the ligand binding parameters including maximal binding (B_{max}) and IC₅₀ as well as the cAMP signaling parameters including maximal response (R_{max}) and EC₅₀. The significance of differences in binding and signaling parameters, receptor cell surface expression levels, and pERK1/2 activities between the WT and mutant

hMC3Rs were determined by Student's *t*-test using GraphPad Prism 4.0.

Results

NDP-MSH, an analog of endogenous α -MSH (Sawyer *et al.* 1980), has been shown to be a superpotent agonist for MC3R and was used in the present study. To determine the function of each residue of the DPLIY motif and helix 8 of MC3R, we generated 20 mutants using alanine-scanning mutagenesis (Table 1, Fig. 1). Since the mutant I335A has been studied previously (Tao 2007), its ligand binding and signaling properties are not included herein.

Cell surface expression of the WT and mutant hMC3Rs

Retention of the mutant receptors in the endoplasmic reticulum (ER) resulting from failing to pass the quality control system has been recognized as the major defect of inactivating mutations in numerous GPCRs (reviewed in Tao (2006) and Tao & Conn (2014)). To investigate the cell surface expression of the mutants, flow cytometry was performed. As shown in Fig. 2, all mutants were expressed normally on the cell surface and no significant differences were observed between the WT hMC3R and the mutants. The data for I335A here were in agreement with previous results using confocal microscopy (Tao 2007). To confirm the results, we further tested the HA-tagged I335S and D158E. Our data were consistent with previous reports that I335S is intracellularly retained (with only ~5% WT hMC3R expression at the cell surface) while D158E is partially retained with decreased cell surface expression compared to the WT hMC3R (Fig. 2) (Tao 2007, Wang *et al.* 2008).

Figure 1

Schematic model of the hMC3R with the 20 residues characterized in the present study highlighted.

Figure 2

Cell surface expression of the WT and mutant hMC3Rs by flow cytometry. The results are expressed as % of cell surface expression level of WT hMC3R after correction of the nonspecific staining in cells transiently transfected with the empty vector as described in Materials and methods. Values are mean $\pm$ S.E.M. of at least three independent experiments.

* indicates significantly different from WT hMC3R ($P < 0.05$).

Ligand binding of the WT and mutant hMC3Rs

To compete with different concentrations of unlabeled NDP-MSH, 125 I-NDP-MSH was used as the radioligand in the binding assay. The result showed that P333A, L343A, and R344A had no detectable binding. Nine mutants (L334A, Y336A, S340A, E342A, F347A, R348A, E349A, I350A, and L351A) showed decreased IC_{50} while only one mutant, L341, had increased IC_{50} . There were no significant differences between the IC_{50} s of all the other mutants and that of the WT hMC3R (Table 2, Fig. 3).

As shown in Fig. 4, nine mutants (D332A, L344A, Y336A, S340A, E342A, F347A, R348A, I350A, and L351A) had significantly decreased maximal binding compared with WT hMC3R while one mutant (F338A) showed significantly, although very slightly, increased maximal binding. All the other mutants had similar maximal binding as the WT hMC3R.

Signaling properties of the WT and mutant hMC3Rs

Whether the hMC3R variants could respond to NDP-MSH stimulation with enhanced cAMP generation was then investigated. As expected, NDP-MSH could stimulate dose-dependent increase of intracellular cAMP in HEK293T cells transfected with WT hMC3R. As shown in Table 2, two mutants (Y336A and E342A), in addition to three binding-defective mutants (P333A, L343A, and R344A), had no measurable signaling. F338A and R339A showed increased EC_{50} while F347A displayed reduced EC_{50} . No significant differences were observed between the EC_{50} s of all the other mutants and that of the WT hMC3R.

Table 2 The ligand binding and signaling properties of WT and mutant hMC3Rs. Values are expressed as the mean $\pm$ S.E.M. of at least four independent experiments. The $R_{\max}$ of WT hMC3R was 2386.00 ± 239.95 pmol/ 10^6 cells upon NDP-MSH stimulation

hMC3R	NDP-MSH binding	NDP-MSH-stimulated cAMP	
	IC ₅₀ (nM)	EC ₅₀ (nM)	R _{max} (100%)
WT	2.63 $\pm$ 0.27	0.47 $\pm$ 0.05	100
D332A	1.59 $\pm$ 0.27	0.43 $\pm$ 0.04	98.50 $\pm$ 18.45
P333A	N/A ^a	N/A ^a	N/A ^a
L334A	1.09 $\pm$ 0.07 ^b	0.39 $\pm$ 0.10	79.50 $\pm$ 12.12
Y336A	0.79 $\pm$ 0.10 ^b	N/A ^a	N/A ^a
A337G	2.32 $\pm$ 0.42	0.57 $\pm$ 0.17	98.25 $\pm$ 11.27
F338A	3.17 $\pm$ 0.79	0.90 $\pm$ 0.24 ^c	94.75 $\pm$ 15.58
R339A	2.38 $\pm$ 0.28	2.34 $\pm$ 0.37 ^d	48.33 $\pm$ 20.00 ^c
S340A	0.84 $\pm$ 0.12 ^b	0.77 $\pm$ 0.22	52.29 $\pm$ 13.18 ^c
L341A	4.81 $\pm$ 1.13 ^c	0.50 $\pm$ 0.07	102.50 $\pm$ 19.31
E342A	0.58 $\pm$ 0.02 ^d	N/A ^a	N/A ^a
L343A	N/A ^a	N/A ^a	N/A ^a
R344A	N/A ^a	N/A ^a	N/A ^a
N345A	1.89 $\pm$ 0.17	0.36 $\pm$ 0.08	107.00 $\pm$ 12.13
T346A	2.08 $\pm$ 0.18	0.48 $\pm$ 0.16	91.80 $\pm$ 8.89
F347A	1.09 $\pm$ 0.19 ^b	0.25 $\pm$ 0.06 ^c	67.25 $\pm$ 8.38 ^c
R348A	0.91 $\pm$ 0.04 ^b	0.42 $\pm$ 0.11	53.40 $\pm$ 5.62 ^b
E349A	1.21 $\pm$ 0.18 ^c	0.47 $\pm$ 0.16	103.00 $\pm$ 23.34
I350A	0.61 $\pm$ 0.07 ^b	0.33 $\pm$ 0.07	15.75 $\pm$ 1.55 ^d
L351A	0.64 $\pm$ 0.03 ^b	0.32 $\pm$ 0.10	17.50 $\pm$ 2.02 ^d

^aNot detectable.

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

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

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

Our data of maximal response in Fig. 5 also showed that six mutants (R339A, S340A, F347A, R348A, I350A, and L351A) had significantly decreased maximal response compared with WT hMC3R. All the other mutants had apparent normal signaling.

Constitutive activity of hMC3Rs

Unlike the human MC4R, which has been previously shown to be constitutively active (Nijenhuis *et al.* 2001, Tao 2014), the hMC3R has been recognized with little or no constitutive activity (Tao 2007). In the present study, the constitutive activities of the 19 mutants of hMC3R (I335A not included) were analyzed. We demonstrated that the F347A had significantly increased basal activity compared with the WT hMC3R. As shown in Fig. 6A, the nearly 4.6 times elevation of basal activity demonstrated that the F347A was a constitutively active mutant, consistent with our previous report (Tao *et al.* 2010). All the other mutants had similar basal activities compared with that of the WT hMC3R. The dose-response curve in Fig. 6B highlights that F347A, although constitutively active, had reduced maximal response compared with the WT hMC3R.

ERK1/2 signaling of the WT and mutant hMC3Rs

To further investigate the ERK1/2 signaling pathway, especially the newly-identified biased signaling of hMC3R, the WT hMC3R together with nine mutants, including one constitutively active mutant (F347A) and eight binding- or signaling-defective mutants (P333A, I335A, Y336A, E342A, L343A, R344A, I350A, and L351A) which led to $< 20\%$ the WT receptor cAMP production, were studied using western blots. Empty vector pcDNA3.1 and WT hMC3R without the $3 \times$ HA tag were also studied, excluding non-specific stimulation.

Our results showed that the basal pERK1/2 levels in cells transfected with either WT MC3Rs (with or without $3 \times$ HA-tag) were similar to that of cells transfected with the empty vector (Fig. 7B). Upon $1 \mu\text{M}$ NDP-MSH stimulation, WT MC3Rs showed significant ERK1/2 phosphorylation but no ERK1/2 activation was detected in cells transfected with an empty vector. There was no significant difference between the pERK1/2 levels of HA-tagged and non-tagged WT MC3Rs (Fig. 7C).

The constitutive activities of WT and mutant hMC3Rs in activating ERK1/2 were studied. Our results showed that all the mutants, including the F347A that was shown to be

Figure 3

Ligand binding properties of the WT and mutant hMC3Rs with NDP-MSH as the ligand. HEK293T cells were transiently transfected with WT and mutant constructs, and the binding properties were measured 48 h later by displacing the binding of ^{125}I -NDP-MSH using different concentrations of unlabeled NDP-MSH as described in Materials and methods. Data are expressed as % of WT binding $\pm$ range from duplicate measurements within one experiment. All experiments were performed at least three times independently.

constitutively active in cAMP pathway, had similar basal pERK1/2 levels as the WT hMC3R (Fig. 7D).

We then investigated the pERK1/2 levels of WT and mutant hMC3Rs after 1 μM NDP-MSH stimulation. As expected, the ERK1/2 phosphorylation was significantly enhanced in HEK 293T cells transfected with WT hMC3R. P333A and L343A did not respond to NDP-MSH stimulation with ERK1/2 activation while all other mutants had significantly increased pERK1/2 levels compared to the corresponding basal activity upon 1 μM NDP-MSH stimulation (Fig. 7E).

Discussion

Although several physiological functions of the MC3R have now been described, it remains the most enigmatic member of the melanocortin receptor family. The recent identification of an additional helix 8, linking the TM7 and the C-terminus of GPCRs, has also aroused great interest. Analysis of the role of helix 8 in both family A and B GPCRs confirmed its importance in receptor expression, ligand binding, signal transduction and internalization (Ernst *et al.* 2000, Marin *et al.* 2000, Tetsuka *et al.* 2004, Faussner *et al.* 2005, Kuwasako *et al.* 2011). In the present study, we performed a detailed study of the 20 residues in helix 8 and the DPLIY motif of hMC3R using the classical

method of site-directed alanine-scanning mutagenesis (Cunningham & Wells 1989).

The flow cytometric analysis demonstrated that all mutants were expressed normally on the cell surface. Receptor retention or mislocalization, which has previously been described as the major defect for human disease caused by GPCR mutations (Tao 2006, Tao & Conn 2014), was not observed in the present study. Similarly, in our studies on the second and third intracellular loops, we also showed that the alanine mutants are expressed normally at the cell surface (Wang & Tao 2013, Huang & Tao 2014).

Ligand binding studies revealed that 11 residues (P333, L334, Y336, S340, E342, L343, R344, F347, R348, I350 and L351) were important for NDP-MSH binding. Although it is uncommon that residues important for ligand binding are located in the cytoplasmic side of receptor, observations have been reported previously in MC3R (Huang & Tao 2014) as well as other GPCRs, such as the gonadotropin-releasing hormone receptor (Lu *et al.* 2005), angiotensin II type 2 receptor (Moore *et al.* 2002), and V2 vasopressin receptor (Pan *et al.* 1994). These residues are not expected to directly participate in ligand binding. Rather they might indirectly participate in ligand-receptor interaction through intramolecular interactions and conformational changes (Kobilka & Deupi 2007, Rovati *et al.* 2007).

The highly conserved DPLIY motif is one of the two crucial motifs (the other one is D/ERY motif in TM3) for receptor stabilization and activation (Park *et al.* 2008). p^{7.50} (the superscript number represents Ballesteros-Weinstein numbering (Ballesteros & Weinstein 1995)) has been regarded as critical in inducing a structurally

Figure 4

Total specific binding of WT and mutant hMC3Rs with NDP-MSH as the ligand. Data are mean $\pm$ S.E.M. of at least three independent experiments. * indicates significantly different from WT hMC3R ($P < 0.05$).

Figure 5

Signaling properties of the WT and mutant hMC3Rs with NDP-MSH as the ligand. HEK293T cells were transiently transfected with WT and mutant constructs, and intracellular cAMP levels were measured by RIA after stimulation with different concentrations of NDP-MSH as described in Materials and methods. Data are mean $\pm$ S.E.M. from triplicate measurements within one experiment. All experiments were performed at least three times independently.

important helical break due to its distinctive cyclic structure of a side chain. Although L^{7.51} was not studied in detail before, its adjacent residue I^{7.52} has been recognized to be important in multiple aspects of MC3R function (Tao 2007). L^{7.51} might contribute to the supposed interaction between the I^{7.52} and hydrophobic residues in helix 8 to maintain the receptor conformation necessary for ligand binding. Y^{7.53} has been shown to be critical for receptor activation and signal transduction in many GPCRs, such as MC4R, gonadotropin-releasing receptor, and 5HT_{2C} receptor (Arora *et al.* 1996, Prioleau *et al.* 2002, Roth *et al.* 2009). The conformational change of tyrosine enables it to insert into the space previously occupied by TM6 and hinder its inward tilt to stabilize the active state (Scheerer *et al.* 2008). Mutations of the tyrosine residue in some other receptors result in either no ligand binding or defective signaling (Feng & Song 2001). Our results here highlighted the importance of Y^{7.53} in hMC3R.

The D/E(x)₇LL motif, composed of a highly conserved di-leucine sequence with an upstream acidic residue, has been shown to be present in the C-terminus of several GPCRs such as α_1 -, α_2 -adrenergic receptors and the dopamine receptor (Schulein *et al.* 1998). The corresponding residue of glutamate (E335^{7.63}) in the V₂ vasopressin receptor was reported as crucial for establishing a transport-competent folding state to support the escape of the receptor from the ER (Schulein *et al.* 1998). The di-leucine motif, which should be strictly defined as a

dihydrophobic pair due to mainly consisting of leucine, isoleucine or valine, was shown to play an important role in cell surface targeting, receptor internalization and protein trafficking (Gabilondo *et al.* 1997, Ho & MacKenzie 1999). The alanine mutation of di-leucine pair was reported to have no effect on ligand affinity in the β_2 -adrenergic receptor (Gabilondo *et al.* 1997), whereas the same mutation of di-isoleucine was shown to impair NDP-MSH binding to MC4R (VanLeeuwen *et al.* 2003). Our data demonstrated that the maximal binding of E342^{7.59}A, I350^{7.67}A, and L351^{7.68}A had decreased by 90, 87 and 83% respectively, compared with WT hMC3R, although all the mutant receptors could be normally expressed on the cell surface.

The interaction between two highly conserved aromatic residues, tyrosine in the D/NPxxY motif and phenylalanine in helix 8, has been highlighted in several studies. However, the conserved phenylalanine in the melanocortin receptor family is substituted by a leucine

Figure 6

Basal activities of WT and mutant hMC3Rs. (A) HEK293T cells were transiently transfected with WT and mutant constructs, and intracellular cAMP levels were measured without ligand stimulation. Data are mean $\pm$ S.E.M. of at least four independent experiments. The basal cAMP level of WT hMC3R was 25.79 ± 2.69 pmol/10⁶ cells. * indicates significantly different from WT hMC3R ($P < 0.05$). (B) A representative dose-response curve of WT and F347A hMC3Rs. Similar results were obtained in at least three independent experiments.

Figure 7

MAPK signaling of WT and mutant hMC3Rs. (A) HEK293T cells were transiently transfected with pcDNA3.1, WT (with or without HA tag) or mutant constructs, and 24 h later cells were starved overnight and then harvested after they were treated with or without 1 μM NDP-MSH stimulation for 5 min. Western blot analysis was performed using antibody against pERK1/2 and β-tubulin as control. (B) Basal pERK1/2 levels of cells transfected with pcDNA3.1 or WT hMC3Rs (with or without HA tag); data are mean ± S.E.M. of three independent experiments. (C) Stimulated pERK1/2 levels in cells transfected with pcDNA3.1 or WT hMC3R (with or without HA tag) after 1 μM NDP-MSH stimulation, expressed as a percentage of each basal pERK1/2 activity from three independent experiments. * indicates significantly different from corresponding basal level ($P < 0.05$) and NS means no statistical difference between HA-tagged and non-tagged WT hMC3R ($P > 0.05$). (D) Data of the basal pERK1/2 of WT and mutant hMC3Rs are mean ± S.E.M. of at least four independent experiments. (E) Data of the pERK1/2 levels of WT and mutant hMC3Rs after 1 μM NDP-MSH stimulation are expressed as percentage of each basal pERK1/2 activity from at least four independent experiments. * indicates significantly different from corresponding basal activity ($P < 0.05$).

residue (L343^{7.60} in hMC3R) and the function of replaced phenylalanine might be taken by another aromatic residue, F347^{7.64}. As previously discussed, the I^{7.52} in DPLIY motif of MC3R might interact with hydrophobic residues in helix 8 to maintain the receptor conformation. L343^{7.60} is a preferable choice for taking part in the hydrophobic interaction since the mutant L343^{7.60}A did

not exhibit any binding or signaling in response to NDP-MSH stimulation in our study.

One residue, R339^{7.56}, was demonstrated to be crucial for normal receptor signaling. Alanine mutation of this arginine resulted in normal ligand binding but severely decreased signaling potency. Missense mutations of the corresponding residue of arginine in hMC4R, including R305^{7.56}S, R305^{7.56}Q, and R305^{7.56}W, have been identified from obese patients (Tao 2009). R305^{7.56}S and R305^{7.56}Q have been shown to have a partial cAMP response to α-MSH stimulation (Stutzmann *et al.* 2008, Calton *et al.* 2009) and R305^{7.56}W exhibits normal binding but severely impaired signaling in response to α-MSH stimulation (Roubert *et al.* 2010), which is in agreement with our results of R339^{7.56}A hMC3R.

The MC3R has been reported to activate the ERK1/2 signaling pathway (see Introduction). Unbalanced cAMP and ERK1/2 signaling was reported in some human MC4R mutants (Huang & Tao 2012, Mo *et al.* 2012, He & Tao 2014) (reviewed in Tao (2014)). In our recent study of the DRY motif and intracellular loop 2 of hMC3R, the presence of this biased signaling was also suggested (Huang & Tao 2014). In the current study to test it further, the ERK1/2 phosphorylation levels of nine hMC3R mutants, including eight binding- or signaling-defective mutants (P333A, I335A, Y336A, E342A, L343A, R344A, I350A, and L351A) which led to <20% cAMP production compared with WT hMC3R, and one constitutively active mutant (F347A) with enhanced basal cAMP activity, were investigated using western blots.

Non-specific effects were excluded in the present study by using the empty vector pcDNA3.1 and non-tagged WT hMC3R. The results indicated that there was no endogenous receptor in HEK293T cells responding to NDP-MSH stimulation and HA tag did not have any effect on MC3R activation of ERK1/2 (Fig. 7A, B and C). All nine mutants had similar basal pERK1/2 levels as the WT MC3R. Notably, F347A, which had increased basal cAMP level, did not show any increase in the basal pERK1/2 level compared with WT hMC3R. Upon 1 μM NDP-MSH stimulation, two binding-defective mutants, P333A and L343A, did not activate ERK1/2. However, all the other tested mutants, especially I335A, Y336A, E342A, and R344A that had almost no cAMP response, had shown significant ERK1/2 activation. Two mutants with no detectable binding, I335A (Tao 2007) and R344A (present study), responded to NDP-MSH stimulation with increased pERK1/2 levels. Although it is uncommon, similar observations have been previously reported in other GPCRs, such as MC4R (Huang & Tao 2012) and

gonadotropin-releasing hormone receptor (Bedecarrats *et al.* 2003). This might be due to a faster dissociation of the ligand from the MC3R mutants compared with the WT receptor making the binding difficult to detect, but ERK1/2 could still be activated by this transient binding (Huang & Tao 2014). NDP-MSH was reported to induce ERK1/2 phosphorylation in HEK293 cells transfected with WT MC3R in a very fast and potent pattern, peaking at only 5 min with an EC_{50} of 3.3 ± 1.5 nM (Chai *et al.* 2007). These data suggest these mutants were biased receptors, supporting two recent studies on biased signaling at the MC3R (Montero-Melendez *et al.* 2015, Yang *et al.* 2015).

In summary, from the systematic study of the DPLIY motif and helix 8 of hMC3R, we have identified residues that were crucial for ligand binding and cAMP generation. One constitutively active mutant was identified and the existence of biased signaling was confirmed in MC3R mutants. The data obtained in our present research indicated that the highly conserved DPLIY motif and helix 8 play important roles in MC3R activation and signal transduction. Our result established a theoretical basis for the structure-functional relationship of MC3R and will be useful for further investigation of its physiological role in energy homeostasis.

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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