Pleiotropic functions of the transmembrane domain 6 of human melanocortin-4 receptor

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Abstract

The melanocortin-4 receptor (MC4R) is a critical regulator of energy homeostasis and has emerged as a premier target for obesity treatment. Numerous mutations in transmembrane domain 6 (TM6) of MC4R resulting in functional alterations have been identified in obese patients. Several mutagenesis studies also provided some data suggesting the importance of this domain in receptor function. To gain a better understanding of the structure–function relationship of the receptor, we performed alanine-scanning mutagenesis in TM6 to determine the functions of side chains. Of the 31 residues, two were important for cell surface expression, five were indispensable for α -melanocyte-stimulating hormone (α -MSH) and β -MSH binding, and six were important for signaling in the Gs–cAMP–PKA pathway. H264A, targeted normally to the plasma membrane, was undetectable by competitive binding assay and severely defective in basal and stimulated cAMP production and ERK1/2 phosphorylation. Nine mutants had decreased basal cAMP signaling. Seven mutants were constitutively active in cAMP signaling and their basal activities could be inhibited by two MC4R inverse agonists, Ipsen 5i and ML00253764. Five mutants were also constitutively active in the MAPK pathway with enhanced basal ERK1/2 phosphorylation. In summary, our study provided comprehensive data on the structure–function relationship of the TM6 of MC4R. We identified residues that are important for cell surface expression, ligand binding, cAMP generation, and residues for maintaining the WT receptor in active conformation. We also reported constitutive activation of the MAPK pathway and biased signaling. These data will be useful for rationally designing MC4R agonists and antagonists for treatment of eating disorders.

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Introduction

The global epidemic of obesity, with a prevalence of 9.8% of adults worldwide (Kelly *et al.* 2008) and 33.8% of adults in the USA (Flegal *et al.* 2010), has become one of the most significant burdens to public health. Obesity is a major independent risk factor for cardiovascular diseases and is often associated with type 2 diabetes mellitus as well as other comorbidities (Guh *et al.* 2009). Though intervention of dietary control and physical activity can successfully contribute to the prevention of weight gain in some groups, it is difficult to reach long-term weight loss maintenance, especially for those carrying genetic alterations (Lemmens *et al.* 2008, Reinehr *et al.* 2009, Kraschnewski *et al.* 2010).

Multiple genes have been identified to be associated with obesity, and mutations in the melanocortin-4 receptor (MC4R) have been characterized as the most frequent cause of monogenic obesity in humans, with a prevalence of from 0 to 6% in different ethnic backgrounds (reviewed in Tao (2010)). Therefore, the MC4R has emerged as a premier target for obesity treatment. Adopting a codominant inheritance model (O’Rahilly *et al.* 2003), MC4R pathogenic mutations exhibit a varied degree of penetrance, interacting with the environment (Stutzmann *et al.* 2008).

The MC4R, which is crucial for regulating both food intake and energy expenditure (Huszar *et al.* 1997), is a G protein-coupled receptor (GPCR) primarily expressed in the CNS. Although the classical G protein for MC4R is the Gs, recent studies suggested that MC4R couple to all three major classes of G proteins (reviewed in Breit *et al.* (2011)). The Gs–cAMP–PKA and ERK1/2 signaling pathways are of particular interest because they are identified to be related to the MC4R function of energy homeostasis *in vivo* (Sutton *et al.* 2005, Czyzyk *et al.* 2008).

Up to now, more than 150 naturally occurring mutations or common alleles of the MC4R have been identified (reviewed in Tao (2009)). They are scattered throughout the receptor, including transmembrane domain 6 (TM6). At least ten naturally occurring mutations or variants have been identified in TM6 and functionally characterized. Some of the mutants (such as L250Q, P260Q, F261S, and I269N) are retained in the endoplasmic reticulum (ER; Proneth *et al.* 2006, Xiang *et al.* 2006, Fan & Tao 2009, Tan *et al.* 2009, Wang & Tao 2011), and L250Q (Proneth *et al.* 2006, Xiang *et al.* 2006) and I251L (Xiang *et al.* 2006) are constitutively active. Most of these mutations are associated with obesity, whereas I251L confers strong protection from obesity (Stutzmann *et al.* 2007,

Mirshahi *et al.* 2011). During the past few years, several studies have demonstrated the functional importance of TM6 in the MC4R (Chen *et al.* 2007a,b, 2009).

Despite these important studies, no systematic investigation of the whole TM6 has been reported. To gain a better understanding of the structure–function relationship of the MC4R, we sought to determine the function of each residue in TM6 of the receptor using alanine-scanning mutagenesis. We generated 31 mutants and used the two endogenous ligands, α -melanocyte-stimulating hormone (α -MSH) and β -MSH, for ligand binding and signaling studies. We also studied the basal activities of the constitutively active mutants (CAMs) in the ERK1/2 signaling pathway.

Materials and methods

Materials

[125 I]-[Nle⁴,D-Phe⁷]- α -MSH (NDP-MSH) was purchased from American Radiolabeled Chemicals (St Louis, MO, USA), α -MSH from Pi Proteomics (Huntsville, AL, USA), and β -MSH from CHI Scientific (Maynard, MA, USA). ML00253764 and Ipsen 5i were synthesized by Enzo Life Sciences, Inc. (Plymouth Meeting, PA, USA). Radiolabeled cAMP was iodinated with chloramine T method. The c-myc-tagged WT hMC4R at the N-terminus was described previously (Tao & Segaloff 2003).

Site-directed mutagenesis

Mutant receptors were generated by QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) using the WT receptor as the template and sequenced by the DNA Sequencing Facility of University of Chicago Cancer Research Center (Chicago, IL, USA) to confirm the presence of desired mutations and the absence of errors in the coding sequences.

Cell culture and transfection

HEK293 and 293T cells were purchased from American Type Culture Collection (Manassas, VA, USA) and were cultured in DMEM with 10% newborn calf serum. The HEK293T cells were plated into six-well clusters coated with 0.1% gelatin, transfected with the WT or mutant constructs at 50% confluence using calcium phosphate precipitation method (Tao *et al.* 2010), and were used for ligand binding and signaling studies ~48 h later. For western blot, HEK293T were seeded into gelatin-coated 100 mm dishes and were transfected using the same method. The transfected HEK293 cells were selected by G418 for imaging the cell surface expression by confocal microscopy.

Ligand binding assays

HEK293T cells were transfected as described earlier. 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 1 ml Waymouth/BSA containing 50 μ l, 100 000 c.p.m. of 125 I-NDP-MSH, and with or without different concentrations of α - or β -MSH giving a final concentration ranging from 10^{-10} to 10^{-5} M for 1 h at 37 °C. Cells were then washed twice with cold Hank's balanced salt solution to terminate the reactions, lysed by 100 μ l 0.5 M NaOH, collected with cotton swabs, and counted in a gamma counter.

cAMP assay

HEK293T cells were transfected as described earlier. On the day of experiment, cells were washed twice with warm Waymouth/BSA and then incubated with 1 ml fresh Waymouth/BSA containing 0.5 mM isobutylmethylxanthine (Sigma–Aldrich) for 15 min at 37 °C. Then cells were treated with or without different concentrations of α - or β -MSH giving a final concentration ranging from 10^{-11} to 10^{-5} M. After incubation at 37 °C for 1 h, cells were lysed by 0.5 M perchloric acid containing 180 μ g/ml theophylline and neutralized by 0.72 M KOH/0.6 M KHCO₃. cAMP levels were measured using RIA (Fan *et al.* 2008, Tao *et al.* 2010).

Confocal microscopy

The method for immunohistochemistry (IH) has been described earlier (Tao & Segaloff 2003). Briefly, HEK293 cells stably expressing c-myc-tagged WT or mutant hMC4Rs were established as described earlier. Cells were seeded into poly-D-lysine-coated eight-well chamber slides (Biocoat cellware from Falcon, BD Systems, Franklin Lakes, NJ, USA) and incubated at 37 °C for ~24 h. Cells were fixed with 4% paraformaldehyde in filtered PBS for IH (PBS-IH) for 30 min and incubated with 5% BSA in PBS-IH for 1 h to block nonspecific binding. Cells were then incubated with the primary antibody, 9E10 monoclonal anti-myc antibody (Developmental Studies Hybridoma Bank, the University of Iowa, Iowa City, IA, USA; 1:100 dilution in PBS-IH with 0.5% BSA) for 1 h, and this was followed by incubation with the secondary antibody, Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen; 1:1000 dilution in PBS-IH 0.5% BSA) for 1 h. The slide was then covered by a coverslip using Vectashield Mounting Media (Vector Laboratories, Burlingame, CA, USA). Fluorescent images were taken using a Bio-Rad confocal microscope.

Protein preparation and western blot

HEK293T cells were seeded and transfected as described earlier. Approximately 24 h after transfection, cells were starved in Waymouth/BSA at 37 °C overnight and then were treated with or without different concentrations of NDP-MSH giving a final concentration ranging from 10^{-9} to 10^{-6} M in 5 ml Waymouth/BSA for 5 min at 37 °C. Cells were placed directly on ice and washed twice with cold 0G (150 M NaCl and 20 mM Hepes, pH 7.4) and then were scraped into lysis buffer in 0G (containing 0.5% NP-40, 2 mM EDTA, 1 mM Na_3VO_4 , and 1 mM NaF). Total protein concentrations were determined by Bradford protein assay, and 30 μg protein samples were separated by 10% SDS-PAGE using the Tris-glycine buffer system and blotted onto pre-wetted PVDF membranes in wet conditions. The membranes were blocked in 10% nonfat dry milk (containing 0.2% Tween 20) for at least 3 h at room temperature with agitation and then immunoblotted with the primary antibodies, rabbit phosphorylation levels of ERK1/2 (p-ERK1/2) antibody (Cell Signaling, Billerica, MA, USA) 1:1000 and mouse β -tubulin antibody (Developmental Studies Hybridoma Bank, the University of Iowa) 1:40 000 diluted in TBST/5% BSA overnight at 4 °C. This was followed by the incubation of the HRP-conjugated secondary antibodies, anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) 1:1000 and anti-mouse IgG (Jackson ImmunoResearch) 1:40 000 diluted in 10% nonfat dry milk for at least 1 h at room temperature. Specific bands were detected with ECL reagent (Thermo Scientific, Rockford, IL, USA) and were analyzed and quantified by Image J Software (NIH, Bethesda, MD, USA).

Statistical analysis

The competitive binding curves and cAMP dose-response curves were fitted to a one-site model using GraphPad Prism 4.0 Software (San Diego, CA, USA). Concentrations that result in 50% inhibition (IC_{50}) and apparent maximal binding (B_{max}) or receptor occupancy (Wang *et al.* 2008) were calculated from competitive binding data. Concentrations that result in 50% maximal responses (EC_{50}) and maximal responses (R_{max}) were calculated from cAMP assays. The significance of differences in p-ERK1/2 levels and binding and signaling parameters between WT and mutant hMC4Rs were analyzed by Student's *t*-test using Prism 4.0 Software.

Results

The two endogenous agonists of MC4R, α -MSH (Fan *et al.* 1997) and β -MSH (Abbott *et al.* 2000), have been shown to be capable of controlling feeding and energy balance through activation of MC4R. Here, we first studied the binding and signaling properties of all 31 hMC4R mutants using α - or β -MSH as the ligand (Fig. 1).

Pharmacology of the WT and mutant hMC4Rs at α -MSH

Competitive binding assays were performed using ^{125}I -NDP-MSH as the radioligand, and different concentrations of α -MSH, from 10^{-10} to 10^{-5} M, were used to displace radioligand in HEK293T cells transiently transfected with WT or mutant MC4Rs. IC_{50} values were calculated and analyzed for the WT and mutant hMC4Rs. Our data showed that H264A had no detectable binding; L265A and Y268A had decreased

Figure 1 Schematic representation of the 31 residues in TM6 of hMC4R. The phenotypes of mutations at several loci that changed cell surface expression, ligand binding, signaling, and constitutive activity are highlighted.

Figure 2 The ligand binding (A) and signaling (B) properties of WT and mutant hMC4Rs with α -MSH as the ligand. HEK293T cells were transiently transfected with WT or mutant constructs, and 48 h later were used for binding and signaling studies. (A) Intact cell surface binding was measured by competitive inhibition of 125 I-NDP-MSH with serial concentrations of α -MSH. Data are depicted as percentage of WT maximal binding. (B) Intracellular cAMP samples were collected with or without stimulation with different concentrations of α -MSH, and cAMP concentrations were determined by RIA. Data points are mean $\pm$ s.e.m. of duplicate (A) or triplicate (B) measurements within one experiment. Results are representative of at least three independent experiments.

affinities, whereas M241A, A244G, T246A, L250A, C257A, P260A, I266A, and F267A had increased affinities for α -MSH (Fig. 2A and Table 1).

To measure the signaling capacities of the mutant receptors, HEK293T cells transiently transfected with WT or mutant hMC4Rs were stimulated with increasing concentrations of α -MSH. Intracellular cAMP levels were measured after 1-h stimulation. Maximal response ($R_{\max}$) and EC_{50} values were calculated from the dose-response curves. Our data showed that H264A only responded to 10^{-5} M α -MSH stimulation. Six mutants (L247A, W258A, P260A, F261A, L265A, and Y268A) had increased EC_{50} values compared with the WT hMC4R (Fig. 2B). One mutant (A259G) had decreased EC_{50} . Four mutants (G243A, T246A, L250A, and F254A) had decreased $R_{\max}$, whereas five mutants (P260A, F261A, L265A, I266A, and I269A) had increased maximal responses compared with the WT hMC4R. The other mutants had similar EC_{50} and $R_{\max}$ as the WT hMC4R (Fig. 2B and Table 1).

Pharmacology of the WT and mutant hMC4Rs at β -MSH

When ligand binding and signaling experiments were performed using β -MSH as the ligand, similar data

were obtained, with some moderate differences. In binding experiments, L265A and Y268A had significantly increased IC_{50} s, whereas T246A, L250A, and P260A had decreased IC_{50} s (Fig. 3A and Table 1). In signaling experiments, H264A was only responsive to 10^{-5} M β -MSH stimulation. Twelve mutants (T246A, L247A, L250A, I251A, G252A, F254A, W258A, P260A, F261A, F262A, L265A, and Y268A) had increased EC_{50} s compared with the WT hMC4R, and two mutants (V253A and A259G) had decreased EC_{50} s (Fig. 3B and Table 1). Three mutants (I245A, F254A, and A259G) had decreased $R_{\max}$ (Fig. 3B and Table 1).

$B_{\max}$ values were also calculated from these binding assays. As described earlier, H264A had no measurable binding. In addition, 12 mutants (G243A, A244G, L250A, I251A, F254A, V255A, C257A, P260A, F261A, L265A, I266A, and I269A) had decreased $B_{\max}$ (Fig. 4).

Cell surface expression of several mutant hMC4Rs

Retention of mutant receptors in the ER by the quality control system is the major defect of inactivating MC4R mutations (reviewed in Tao (2005)). For the six mutants (L250A, C257A, P260A, F261A, H264A, and

Table 1 The ligand binding and signaling properties of WT and mutant hMC4Rs. Values are mean $\pm$ S.E.M. of at least three independent experiments. The $R_{\max}$ of WT hMC4R was $2744 \cdot 11 \pm 373 \cdot 20$ pmol/ 10^6 cells with α -MSH stimulation and was $2848 \cdot 15 \pm 377 \cdot 85$ pmol/ 10^6 cells with β -MSH stimulation

hMC4R construct	α -MSH binding	α -MSH-stimulated cAMP		β -MSH binding	β -MSH-stimulated cAMP	
	IC ₅₀ (nM)	EC ₅₀ (nM)	$R_{\max}$ (% WT)	IC ₅₀ (nM)	EC ₅₀ (nM)	$R_{\max}$ (% WT)
WT	1120.36 $\pm$ 157.69	4.29 $\pm$ 0.79	100	858.18 $\pm$ 216.64	2.34 $\pm$ 0.17	100
N240A	415.00 $\pm$ 68.27	3.18 $\pm$ 1.14	89.22 $\pm$ 15.27	240.49 $\pm$ 79.69	2.82 $\pm$ 1.45	102.60 $\pm$ 23.91
M241A	202.70 $\pm$ 14.63 ^a	1.54 $\pm$ 0.44	131.42 $\pm$ 22.37	126.41 $\pm$ 30.19	1.30 $\pm$ 0.29	74.76 $\pm$ 13.74
K242A	614.50 $\pm$ 235.71	35.05 $\pm$ 10.77	112.32 $\pm$ 23.61	387.53 $\pm$ 122.68	10.88 $\pm$ 4.00	74.39 $\pm$ 17.02
G243A	730.80 $\pm$ 268.06	12.55 $\pm$ 4.26	65.83 $\pm$ 5.91 ^b	216.57 $\pm$ 53.71	4.043 $\pm$ 0.99	106.61 $\pm$ 29.00
A244G	183.10 $\pm$ 45.30 ^a	4.23 $\pm$ 1.44	87.11 $\pm$ 13.98	115.03 $\pm$ 9.56	1.48 $\pm$ 0.47	136.98 $\pm$ 24.91
I245A	828.10 $\pm$ 190.35	2.62 $\pm$ 0.94	133.69 $\pm$ 25.68	192.70 $\pm$ 34.30	3.24 $\pm$ 1.15	65.33 $\pm$ 3.51 ^b
T246A	23.23 $\pm$ 2.39 ^a	6.09 $\pm$ 1.66	68.52 $\pm$ 3.22 ^b	31.11 $\pm$ 3.46 ^b	38.05 $\pm$ 12.9 ^a	128.87 $\pm$ 43.23
L247A	1337.97 $\pm$ 463.56	37.82 $\pm$ 3.23 ^b	76.84 $\pm$ 10.34	723.97 $\pm$ 149.75	30.18 $\pm$ 4.82 ^b	143.71 $\pm$ 41.51
T248A	2502.67 $\pm$ 382.82	1.86 $\pm$ 0.39	98.35 $\pm$ 16.20	642.17 $\pm$ 83.42	2.65 $\pm$ 0.72	79.52 $\pm$ 11.07
I249A	423.63 $\pm$ 67.09	3.36 $\pm$ 1.24	118.89 $\pm$ 34.01	280.73 $\pm$ 19.34	4.34 $\pm$ 0.93	95.39 $\pm$ 27.75
L250A	23.85 $\pm$ 15.89 ^a	4.13 $\pm$ 1.57	43.67 $\pm$ 6.72 ^a	50.57 $\pm$ 6.54 ^a	9.51 $\pm$ 2.2 ^a	94.73 $\pm$ 5.54
I251A	1147.97 $\pm$ 450.20	22.08 $\pm$ 7.02	78.00 $\pm$ 11.17	834.33 $\pm$ 217.62	31.76 $\pm$ 9.12 ^a	115.50 $\pm$ 9.44
G252A	1717.98 $\pm$ 512.41	5.64 $\pm$ 1.06	91.99 $\pm$ 25.52	867.10 $\pm$ 251.24	14.82 $\pm$ 2.18 ^b	117.70 $\pm$ 5.97 ^a
V253A	889.45 $\pm$ 308.28	2.23 $\pm$ 0.30	89.49 $\pm$ 14.29	981.70 $\pm$ 231.31	0.77 $\pm$ 0.28 ^a	86.58 $\pm$ 12.45
F254A	277.57 $\pm$ 109.98	5.36 $\pm$ 1.61	54.82 $\pm$ 11.67 ^a	173.77 $\pm$ 20.73	6.18 $\pm$ 1.01 ^a	69.12 $\pm$ 8.28 ^a
V255A	501.77 $\pm$ 79.09	2.28 $\pm$ 0.68	74.86 $\pm$ 11.6	1010.53 $\pm$ 192.72	1.71 $\pm$ 0.55	81.47 $\pm$ 7.56
V256A	1043.67 $\pm$ 314.14	4.52 $\pm$ 1.77	90.5 $\pm$ 11.95	1532.90 $\pm$ 503.43	1.71 $\pm$ 0.31	80.25 $\pm$ 13.46
C257A	198.33 $\pm$ 58.36 ^a	3.98 $\pm$ 0.94	98.12 $\pm$ 31.62	364.03 $\pm$ 74.94	1.99 $\pm$ 0.24	101.55 $\pm$ 20.04
W258A	1638.00 $\pm$ 532.63	36.78 $\pm$ 11.84 ^a	132.24 $\pm$ 14.59	1461.13 $\pm$ 404.94	29.94 $\pm$ 4.11 ^b	95.60 $\pm$ 10.70
A259G	886.73 $\pm$ 226.82	1.29 $\pm$ 0.48 ^a	139.67 $\pm$ 23.16	1233.60 $\pm$ 280.59	0.75 $\pm$ 0.36 ^a	64.93 $\pm$ 13.20 ^a
P260A	90.28 $\pm$ 6.48 ^a	47.24 $\pm$ 16.79 ^a	157.82 $\pm$ 22.42 ^a	95.51 $\pm$ 28.85 ^a	41.06 $\pm$ 5.97 ^b	134.20 $\pm$ 21.77
F261A	1154.50 $\pm$ 324.91	39.41 $\pm$ 14.57 ^a	186.61 $\pm$ 15.41 ^b	829.20 $\pm$ 196.47	37.36 $\pm$ 7.06 ^b	174.82 $\pm$ 34.61
F262A	2201.67 $\pm$ 298.8	11.80 $\pm$ 3.61	179.57 $\pm$ 59.19	1624.25 $\pm$ 429.92	13.88 $\pm$ 1.73 ^b	121.68 $\pm$ 22.23
L263A	854.47 $\pm$ 61.23	4.09 $\pm$ 1.36	237.90 $\pm$ 63.41	768.05 $\pm$ 295.29	2.83 $\pm$ 0.60	100.97 $\pm$ 2.77
H264A	N/A ^c	N/A ^d	N/A ^d	N/A ^c	N/A ^d	N/A ^d
L265A	4893.67 $\pm$ 922.79 ^b	196.76 $\pm$ 58.24 ^b	204.86 $\pm$ 23.85 ^a	3255.33 $\pm$ 682.79 ^b	164.80 $\pm$ 31.35 ^b	98.06 $\pm$ 12.54
I266A	200.47 $\pm$ 33.83 ^a	2.42 $\pm$ 1.08	164.88 $\pm$ 15.52 ^a	246.51 $\pm$ 94.79	1.60 $\pm$ 0.10	96.29 $\pm$ 27.55
F267A	415.27 $\pm$ 40.69 ^a	4.09 $\pm$ 1.26	148.20 $\pm$ 22.56	378.08 $\pm$ 127.62	1.88 $\pm$ 0.31	94.54 $\pm$ 4.13
Y268A	2823.00 $\pm$ 605.36 ^a	31.12 $\pm$ 12.24 ^a	110.29 $\pm$ 13.04	4440.00 $\pm$ 1010.88 ^b	46.53 $\pm$ 8.68 ^b	86.18 $\pm$ 13.16
I269A	212.43 $\pm$ 43.15	4.32 $\pm$ 0.63	139.46 $\pm$ 14.59 ^a	244.15 $\pm$ 60.04	5.61 $\pm$ 1.49	168.04 $\pm$ 25.97
S270A	665.87 $\pm$ 134.75	4.95 $\pm$ 1.77	124.34 $\pm$ 16.30	799.63 $\pm$ 166.75	1.77 $\pm$ 0.27	125.15 $\pm$ 32.30

^aSignificantly different from WT hMC4R, $P < 0.05$.

^bSignificantly different from WT hMC4R, $P < 0.01$.

^cNot detected.

^dOnly responsive to 10^{-5} M α - or β -MSH stimulation.

L265A) that caused dramatic decrease in $B_{\max}$, by more than 50% of WT, F261A and H264A were previously reported to be targeted to the plasma membrane normally (Chen *et al.* 2007b, 2009), and L250A was described to be expressed at the cell surface at 57% of WT (Proneth *et al.* 2006). C257A showed increased affinity for ligand binding and normal signaling properties; therefore, it was not studied here.

We selected the two mutants (P260A and L265A) not studied before and one mutant (H264A) lacking ligand binding to investigate their expression at the plasma membrane. HEK293 cells stably expressing c-myc-tagged WT or mutant hMC4Rs were used and imaged by confocal microscopy at the nonpermeabilized status. Immunostaining of H264A and L265A was at least as strong as the WT hMC4R, demonstrating

that the two mutants were indeed successfully transported to the plasma membrane. The immunostaining of P260A was weaker than that of the WT (Fig. 5).

Constitutive activity of WT and mutant hMC4Rs in the cAMP pathway

The MC4R was previously described to be constitutively active (Nijenhuis *et al.* 2001), and the extracellular N-terminus of the receptor, which could be positioned similar to the endogenous agonists (Pogozheva *et al.* 2005), acted as a tethered intramolecular ligand (Srinivasan *et al.* 2004). Impairment in the fine-tuning of constitutive activity, the basal signaling in the absence of any ligand, was reported to affect the long-term energy balance (Srinivasan *et al.* 2004). Herein, the

Figure 3 The ligand binding (A) and signaling (B) properties of WT and mutant hMC4Rs with β -MSH as the ligand. HEK293T cells were transiently transfected with WT or mutant constructs and 48 h later were used for binding and signaling studies. (A) Intact cell surface binding was measured by competitive inhibition of 125 I-NDP-MSH with serial concentrations of β -MSH. Data are depicted as percentage of WT maximal binding. (B) Intracellular cAMP samples were collected with or without stimulation with different concentrations of β -MSH, and cAMP concentrations were determined by RIA. Data points are mean $\pm$ S.E.M. of duplicate (A) or triplicate (B) measurements within one experiment. Results are representative of at least three independent experiments.

constitutive activities of the 31 mutants were measured and analyzed. The basal cAMP levels of the WT receptor were 32.60 ± 4.39 pmol/ 10^6 cells. We found that nine mutants (K242A, G243A, T246A, L247A, I251A, G252A, W258A, P260A, and H264A) had significantly lower basal cAMP levels compared with that of the WT receptor. Eight mutants (N240A, M241A, A244G, L250A, A259G, I266A, F267A, and I269A) displayed a modest elevation of basal activities and hence were identified to be CAMs (Fig. 6A).

Both Ipsen 5i (Ki 2 nM; Poitout *et al.* 2007) and ML00253764 (Ki 0.16 μ M; Vos *et al.* 2004) were synthesized and identified as selective antagonists of MC4R and subsequently were characterized to exert partial inverse agonistic actions at MC4R (Tao 2010, Tao *et al.* 2010). We studied the inverse agonistic properties of these two small molecules at the eight CAMs described earlier. HEK293T cells transiently transfected with WT or mutant hMC4Rs were incubated in the presence or absence of 10^{-6} M Ipsen 5i or 10^{-5} M ML00253764, and cAMP levels were measured. The basal activities of the WT and all the mutants were significantly decreased by the treatment of either Ipsen 5i or ML00253764. The maximal inhibition of L250A was 41% for Ipsen 5i and 60% for ML00253764. Of the WT and other mutants, the maximal inhibition

was 65 to 85% for Ipsen 5i and from 80 to 88% for ML00253764 (Fig. 6B).

Constitutive activity of WT and mutant hMC4Rs in the ERK1/2 pathway

MC4R activation results in phosphorylation of ERK1/2 (reviewed in Tao (2010)). We investigated whether seven mutant hMC4Rs that were constitutively active in cAMP pathway were also constitutively active in the ERK1/2 signaling pathway (N240A was not studied).

Figure 4 Total specific binding of WT and mutant hMC4Rs (% WT). Data are mean $\pm$ S.E.M. of at least six independent experiments. *Significantly different from WT hMC4R, $P < 0.05$.

Figure 5 Confocal imaging of WT and mutant hMC4Rs. The intact HEK293 cells stably expressing WT or mutant receptors were stained with fluorescein-conjugated anti-myc monoclonal antibody and cell surface fluorescence were detected by confocal microscopy.

The p-ERK1/2 were measured through western blots. Data from the experiments showed that five of these mutants (M241A, L250A, I266A, F267A, and I269A) were also constitutively active in the MAPK pathway with significantly enhanced basal ERK1/2 phosphorylation. Two mutants (A244G and A259G) displayed similar basal p-ERK1/2 levels as the WT hMC4R (Fig. 7).

Signaling property of H264A hMC4R in the ERK1/2 pathway

H264A, undetectable in competitive binding assays, was only responsive to 10^{-5} M α - or β -MSH stimulation with increased cAMP production. One previous study reported that the EC_{50} of H264A was $0.14 \mu\text{M}$ for NDP-MSH (Chen *et al.* 2007b). To investigate whether it also affects the ERK1/2 signaling pathway, we studied p-ERK1/2 in the absence or presence of different concentrations of NDP-MSH, giving a final concentration ranging from 10^{-9} to 10^{-6} M. H264A was only responsive to 10^{-6} M NDP-MSH stimulation. Compared with the WT hMC4R, H264A was significantly defective in both basal and stimulated ERK1/2 phosphorylation (Fig. 8).

Discussion

In rhodopsin-like GPCRs, TM6 has been demonstrated to be able to directly contact the ligand (Jaakola *et al.* 2008, Chien *et al.* 2010, Xu *et al.* 2011, Hanson *et al.* 2012), alter receptor conformations through intramolecular interactions (Palczewski *et al.* 2000, Park *et al.* 2008), and interact with cognate G proteins

(Abell & Segaloff 1997, Murakami & Kouyama 2008, Scheerer *et al.* 2008). In the β_2 adrenoceptor, TM6 has been shown to cause the largest changes between agonist- and inverse agonist-bound conformations (Rasmussen *et al.* 2007) and also been suggested to be involved in regulating the high basal activity of this receptor (Rasmussen *et al.* 2007, Rosenbaum *et al.* 2007). For the current study, we performed detailed pharmacological study of 31 alanine mutants of each residue in the TM6 of hMC4R.

Two residues (L250 and P260) were important for cell surface expression of hMC4R. Alanine mutations of the two residues (L250A and P260A) severely impaired normal plasma targeting of the receptor. At these positions, two naturally occurring mutations, L250Q (Proneth *et al.* 2006, Xiang *et al.* 2006) and P260Q (Wang & Tao 2011), were also reported to cause intracellular retention of hMC4R, characterized by decreased cell surface expression but normal total expression levels. Further studies on the interactions between these mutants and molecular chaperones might help to understand the forward trafficking mechanism of MC4R. In the related MC3R, we showed that mutations at T280, corresponding to

Figure 6 Basal activities of WT and mutant hMC4Rs. (A) HEK293T cells were transiently transfected with WT or mutant receptors and intracellular cAMP levels were measured without any stimulation of the ligand. Data expressed as percentage of WT basal activity are shown as mean $\pm$ s.e.m. of at least six independent experiments. The cAMP levels of WT hMC4R were $32.60 \pm 4.39 \text{ pmol}/10^6 \text{ cells}$. *Significantly higher or lower than WT hMC4R, $P < 0.05$. (B) Partial inverse agonism of Ipsen 5i and ML00253764. HEK293T cells were transiently transfected with WT or mutant receptors, and 48 h later were treated with 10^{-6} M Ipsen 5i or 10^{-5} M ML00253764. cAMP levels were measured with RIA.

Figure 7 Constitutive activation of MAPK pathway. (A) HEK293T cells were transiently transfected with pcDNA 3.1, WT, or mutant hMC4Rs, and 24 h later cells were starved overnight and then harvested. Western blot analysis was performed using antibody against p-ERK1/2 and β -tubulin as a control. (B) Values are mean $\pm$ S.E.M. of at least three independent experiments. *Significantly different from WT hMC4R, $P < 0.05$.

T246 in the MC4R, decreased cell surface expression (Yang & Tao 2012).

Five residues (F254, F261, H264, L265, and Y268) were indispensable for α - and β -MSH binding. F254^{6,44}A, which signaled normally with NDP-MSH stimulation (Chen *et al.* 2007b), had impaired interactions with α - and β -MSH. The corresponding residue of Phe^{6,44} in other GPCRs was reported to be important for the rotation of TM6, which led to an outward shift of the cytoplasmic end of this TM domain (Lebon *et al.* 2011, Rasmussen *et al.* 2011), and this movement opened the binding site for the carboxyl terminus of $G\alpha$ (Scheerer *et al.* 2008). Previously, F261A and H264A were reported to severely impair the ligand binding and signaling processes of NDP-MSH (Yang *et al.* 2000, Haskell-Luevano *et al.* 2001, Chen *et al.* 2007b), though another study reported that F261A had no effect on NDP-MSH binding affinity but restored the signaling efficacy of modified NDP-MSH (Fleck *et al.* 2007). The residues important for the binding and signaling of α - and β -MSH are mostly located in the upper region of the TM domain. They probably could directly participate in the hydrophobic binding pocket (F261, H264, L265, and Y268) respectively, interacting with the pharmacophore His⁶-Phe⁷-Arg⁸-Trp⁹ of α - and β -MSH.

Six residues (T246, L247, I251, G252, W258, and F262) were crucial for normal receptor signaling upon α - and β -MSH stimulation. Alanine mutations of these residues led to severely decreased signaling potencies and reduced basal signaling activities (except for F262A). These residues, primarily located at the cytoplasmic half of the TM domain, probably maintain the active conformation of the receptor. Serine mutation of G252 (G252S) caused significantly decreased signaling potency with α -MSH and ACTH

(1–24) but not β -MSH (Xiang *et al.* 2007). However, alanine mutation of G252 (G252A) acted differently; it exhibited impaired signaling properties for both α - and β -MSH. W258A was previously reported to cause a threefold increase in K_i and >10 -fold increase in EC_{50} with NDP-MSH stimulation by some studies (Chen *et al.* 2007b, 2009) and was described not to affect interactions with NDP-MSH by another study (Pogozheva *et al.* 2005). This mutant showed normal ligand binding but severe defect in signaling to α - and β -MSH in our study. The homologous residues of Trp^{6,48} were described to directly contact with the ligand in A_{2A} adenosine receptor (Jaakola *et al.* 2008) and rhodopsin (Cherezov *et al.* 2007) but not in β_2 -adrenoceptor (Cherezov *et al.* 2007, Rasmussen *et al.* 2007).

Although no binding could be detected for H264A, with the more sensitive signaling assays, we showed that it responded to high concentrations of ligand stimulation with increased cAMP levels (10^{-5} M α - or β -MSH stimulation) and ERK1/2 phosphorylation (10^{-6} M NDP-MSH stimulation). Basal signaling in both pathways was also decreased compared with the WT receptor. No apparent biased signaling (see below) was observed.

The basal activities of the MC4R have been suggested to be of physiological and pathophysiological significance. Defects in basal signaling have been suggested to be a potential cause of genetic obesity caused by MC4R mutations (Srinivasan *et al.* 2004). We also showed that some naturally occurring MC4R mutations decrease basal signaling activity (Tao & Segaloff 2005, Fan & Tao 2009, Wang & Tao 2011). Unlike the related MC3R that has no basal activity (Tao 2007), the WT hMC4R has modest basal activity that can be decreased by agouti-related protein (Nijenhuis *et al.* 2001,

Figure 8 MAPK signaling of hMC4R H264A. In (A), HEK293T cells were transiently transfected with WT or H264A hMC4R, and 24 h later cells were starved overnight and stimulated with different concentrations of NDP-MSH ranging from 10^{-9} to 10^{-6} M for 5 min. Cell lysates were harvested. Western blot analysis was performed using antibody against p-ERK1/2 and β -tubulin as a control. In (B), values are mean $\pm$ S.E.M. of three independent experiments. *Significantly different from WT hMC4R treated with the same concentration of NDP-MSH, $P < 0.05$.

Tao *et al.* 2010). In this study, we showed that of the 31 mutations generated, nine mutants had significantly decreased basal activities (Fig. 6). Eight mutants (N240A, M241A, A244G, L250A, A259G, I266A, F267A, and I269A) had increased basal cAMP levels and the basal activities of these mutants could be partially inhibited by the treatment of either Ipsen 5i or ML00253764 (Fig. 6), two MC4R inverse agonists (Nicholson *et al.* 2006, Tao *et al.* 2010), suggesting that these mutants are CAMs. They are numbered as 6·30, 6·31, 6·34, 6·40, 6·49, 6·56, 6·57, and 6·59 according to the numbering scheme of Ballesteros & Weinstein (Ballesteros & Weinstein 1995). Previous studies with the glycoprotein hormone receptors showed that mutations at some of these loci (such as 6·30, 6·34, and 6·40) were found to cause constitutive activation that cause human diseases (reviewed in Tao (2008)). However, other loci found to cause constitutive activation in the glycoprotein hormone receptors, including the hotspot 6·44 and surrounding residues 6·37, 6·38, 6·41, 6·42, 6·43, and 6·45 (see Tao (2008) for original references), did not cause constitutive activation in hMC4R. These results suggested that although TM6 is a domain that is important for constraining the WT in inactive conformation in many GPCRs through interhelical interactions with TM5 and TM7 (Tao *et al.* 2002, Schneider *et al.* 2010), there are important differences in the local environment that contributed to the differences in constitutive activities.

It has been suggested that in family A GPCRs, an acidic residue at position 6·30 and R3·50 of the DRY motif in TM3 form a salt bridge constraining the WT receptor in inactive conformation. Mutations of 6·30 cause constitutive activation in rhodopsin (Ramon *et al.* 2007), β_2 -adrenoceptor (Ballesteros *et al.* 2001), and lutropin receptor (Angelova *et al.* 2002). The salt bridge was indeed observed in the crystal structure of rhodopsin (Palczewski *et al.* 2000). However, the ionic lock is not observed in the crystal structures of the turkey β_1 -adrenoceptor (Warne *et al.* 2008), the human β_2 -adrenoceptor (Cherezov *et al.* 2007, Rosenbaum *et al.* 2007), human A_{2A} adenosine receptor (Jaakola *et al.* 2008), and human κ -opioid receptor (Wu *et al.* 2012). Mutagenesis data also suggested that this ionic lock is not important in other GPCRs (Schneider *et al.* 2010). N240 in hMC4R cannot form a salt bridge with R3·50, although its mutation led to constitutive activation.

MC4R also activates the ERK1/2 signaling pathway. We asked whether the mutants that had increased basal cAMP levels also constitutively activated the ERK1/2 pathway. Transfected cells were starved overnight in serum-free media and levels of phosphorylated ERK1/2 were measured by western blots. We showed herein that five of these mutants (M241A, L250A, I266A, F267A,

and I269A) were also constitutively active in the MAPK pathway with significantly enhanced basal ERK1/2 phosphorylation. To the best of our knowledge, this is the first study to show that mutations in the MC4R could constitutively activate the ERK1/2 pathway. We showed that two mutants (A244G and A259G) had increased basal cAMP levels but had normal basal p-ERK1/2 levels. The results suggested that these mutants preferentially stabilize certain active conformations of the receptor. Mutations displaying biased signaling have already been identified in MC4R and several other GPCRs, such as MC4R-D90N (Buch *et al.* 2009), MC1R-E92K (Benned-Jensen *et al.* 2011), and M_2 muscarinic acetylcholine receptor-Y104A and -Y177A (Gregory *et al.* 2010). Phosphorylation of ERK1/2 induced by MC4R has been described to be mediated by protein kinase A (Sutton *et al.* 2005), protein kinase C (Chai *et al.* 2006), or phosphoinositide 3-kinase (Vongs *et al.* 2004), depending on the cell lines used, and also may be initiated independently by β -arrestins as suggested in other GPCRs (reviewed in Violin & Lefkowitz (2007) and Reiter *et al.* (2012)). More studies are needed to elucidate the biased signaling mechanisms of MC4R.

In summary, of the 31 residues in the TM6 of hMC4R, we have identified residues that were important for cell surface expression, ligand binding, cAMP production, and maintaining the WT receptor in active conformation. We also reported the constitutive activation of the ERK1/2 pathway. These data provided comprehensive information on the structure–function relationship of the TM6 of MC4R and will be useful for rationally designing MC4R agonists and antagonists for treatment of energy balance disorders.

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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