

Identification of Domains Directing Specificity of Coupling to G-proteins for the Melanocortin MC3 and MC4 Receptors*

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The melanocortin receptors, MC3R and MC4R, are G protein-coupled receptors that are involved in regulating energy homeostasis. Using a luciferase reporter gene under the transcriptional control of a cAMP-responsive element (CRE), the coupling efficiency of the MC4R and MC3R to G-proteins was previously shown to be different. MC4R exhibited only 30–50% of the maximum activity induced by MC3R. To assess the role of the different MC3R and MC4R domains in G-protein coupling, several chimeric MC3R/MC4R receptors were constructed. The relative luciferase activities, which were assessed after transfecting the chimeric receptors into HEK 293T cells, showed that the i3 (3rd intracellular) loop domain has an essential role in the differential signaling of MC3R and MC4R. To reveal which amino acid residue was involved in the MC4R-specific signaling in the i3 loop, a series of mutant MC4Rs was constructed. Reporter gene analysis showed that single mutations of Arg²²⁰ to Ala and Thr²³² to either Val or Ala increased the relative luciferase activities, which suggests that these specific amino acids, Arg²²⁰ and Thr²³², in the i3 loop of MC4R play crucial roles in G-protein coupling and the subtype-specific signaling pathways. An examination of the inositol phosphate (IP) levels in the cells transfected with either MC3R or MC4R after being exposed to the melanocortin peptides revealed significant stimulation of IP production by MC3R but no detectable increase in IP production was observed by MC4R. Furthermore, none of the MC4R mutants displayed melanocortin peptide-stimulated IP production. Overall, this study demonstrated that MC3R and MC4R have distinct signaling in either the cAMP- or the inositol phospholipid-mediated pathway with different informational requirements.

Melanocortins are peptide hormones that are derived from the precursor peptide pro-opiomelanocortin, by a series of proteolytic cleavages (1). The melanocortins are known to have a broad spectrum of physiological actions, which include the regulation of melanocyte pigmentation (2), thermoregulation (3), obesity (4), control of the cardiovascular system (5), and learning and memory (6), and have also been found to have immunomodulatory effects (7). These hormones mediate their effects through G protein-coupled receptors by stimulating adenylate cyclase (8). To date five melanocortin receptor subtypes, with different patterns of tissue expression in the brain and peripheral body, have been cloned and characterized (8–12).

It has been reported that the activation of melanocortin 4 receptor (MC4R)¹ by α -melanocyte-stimulating hormone (MSH) increases energy expenditure and decreases food intake. Moreover, the genetic disruption of MC4R was found to cause obesity in mice (13). Recent experiments in MC3R-null mice indicate that the inactivation of MC3R results in increased fat mass and reduced body mass, despite the fact that the animals were hypophagic and maintained normal metabolic rates (14, 15). These results suggest the nonredundancy of the MC3R and MC4R melanocortin receptors in the regulation of energy homeostasis (14, 15).

In previous studies, we and others have demonstrated that heterologously expressed MC3R and MC4R are coupled to the cAMP pathway. We analyzed several α -MSH analogues upon stimulation of MC3R and MC4R using a CRE (cAMP responsive element)-mediated reporter gene transcription activity assay (16), and were able to show that both MC3R and MC4R, expressed in human cell line HEK 293T, stimulate transcription when stimulated using different analogues of melanocortin at different levels. Our previous studies have shown that MC3R and MC4R may have differential efficiencies and/or modes of signaling in terms of G-protein coupling, in addition to their specific ligand-receptor interactions, which can specify subtype-specific signaling pathways *in vivo* (16).

The role of the third intracellular (i3) loop in G-protein coupling specificity has been investigated extensively for many seven-transmembrane domain receptors, including adrenergic, serotonergic, muscarinic, and dopaminergic receptors (17–21). For example, swapping experiments performed upon two different G protein-coupled receptors demonstrated the importance of this loop in selective coupling to specific G-protein/effecter systems (22, 23).

To identify the role(s) of the third intracellular loop of the

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¹ The abbreviations used are: MC4R, melanocortin 4 receptor; MSH, α -melanocyte-stimulating hormone; CRE, cAMP-responsive element; i3 loop, 3rd intracellular loop; IP, inositol phosphate.

TABLE I

Sequences of overlapping primer for chimeric receptor construction

Overlapping primers were designed from the junction of the third intracellular loop domain and the carboxyl terminus in each rat MC3R and human MC4R. Nucleotide sequences from MC3R are shown in bold font and sequences from MC4R are shown in italics.

Primers	
MC3R-F (Vector primer)	5' CGA GCT CGG ATC CCC GGG CTG CAG GAA TTC 3'
MC4R-R (Vector primer)	5' ATG CAT GCT CGA GGT CGA CGG TAT CGA TAA 3'
MC4R-F	5' CACA <i>GTTCCTC</i> <i>ATGCCAGGCTTCACATT</i> AAGAGG 3'
	734 745 652 675
MC3R-R	5' <i>AAGCCTGGCCAT</i> GAGGAACATGTGGATATATAGGGT 3'
	663 652 745 722
Chm2-3R-F	5' <i>GGAGCGATTACC</i> <i>ATCACTATCCTGCTGGGTGTTTTTC</i> 3'
	727 738 839 862
Chm2-4R-R	5' <i>CAGGATAGTGAT</i> <i>GGTAATCGCTCCCTTCATATTGGC</i> 3'
	850 839 738 715
Chm3-4R-F	5' <i>ATCTACGCCTTC</i> <i>CGGAGTCAAGAACTGAGGAAAACC</i> 3'
	1001 1012 913 936
Chm3-3R-R	5' <i>TTCTTGACTCCG</i> GAAGGCCTAGATGAGGGGGCTCGAT 3'
	924 913 1012 989

MC3R and MC4R receptors in terms of G-protein coupling specificity and receptor activation, several chimeras were constructed and characterized. We used the CRE-luciferase reporter gene assay to score the efficacy of receptor-G proteins coupling (24, 25). In parallel, amino acid mutations were generated in the third intracytoplasmic loop of MC4R to identify the residues that play a role in G-protein coupling. These mutant receptors were examined in terms of their abilities to bind melanocortin receptor-specific ligands and with respect to signal transduction at the cAMP level.

EXPERIMENTAL PROCEDURES

Construction of Chimeric Receptors—Chimeric receptors were made by using the polymerase chain reaction with sequence substitution of the i3 and carboxyl-terminal domain in rat MC3R (GenBank™ accession number X70667) and human MC4R (GenBank™ accession number S77415). Overlapping primers were designed from the junction of i3 and the carboxyl-terminal in MC3R and MC4R (Table I). To construct the chimeras, fragments originating from each receptor were first amplified in separate reactions with the indicated primers, namely, a vector primer and an overlapping primer directed against the desired junction sites between MC3R and MC4R receptors. Fragments were isolated and purified from agarose gel and used as templates in a second polymerase chain reaction performed with two outer vector primers. For example, to construct chimera 1, the MC3R sequence from the amino terminus to the carboxyl-terminal end of transmembrane domain V was amplified using a vector primer (MC3R-F, Table I) and an overlapping 36-mer primer (MC3R-R, Table I). This overlapping primer contained 24 bases (in bold, Table I) from the template sequence (MC3R) and an additional 12 bases from MC4R (in italics, Table I). In the same way, a second PCR was performed using two primers (MC4R-F and MC4R-R) with an MC4R template. These PCR products were purified and used as templates for another PCR, which was performed using the MC3R-F and MC4R-R primers. Final PCR products of each chimera were isolated and purified from agarose gel. All chimeric inserts were subsequently subcloned into pSV (16, 25) using the *Bam*HI and *Xho*I enzyme restriction sites. All

TABLE II

Oligonucleotide primers for MC4R mutant receptor construction

MC4R mutants were constructed by site-directed mutagenesis with the indicated primers. The nucleotides of mutant amino acid residues are shown in bold italics.

Mutant	Primers
R220A	(F) 5' <i>gttctgatggcgcgcttcacattaag</i> 3' (R) 5' <i>ctcttaatggaagcgcgccatcaggaac</i> 3'
K224A	(F) 5' <i>gccaggcttcacattgagggattgctgc</i> 3' (R) 5' <i>gacagcaatcctcgcaatggaagcctgc</i> 3'
R225A	(F) 5' <i>cttcacattaaggcattgctgctctccc</i> 3' (R) 5' <i>ggggaggacagcaatcgcttaatggaag</i> 3'
T232A	(F) 5' <i>gtctccccggcctgggctccatccc</i> 3' (R) 5' <i>gcgatggaccagcggccgggaggac</i> 3'
T232V	(F) 5' <i>gtctccccggcctgggctccatccc</i> 3' (R) 5' <i>gcgatggaccacgccgggaggac</i> 3'
I235P	(F) 5' <i>ggcactggtgccccaccgcaaggtgc</i> 3' (R) 5' <i>ggcacttggcgtgggaccaccagtgcc</i> 3'
R236Q	(F) 5' <i>cactggtgccatcagcaaggtgccaatg</i> 3' (R) 5' <i>catattggcacttctgctgatggcaccagtg</i> 5'
R236A	(F) 5' <i>cactggtgccatcgcgcaaggtgccaatg</i> 3' (R) 5' <i>catattggcacttgcgatggcaccagtg</i> 5'
G238H	(F) 5' <i>gccatccgcaaacacgccaatgaggg</i> 3' (R) 5' <i>ccctcatattgctgttggcggatggc</i> 3'
A239S	(F) 5' <i>gccatccgcaaggttcgaatgaggg</i> 3' (R) 5' <i>ccctcatattcgacaccttggcggatggc</i> 3'
N240C	(F) 5' <i>ccgccaaggtgctcatgaaggaggcg</i> 3' (R) 5' <i>cgctcccttcagcagcacttggcg</i> 3'

chimeric inserts were sequenced on an automatic sequencer, ALF-express (Amersham Biosciences), using a dideoxy terminator cycle sequencing kit (Amersham Biosciences). No point mutations were observed, and exact substitutions of all domains were found.

Construction of MC4R Mutant Receptors—The MC4R and MC3R sequences were extracted from the NCBI data base and aligned using the ClustalW and GENE DOC programs. Primers were designed from the sequences in the I3 loop domain of MC4R using a recommended protocol (QuikChange™ Site-directed mutagenesis Kit, Stratagene) (Table II). Briefly, denatured double stranded plasmid DNA was annealed to a mutagenic primer pair of forward and reverse oligonucleotides and the new strand of DNA so obtained was synthesized with *pfu* DNA polymerase (Pfu Turbo DNA polymerase, Stratagene) by polymerase chain reaction (95 °C for 30 s, 55 °C for 1 min, and 68 °C for 12 min for 16 cycles). Methylated template DNA was digested with *Dpn*I enzyme for 1 h at 37 °C. Reaction mixtures were then used to transform competent XL-1 blue *Escherichia coli* and the plasmid DNA was prepared. Inserts of plasmid DNAs were sequenced entirely and all amino acid changes were confirmed by full double stranded sequencing.

Expression of Melanocortin Receptors and Luciferase Reporter Gene Assay—Rat MC3R and human MC4R cDNA, kindly provided by Dr. Roger D. Cone, were cloned into pSV expression vector (16, 25). For receptor expression, HEK 293T cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and transfected with pSV-rMC3R and pSV-hMC4R, respectively, using FuGENE 6 transfection reagent (Roche Molecular Biochemicals) using the procedure recommended by the manufacturer. Briefly, 5–7 × 10⁵ cells were plated per six-well culture dish and transfected with 1 μg of pCRE-luc (Stratagene), 0.5 μg of plasmid pCH110 carrying the β-galactosidase gene, and 1 μg of each chimeric receptor or MC4R mutant plasmid DNA. After 6 h, the transfection mixture was replaced with fresh medium. Twenty-four hours after transfection, HEK 293T cells were treated for 3 h with various concentrations of α-MSH-ND or NDP-MSH in Dulbecco's modified Eagle's medium supplemented with 0.5% bovine serum albumin. After treatment, the cells were lysed and assayed for luciferase activity using the luciferase assay system (Promega), and luminescence was measured using a 96-well luminometer (Microumat;

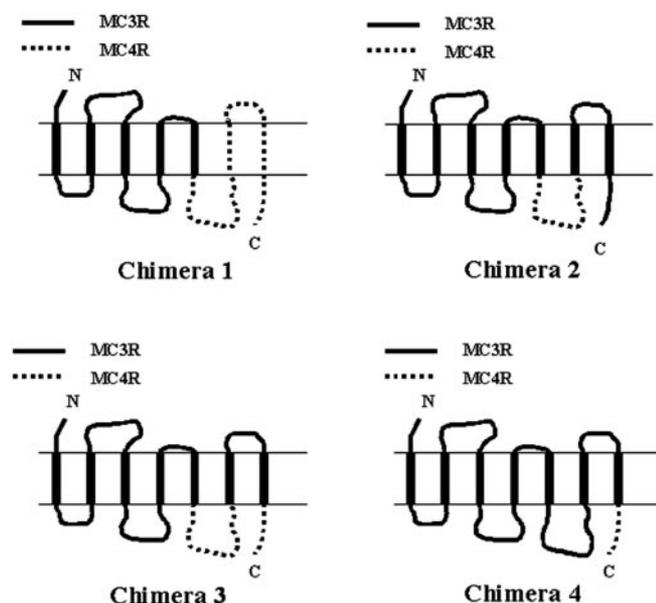


FIG. 1. Schematic representation of MC3/MC4R chimeric receptors. Bold lines denote the sequence of MC3R and dotted lines the sequence of MC4R. Black rectangles represent putative transmembrane domains.

EG & G Berthold, Bad Wilbad, Germany). The expression of the reporter gene was normalized (26) using β -galactosidase activity (27). Transfection in the control group was performed under the transfection conditions described above with 1 μ g of pCRE-luc, 0.5 μ g of plasmid pCH110 carrying the β -galactosidase gene, and 1 μ g of chimeric receptor or MC4R mutant plasmid DNA, but without stimulating by the melanocortin peptides. Results are expressed as the ratio of luciferase activity of the transfected cells to that of the unstimulated controls. The mean values of the data obtained were fitted to a sigmoid curve with a variable slope factor using nonlinear squares regression in GraphPad Prism software. EC_{50} values (nM) are described as mean \pm S.E.

Ligand Binding Study—Iodinated NDP-MSH, 125 I(iodotyrosyl-2)-[Nle⁴,D-Phe⁷] α -MSH, was prepared using the chloramine-T method, as follows. 1 mCi (10 μ l) of Na 125 I (PerkinElmer Life Sciences) was added to 5 μ g of NDP-MSH in 100 μ l of 200 mM sodium phosphate buffer (pH 7.2), and 20 μ l of 2.8 mg/ml chloramine T solution in 200 mM sodium phosphate (pH 7.2) was then added for 15 s, at which time the reaction was stopped with 50 ml of 3.6 mg/ml sodium metabisulfate. The reaction mixture was then diluted in 1 ml of 0.1% bovine serum albumin solution containing 0.1% trifluoroacetic acid and purified using a Sep-Pak C₁₈ cartridge (Waters) and Sephadex G-25 gel filtration chromatography. A 100- μ l aliquot of a 0.1% solution of bovine serum albumin was added to all radioactive fractions. Specific activity of the [125 I]NDP-MSH was measured, which was \sim 0.59 pmol/100,000 cpm. For the binding assay, 24 h after transfection in HEK 293T cells, medium was removed and the cells were washed twice with washing buffer (50 mM Tris, 100 mM NaCl, 5 mM KCl, 2 mM CaCl₂, pH 7.2). The cells were then immediately incubated in wells at 37 $^{\circ}$ C for 2 h with 0.25 ml of binding buffer (containing 50 mM Tris, 100 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 5% Hanks' balanced salt solution, and 0.5% bovine serum albumin, at pH 7.2) containing a constant concentration of [125 I]NDP-MSH and appropriate concentrations of the competing unlabeled ligand in each well. At the end of the incubation, the plates were placed on ice for 15 min and the cells were washed twice with 0.5 ml of ice-cold binding buffer. Cells were detached from the plates using 0.5 ml of 0.5 N NaOH twice. Radioactivity was measured (Workman automatic γ -counter) and K_d and B_{max} values were calculated, to a 95% confidence interval, using GraphPad Prism software.

Determination of Total Inositol Phosphate—HEK 293T cells were plated at a density of 3×10^5 cells/well in six-well plates and allowed to recover for 24 h. The cells were then transfected with pSV-rMC3R and pSV-hMC4R, respectively, using the FuGENE 6 transfection reagent (Roche Molecular Biochemicals) as described above, after which 2 ml of Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum was added to each well and the cells were incubated for an additional 24 h. The medium was then aspirated, and cells were incubated for 16 h in *myo*-inositol-free Dulbecco's modified Eagle's medium

TABLE III
 125 I-Labeled NDP-MSH binding to MC3R and MC4R, and to chimeric receptors

K_d and B_{max} values were calculated at a 95% confidence interval using GraphPad Prism software for binding assay using 125 I-labeled NDP-MSH upon MC3R and MC4R, and upon the chimeric receptors expressed in HEK 293T cells. Values are mean \pm S.E. from at least three independent experiments.

	B_{max}	K_d
	fmol/mg protein	nM
MC3R	361.7 \pm 20.47	3.775 \pm 0.4906
MC4R	375.9 \pm 40.07	4.269 \pm 1.0090
Chimera 1	329.2 \pm 21.56	3.310 \pm 0.5175
Chimera 2	329.3 \pm 18.82	3.558 \pm 0.4753
Chimera 3	298.7 \pm 23.34	3.164 \pm 0.5977
Chimera 4	381.2 \pm 53.52	4.298 \pm 1.3350

supplemented with *myo*-[3 H]inositol (1 μ Ci/ml, 25 mCi/mmol) (DuPont Biotechnology Systems). The cells were then pretreated with Dulbecco's modified Eagle's medium supplemented with 20 mM LiCl for 30 min, and then incubated in the absence or presence of different concentrations of α -MSH for 5 s to 3 or 45 min. The reaction was stopped by the addition of perchloric acid to a final concentration of 5% (w/v). Cells were extracted and [3 H]inositol polyphosphates were analyzed by anion-exchange high performance liquid chromatography, using a Partisphere SAX column (Whatman), as described previously (28, 29). All experiments were performed in triplicate.

Statistical Analysis—Cellular responses to the various peptides were compared using one-way analysis of variance and the Student's *t* test with Instat software (GraphPad).

RESULTS

MC3R and MC4R share 60% overall amino acid identity and 76% similarity. The transmembrane regions show the highest degree of homology, whereas the intra- and extracellular loops show the lowest. As depicted in Fig. 1, four types of MC3R/MC4R chimeric receptors were constructed to investigate the role of the different domains of MC3R and MC4R in G-protein coupling. These chimeric receptors were designed to determine the contribution made by the i3 domain to G-protein coupling efficiency. Thus, the i3 loop and the remainder of the COOH-terminal domain of MC3R were swapped with the corresponding domain of the MC4R (named chimera 1), and in chimera 2, only the i3 loop of MC3R was swapped with that of MC4R. In chimera 3, the i3 loop and the cytoplasmic COOH-terminal loop of MC3R were swapped with that of MC4R, and in chimera 4, only the cytoplasmic loop of MC3R (named chimera 3 or 4, respectively) was swapped with the corresponding region of the MC4R.

Binding and CRE-mediated Reporter Gene Activity of Chimeric Receptors—Wild type MC3R, MC4R, and the chimeric MC3/MC4R receptors were transiently transfected into HEK 293T cells. MC3R, MC4R, and all chimeric receptors all bound [125 I]NDP-MSH with high affinity, as shown in Table III. The affinities of the expressed MC3R and MC4R receptors for [125 I]NDP-MSH were 3.775 \pm 0.4906 and 4.269 \pm 1.0090 nM, respectively, and the B_{max} values estimated for the two receptors were very similar (361.7 \pm 20.47 and 375.9 \pm 40.07 fmol/mg of protein for MC3R and MC4R, respectively, Table III). The respective chimeric receptors were found to have affinities that were similar to those of the parent receptors. These data indicate that substitution of the third intracellular loop had no significant conformational influence on ligand-binding domains, which are localized mainly within the transmembrane domain regions.

In parallel, wild type and chimeric MC3/MC4R receptors were transfected with the CRE-luciferase reporter gene into HEK 293T cells, as described earlier (16). In our previous study (16), using a luciferase reporter plasmid containing a CRE in its promoter (pCRE-Luc), we were able to show that it is pos-

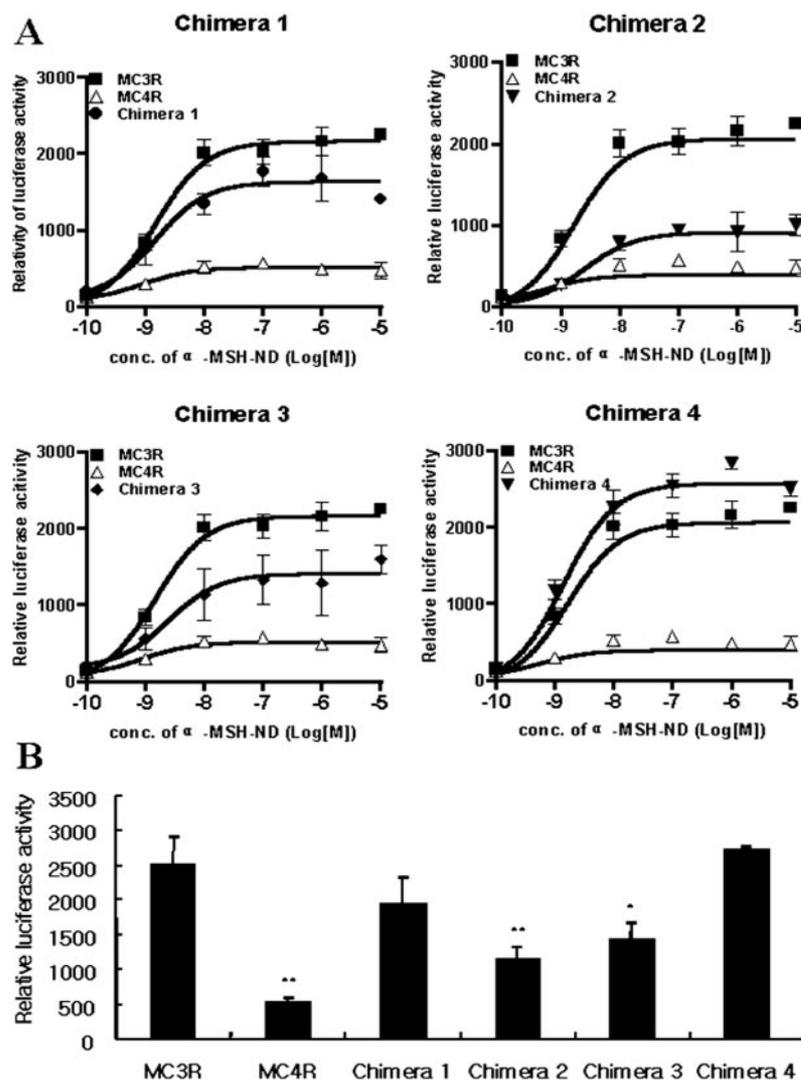


FIG. 2. Relative cAMP-mediated luciferase gene transcriptional activities of MC3R, MC4R, and MC3/MC4R chimeric receptors by melanocortin agonist α -MSH-ND in HEK 293T cells. A, cAMP-mediated luciferase activity was determined after stimulating MC3R and MC4R and different MC3/MC4R chimeric receptors in HEK 293T cells with α -MSH-ND. Data shown represent mean \pm S.E. of at least five independent experiments. B, maximal cAMP-mediated transcriptional activity stimulated by α -MSH-ND at 10^{-6} M on MC3R, MC4R, and chimera mutants in HEK 293T cells. Results are mean \pm S.E. from four independent experiments. *, $p < 0.05$ and **, $p < 0.01$, significantly different from the corresponding MC3R value.

sible to monitor variations in intracellular cAMP levels induced by transient transfections of MC3 and MC4 receptors. When increasing concentrations of α -MSH analogues were added, a typical dose-dependent and saturable induction of luciferase activity was observed (Fig. 2). However, we detected no significant effect of the α -MSH analogues on cells transfected with the reporter plasmid alone, which rules out the possibility of the presence of endogenous melanocortin receptors in the cell lines used for transfection, as the presence of such endogenous species might have affected the measured luciferase activity levels (data not shown). Another control transfection, using a luciferase reporter vector devoid of a CRE sequence, showed no significant response, demonstrating that the luciferase activity measured was dependent of the presence of the CRE site in the reporter plasmid (data not shown). These experiments demonstrate that the measured CRE-luciferase activity levels reflect the effects elicited by the receptors on changes in the intracellular cAMP levels, as represented by changes in cAMP-mediated gene expression, and thus provided evidence of receptor-G protein-effector interaction. Alterations in the abilities of different chimeric receptors to induce luciferase reporter gene activity were measured.

As described previously the luciferase activity induced by MC4R was found to be about 30–50% of the level induced by MC3R. Interestingly, chimera 1, where the i3 loop with the rest of the COOH-terminal domain of the MC3R were swapped with the corresponding domain of the MC4R, was similar to native

MC3R in terms of its maximal reporter gene activity. Chimera 4 showed a slight increase in maximal reporter gene activity. However, the abilities of chimeras 2 and 3 to induce luciferase reporter gene activity were significantly affected, and decreased by 60 and 45% in maximal reporter gene activity, respectively (Fig. 2, A and B). The EC_{50} calculated from the dose-response curves was in general highly reproducible over several experiments, results are summarized in Table IV. The ability to induce the luciferase reporter gene activity of chimera 2 receptors upon stimulation with α -MSH-ND was significantly affected by 8.7-fold ($EC_{50} = 11.37$ nM) versus MC3R ($EC_{50} = 1.3$ nM), whereas the EC_{50} values of other chimeric receptors were not significantly changed (Table IV). Therefore, our analysis of the four chimeras suggests that the dominant loss of G-protein coupling efficiency was associated mainly with the 3rd intracytoplasmic loop of the MC3R and MC4R receptors.

Binding and CRE-mediated Reporter Gene Transcription Activity of Mutant MC4R—To assess the role of the i3 loop and to establish whether mutations in the i3 loop of MC4R alter the coupling profiles of the receptor, amino acid mutations were generated in the i3 loop of MC4R (Fig. 3). Charged amino acids, such as, arginine 220, lysine 224, arginine 225, and arginine 236 were substituted with alanine (R220A, K224A, R225A, and R236A respectively), which lacks an amino acid chain beyond the β carbon and also avoids the introduction of steric hindrance or unwanted ionic interactions. We also substituted threonine 232 with valine (T232V), a nonpolar amino acid, or

TABLE IV

Summary of estimated EC_{50} values of the melanocortin agonist α -MSH-NDP for MC3R, MC4R and chimeric receptors

K_d and B_{max} values were calculated at a 95% confidence interval using GraphPad Prism software for binding assay using ^{125}I -labeled NDP-MSH upon MC3R and MC4R, and upon chimeric receptors expressed in HEX 293T cells. Values are the mean \pm S.E. from at least three independent experiments.

Receptor	EC_{50}
	<i>nM</i>
MC3R	1.309 \pm 0.367
MC4R	1.007 \pm 0.564
Chimera 1	3.303 \pm 0.782 ^a
Chimera 2	11.372 \pm 6.128
Chimera 3	3.163 \pm 1.475
Chimera 4	2.988 \pm 0.725

^a $p < 0.01$ significantly different from corresponding MC3R value.

bMC5R Bos	: LAR TH IRRIAT	FGHS--SVR Q RTGV	GAIT	: 29
oMC5R Ovis	: LAR TH VRRITAA	FGHS--SVR Q RTGV	GAIT	: 29
hMC5R Homo	: LAR TH VKRITAA	FGAS--SAR Q RTSMOGAVT	: 29	
pMC5R Pan	: LAR TH VKRITAA	PRAS--SAR Q RTSMOGAVT	: 29	
sMC5R Sus	: -AR AH VRRITAA	PGYR--SAR Q RTSMOGAVT	: 28	
rMC5R Ratt	: LARN HV KRI AAS	PRYN--SVR Q RASMGAVT	: 29	
mMC5R Mus	: LARN HV KRI AAS	PRYN--SVR Q RTSMOGAVT	: 29	
cMC5R Gall	: LAR TH VKRITAA	PGYN--SVH Q RTSMOGAVT	: 29	
rMC4R Ratt	: MAR LHI KRI AV	PGTG--TI R OGANMGAVT	: 29	
bMC4R Bos	: MAR LHI KRI AV	PGSG--TI R OGANMGAVT	: 29	
hMC4R homo	: MAR LHI KRI AV	PGTG--AI R OGANMGAVT	: 29	
oMC4R Ovis	: MAR LHI KRI AV	PGTG--AI R OGANMGAVT	: 29	
sMC4R Sus	: MAR LHI KRI AV	PGTG--AI R OGANMGAVT	: 29	
cMC4R Gall	: MAR MHI KRI AV	PGTG--PI R OGANMGAVT	: 29	
mMC4R Mus	: MAR LHI KRI AV	PGTG--TI R OGTNMGAVT	: 29	
hMC3R Homo	: -AR LHV KRI AA	PPADGVAP Q CHSCMGAVT	: 30	
oMC3R Ovis	: -AR LHV KRI AA	PPADGAAP Q CHSCMGAVT	: 30	
rMC3R Ratt	: -AR LHV QRI AA	PPADGVAP Q CHSCMGAVT	: 30	
mMC3R Mus	: -AR LHV QRI AA	PPAGVAP Q CHSCMGAVT	: 30	
	AR H6 4IA 1P	Q	6kGA6T	

FIG. 3. Alignment of MC3Rs and MC4Rs focusing on the i3 loop. Black boxes indicate identities and gray boxes conservative substitutions. *hMC4R*, human MC4R; *mMC4R*, mouse MC4R; *sMC4R*, *Sus scrofa* MC4R; *bMC4R*, *Bos taurus* MC4R; *rMC3R*, rat MC3R; *hMC3R*, human MC3R. Mutations generated by site-directed mutagenesis in the i3 loop domain of MC4R are indicated.

with alanine (T232A). In addition, isoleucine 235 and arginine 236 in MC4R were substituted with proline and glutamine, respectively (I235P and R236Q), which are native amino acids located at these positions in MC3R (Fig. 3). Other MC4R-specific amino acids in the i3 loop, such as glycine 238, alanine 239, and asparagine 240 were also substituted with the amino acids present at the same position in the i3 loop of MC3R, respectively (G238H, A239S, and N240C). These mutant receptors were examined in terms of their abilities to bind melanocortin receptor-specific ligands and for their abilities to transduce signals at the cAMP level.

Table 5 summarizes ligand affinities for MC3R, MC4R, and the 11 mutant MC4 receptors. All mutant receptors were found to bind [^{125}I]NDP-MSH, demonstrating that they were all expressed on the plasma membrane. In general, no significant differences in the binding of [^{125}I]NDP-MSH to the mutant receptors were observed.

To assess whether the G-protein coupling efficiencies of the mutant receptors differed from that of MC4R, we measured their abilities to induce CRE-luciferase reporter gene activity. Mutants R220A, T232V, and T232A showed enhanced reporter gene activity, which was very near that of MC3R, whereas the other mutant receptors had CRE-luciferase reporter gene activities similar to that of MC4R (Fig. 4, A and B). The EC_{50} value of R220A was 12-fold lower than that of MC4R, and the EC_{50} value of T232V and T232A was 6.5- and 5.9-fold, respectively, lower than that of MC4R (Table VI). The mutant receptors K224A (2.1-fold), R225A (3.5-fold), R236A (3.9-fold),

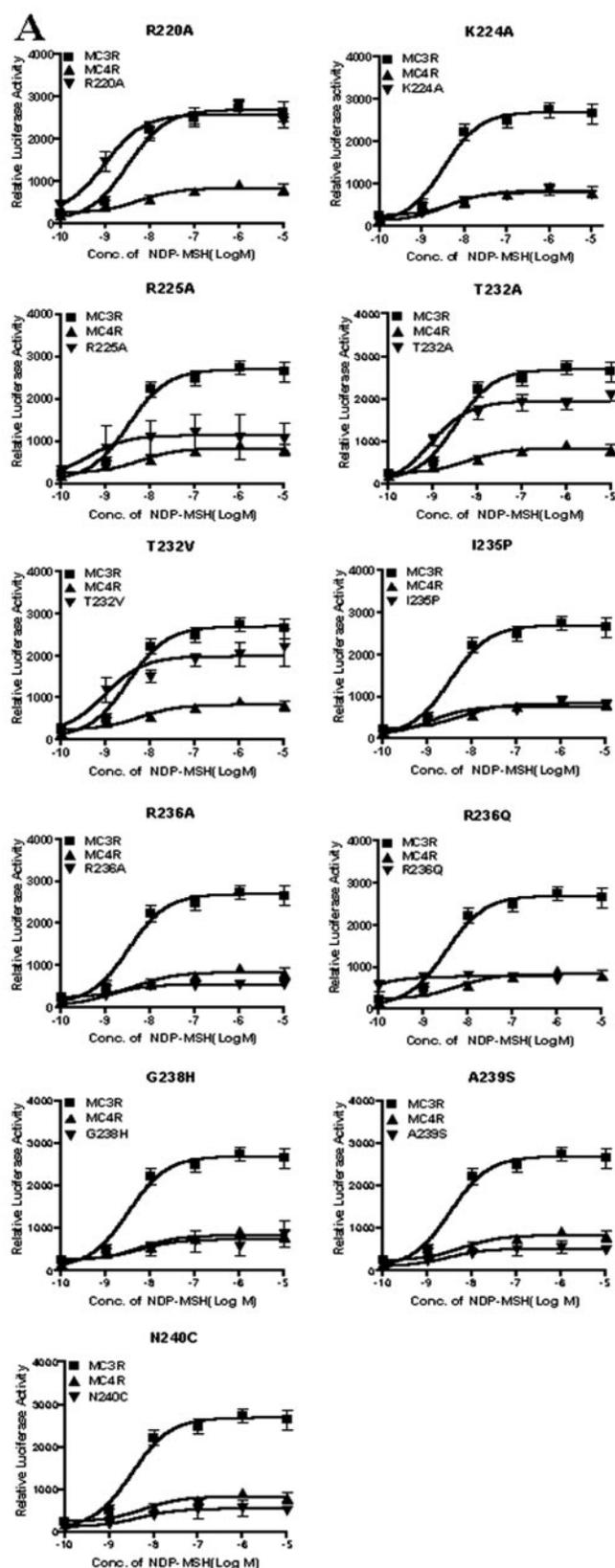


FIG. 4. Relative cAMP-mediated transcriptional activity of MC3R, MC4R, and mutant receptors by NDP-MSH in HEK 293T cells. A, cAMP-mediated transcriptional activity stimulated by NDP-MSH on MC3R and MC4R, and on mutant receptors in HEK 293T cells. Results are mean \pm S.E. from four independent experiments. B, maximal cAMP-mediated transcriptional activity stimulated by NDP-MSH at 10^{-6} M on MC3R, MC4R, and mutant receptors in HEK 293T cells. Results are mean \pm S.E. from four independent experiments. ***, $p < 0.001$, significantly different from the corresponding MC4R value.

FIG. 4—continued

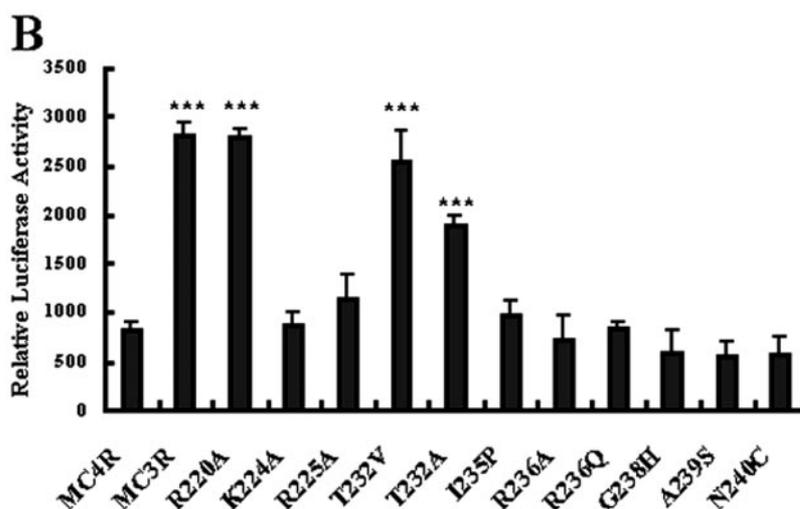


TABLE V
¹²⁵I-Labeled NDP-MSH binding to MC3R, MC4R,
 and MC4R mutants

K_d and B_{max} values were calculated with 95% confidence interval using GraphPad Prism software for binding assay with the ¹²⁵I-labeled NDP-MSH on MC3R, MC4R, and MC4R mutants expressed in HEK 293T cells. Values are the mean \pm S.E. from at least three independent experiments.

	B_{max}	K_d
	<i>fmol/mg protein</i>	<i>nM</i>
MC3R	361.7 \pm 20.47	3.775 \pm 0.4906
MC4R	375.9 \pm 40.07	4.269 \pm 1.0090
R220A	325.9 \pm 25.23	3.893 \pm 0.6862
K224A	297.9 \pm 18.47	4.967 \pm 0.6529
R225A	323.8 \pm 26.67	4.066 \pm 0.7527
T232A	334.8 \pm 28.21	3.559 \pm 0.7007
T232V	304.8 \pm 34.29	3.514 \pm 0.9270
I235P	309.6 \pm 15.92	3.621 \pm 0.4330
R236A	296.3 \pm 38.75	4.081 \pm 1.1980
R236Q	282.0 \pm 41.17	4.529 \pm 1.4400
G238H	397.5 \pm 16.73	3.839 \pm 0.3505
A239S	339 \pm 28.43	4.858 \pm 1.303
N240C	346.5 \pm 32.56	3.005 \pm 0.5872

R236Q (3.9-fold), and G238H (1.9-fold) showed slightly enhanced EC_{50} efficiencies, although their maximum CRE-luciferase reporter gene activities were unchanged (Table VI). The mutants I235P, A239S, and N240C showed no significant changes in EC_{50} values or maximal reporter gene activity (Fig. 4, Table VI). These data suggest that Arg²²⁰ and Thr²³² are critical amino acid residues, and are implicated in the specific G-protein coupling properties of MC4R.

Distinct Inositol Phosphate Signaling by MC3R and MC4R—To determine the basis of the differential CRE-luciferase activity mediated by MC3R and MC4R more closely, we assessed whether or not these receptors were coupled to other signaling pathways, for example, the pathway involving phospholipase C activation. An examination of the inositol phosphate (IP) levels in the cells transfected with either MC3R or MC4R following exposure to the melanocortin peptides revealed a significant stimulation of IP production by MC3R but no detectable increase in IP production was observed by MC4R stimulation, as presented in Fig. 5A. We also measured the IP levels for the mutant MC4 receptors, however, none of these mutants displayed melanocortin peptide-stimulated IP production (Fig. 5B).

TABLE VI
 Summary of estimated EC_{50} values of the melanocortin agonist
 NDP-MSH for MC3R, MC4R and mutant MC4 receptors

EC_{50} values were determined for the cAMP-mediated luciferase reporter gene activity stimulated by NDP-MSH on MC3R and MC4R, and MC4R mutants expressed in HEK293T cells. Data are mean \pm S.E. from at least four independent experiments.

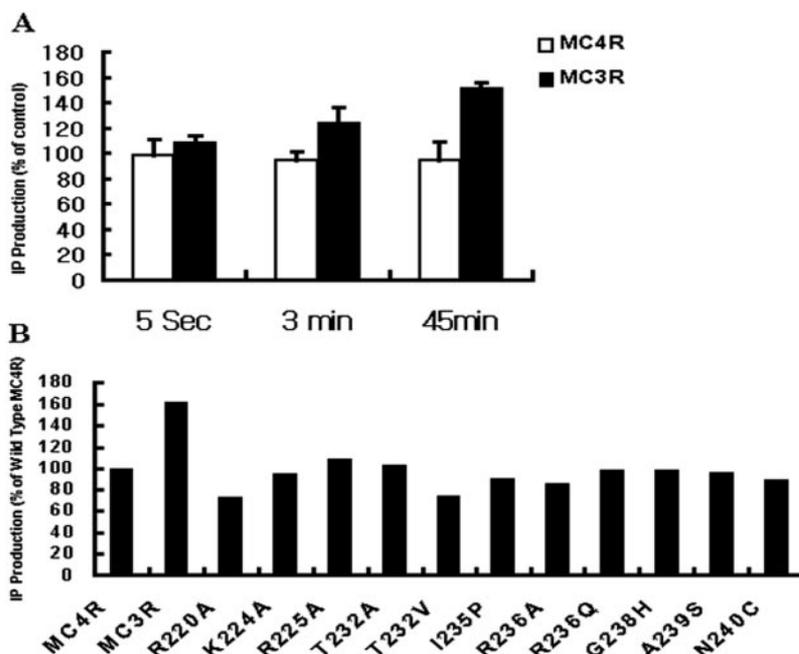
Mutant receptor	EC_{50}
	<i>nM</i>
MC4R	4.86 \pm 1.18
MC3R	3.945 \pm 0.472
R220A	0.405 \pm 0.0939 ^a
K224A	2.36 \pm 1.760
R225A	1.39 \pm 0.7258
T232A	0.870 \pm 0.201 ^a
T232V	0.744 \pm 0.4138 ^a
I235P	3.61 \pm 1.851
R236A	1.24 \pm 0.5365
R236Q	1.26 \pm 0.9031
G238H	2.442 \pm 1.205
A239S	4.783 \pm 0.6933
N240C	4.363 \pm 1.478

^a $p < 0.05$, significantly different from the EC_{50} values of MC4R.

DISCUSSION

MC3R and MC4R are closely related melanocortin receptor subtypes, which share 60% overall sequence homology (30, 31). However, these two MCR subtypes are biologically distinguishable in a number of respects, for example, they show distinctive agonist and antagonist binding affinities. Although both subtypes couple to G_s and activate adenylyl cyclase on agonist stimulation, the G_s coupling efficacy of MC4R is different from that of MC3R (16). Moreover, MC3R and MC4R display distinctive tissue distribution patterns, suggesting that they have different subtype-specific physiological roles. Gene “knock-out” mouse models (13–15) have been established for MC3R and MC4R resulting in different physiological outcomes. Recent experiments in MC3R-null mice indicate that the inactivation of MC3R results in an increased fat mass and a reduced body mass despite being the fact that the animals were hypophagic and maintained normal metabolic rates. This suggests the non-redundancy of MC3R and MC4R in the regulation of energy homeostasis (14, 15). A comparison of MC3R- and MC4R-null mice phenotypes supports the idea that this melanocortin receptor subtype-specific mediated regulation of feeding behavior appears to be controlled in a finely tuned manner. For example, it has been suggested that in contrast to MC4R, which mainly controls food intake, MC3R might regulate fat stores by some specific metabolic pathway (31, 32).

FIG. 5. Generation of IP by stimulation with MC3R and MC4R in HEK293T cells. A, time-dependent generation of [³H]IP in HEK 293T cells transfected with MC3R and MC4R. The receptors were stimulated by 10⁻⁸ M α-MSH. B, generation of [³H]IP by MC3R, MC4R, and the different mutants receptors at 45 min after stimulation by the 10⁻⁸ M α-MSH in the HEK 293T cells.



In our previous study, we compared the CRE-mediated reporter gene activity of MC3R *versus* MC4R and found that MC4R showed 30–50% of the maximum activity induced by MC3R. Moreover, this was not because of a difference in receptor expression, as was shown by a receptor binding assay. Differential CRE-mediated reporter gene transcription by MC3R and MC4R suggests that MC3R and MC4R may have different signaling efficiencies, in terms of G-protein coupling, in addition to their specific ligand-receptor interactions, which can specify a subtype-specific signaling pathway *in vivo*.

It has been shown that the third intracellular loop of several G protein-coupled receptors is an important site for G-protein coupling and specificity (10). Moreover, highly charged regions of the third loop are strikingly conserved between the many different seven-transmembrane receptors, and point mutations or deletions affecting these regions disrupt normal signal transduction by these receptors by altering their binding to G-proteins (17–21). In the present study, by chimeric MC3R/MC4R receptor study, we have demonstrated that the i3 loop in MC3R and MC4R plays a pivotal role in G-protein coupling specificity. Indeed, the i3 loop appears to be critical for full activation of G_s protein for at least MC3R. Replacement of the entire i3 loop of MC3R by MC4R resulted in a ~60% decrease in maximal reporter gene activity, which is similar to the activity induced by MC4R. These findings indicate that the i3 loop is a critical structural determinant for the G-protein coupling property in MC3R and MC4R.

Impaired G_s-mediated cAMP responsiveness was observed in experiments with chimeric MC3R/MC4R receptor constructs, especially in chimera 2, which had a reduced maximal response to melanocortin. In addition its maximal cAMP response to melanocortin was reduced, and its dose-response curve (EC₅₀) for melanocortin was shifted to the right. Maximal response reflects the maximal number of melanocortin receptors that are coupled to G_s and can be activated by melanocortin. The EC₅₀ here represents the ability of the receptor to promote G_s coupling to downstream effectors. The observation that chimera 2 exhibited a 60% decrease in maximal response, with a significant change in EC₅₀, suggests that the i3 loop determines the G-protein coupling efficiency. Chimera 3, where the i3 loop and the cytoplasmic COOH-terminal loop of MC3R were swapped with the corresponding region of MC4R, also showed a signif-

icant decrease in maximal response but without a significant change in EC₅₀. It is possible that the interaction between the i3 loop and the COOH-terminal is important for receptor-G protein interactions, but that the i3 loop is the critical determinant of G-protein coupling efficiency for MC3 and MC4 receptors. Indeed, chimera 4, in which only the cytoplasmic COOH-terminal was swapped with that of MC4R did not significantly affect CRE-luciferase reporter gene activity.

In many cases of G protein-coupled receptors, such as the rhodopsin/β-adrenergic receptor subfamily, the i3 loop has been identified as an important structural domain, the residues of which couple to G-proteins and determine the specificity of receptor-G protein interactions (33–35). Our present results show that the replacement of the i3 loop of MC3R, with that of MC4R, suppresses its G-protein coupling efficiency, as evidenced by the reduced CRE-luciferase activity.

The analysis of the amino acid composition of the third loop of the known seven-transmembrane receptors shows the presence of highly charged residues in the loop. It is known that an alteration of hydrophobic/hydrophilic amino acids can influence the secondary structure of proteins (36–38). More refined analysis of the sequences of the i3 loop in different melanocortin receptors has revealed that homologies are shared by MC3R, MC4R, and MC5R. In the present study, the effects of replacing charged amino acids, namely, Arg²²⁰, Lys²²⁴, and Arg²²⁵, which are conserved in MC3R, MC4R, and MC5R, were examined. A mutation introduced by replacing Arg²²⁰ with alanine enhanced the EC₅₀ 13.9-fold *versus* native MC4R. Similarly, replacing Thr²³², which is specific to MC4R, with valine or alanine also greatly enhanced CRE-luciferase activity by 7.6-fold (Table VI). These data indicate that these mutants acquired an increased G-protein coupling efficiency. However, when Ile²³⁵ or Arg²³⁶ were substituted with proline or glutamine, the amino acid residues natively present in MC3R, respectively, no significant changes in CRE-luciferase activity *versus* MC4R were observed. The mutants I235P, A239S, and N240C, where MC4R-specific amino acids were substituted by the amino acids present at the same position in MC3R, did not display significant changes in the CRE-luciferase activity by the mutation. Therefore, the amino acid residues in the i3 loop seem to be involved in the generation of structure or the modification of the environment of the protein, which confers G-

protein coupling selectivity. On the other hand, Thr²³² might represent a candidate site for phosphorylation, for example, the phosphorylation involved in desensitization of the G protein-coupled receptor.

To determine the basis of the differential CRE-luciferase activity mediated by MC3R and MC4R more closely, we assessed whether or not these receptors were coupled to other signaling pathways, for example, the pathway involving phospholipase C activation. It has been reported that MC3R is also able to couple to G_q (39). An examination of the IP levels in the cells transfected with either MC3R or MC4R following exposure to the melanocortin peptides revealed a rapid stimulation of IP production by MC3R but no detectable increase in IP production was observed by MC4R stimulation, as presented in Fig. 5. Therefore, it is suggested that this differential inositol phospholipid signaling by MC3R and MC4R may also contribute to the differential CRE-luciferase activity mediated by these two receptors. To a certain extent, because a CRE-reporter gene assay can effectively monitor both G_s and G_q activation by CREB-mediated gene expression (24, 25), this distinct inositol phospholipid signaling would be one of the possible explanations for the differential CRE-reporter gene activation profile between MC3R and MC4R. On the other hand, the basal level of the reporter gene activity with MC4R in the absence of stimulation with the melanocortin analogue is actually higher than that of MC3R, suggesting that MC4R could be constitutively activated.² Our observation is consistent with a recent report by Nijenhuis *et al.* (40), which also suggests the relevance of the constitutive activity of MC4R. This aspect would contribute to the distinct signaling pathway mediated by MC3R and MC4R that was observed. Furthermore, this suggests that MC3R and MC4R signaling via the G_s would be more complex and regulated in a fine way.

In conclusion, our study demonstrates that the activation of different signaling pathways by the MC3R and MC4R receptors have distinct conformational requirements. The third intracellular loop of these receptors plays a crucial role in the acquisition of these conformations, inasmuch as, depending on the mutation in this region, it is possible to modify the selectivity of the coupling and to selectively impair the ability of the receptor to transduce a precise effect of melanocortin. Furthermore, our results suggest that MC3R and MC4R have distinct signaling in either the cAMP- or the inositol phospholipid-mediated pathway, and this appears to be regulated in a fine tuned way, possibly to adapt the complex and tonic signaling requirements for their physiological role in fuel homeostasis.

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REFERENCES

- Smith, A. I. & Funder, J. W. (1988) *Endocr. Rev.* **9**, 159–179
- De Wied, D. & Jolles, J. (1982) *Physiol. Rev.* **62**, 976–1059
- Feng, J. D., Dao, T. & Lipton, J. M. (1987) *Brain Res. Bull.* **18**, 473–477
- Fan, W., Boston, B. A., Kesterson, R. A., Hruby, V. J. & Cone, R. D. (1997) *Nature* **385**, 165–168
- Li, S. J., Vargra, K., Archer, P., Hruby, V. J., Sharma, S. D., Kesterson, R. A., Cone, R. D. & Kunos, G. (1996) *J. Neurosci.* **16**, 5182–5188
- De Wied, D. & Croiset, G. (1991) *Methods Achiev. Exp. Pathol.* **15**, 167–199
- Smith, E. M., Hughes, T. K., Hashemi, F. & Stefano, G. B. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 782–786
- Mountjoy, K. G., Robbins, L. S., Mortrud, M. T. & Cone, R. D. (1992) *Science* **257**, 1248–1251
- Chhajlani, V. & Wikberg, J. E. S. (1992) *FEBS Lett.* **309**, 417–420
- Chhajlani, V., Muceniec, R. & Wikberg, J. E. S. (1993) *Biochem. Biophys. Res. Commun.* **195**, 866–973
- Gantz, I., Konda, Y., Tashiro, T., Shimoto, Y., Miwa, H., Munzert, G., Watson, S. J., Del Valle, J. & Yamada, T. (1993) *J. Biol. Chem.* **268**, 8246–8250
- Gantz, I., Miwa, H., Konda, Y., Shimoto, Y., Tashiro, T., Watson, S. J., Del Valle, J. & Yamada, T. (1993) *J. Biol. Chem.* **268**, 15174–15179
- Huszar, D., Lynch, C. A., Fairchild-Huntress, V., Dunmore, J. H., Fand, Q., Berkemeier, L. R., Boston, B. A., Cone, R. D., Smith, F. J., Campfield, L. A., Burn, P. & Lee, F. (1997) *Cell* **88**, 131–141
- Butler, A. A., Kesterson, R. A., Khong, K., Cullen, M. J., Pellemounter, M. A., Dekoning, J., Baetscher, M. & Cone, R. D. (2000) *Endocrinology* **141**, 3518–3521
- Chen, A. S., Marsh, D. J., Trumbauer, M. E., Frazier, E. G., Guan, X. M., Yu, H., Rosenblum, C. I., Vongs, A., Feng, Y., Cao, L., Metzger, J. M., Strack, A. M., Camacho, R. E., Mellin, T. N., Nunes, C. N., Min, W., Fisher, J., Gopal-Truter, S., MacIntyre, D. E., Chen, H. Y. & Van Der Ploeg, L. H. (2000) *Nat. Genet.* **26**, 97–102
- Lee, E. J., Lee, S. H., Jung, J. W., Lee, W., Kim, B. J., Park, K. W., Lim, S. K., Yoon, C. J. & Baik, J. H. (2001) *Eur. J. Biochem.* **268**, 582–591
- Cotecchia, S., Ostrowski, J., Kjelsberg, M. A., Caron, M. G. & Lefkowitz, R. J. (1992) *J. Biol. Chem.* **267**, 1633–1639
- Lembo, P. M., Ghahremani, M. H., Morris, S. J. & Albert, P. R. (1997) *Mol. Pharmacol.* **52**, 164–171
- Lee, N. H. & Fraser, C. M. (1993) *J. Biol. Chem.* **268**, 7949–7957
- Picetti, R., Saiardi, A., Abdel Samad, T., Bozzi, Y., Baik, J. H. & Borrelli, E. (2000) *Crit. Rev. Neurobiol.* **11**, 121–142
- Kunkel, T. A., Roberts, J. D. & Zakour, R. A. (1987) *Methods Enzymol.* **154**, 367–382
- Liggett, S. B., Freedman, N. J., Schwinn, D. A. & Lefkowitz, R. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 3665–3669
- Kozell, L. B., Machids, C. A., Neve, R. L. & Neve, K. A. (1994) *J. Biol. Chem.* **269**, 30199–30206
- Chen, W., Shields, T. S., Stork, P. J. & Cone, R. D. (1995) *Anal. Biochem.* **226**, 349–354
- Baik, J. H., Bahtia, M., Picetti, R., Thiriet, G. & Borrelli, E. (1996) *Methods Neurosci.* **29**, 75–89
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Herbomel, P., Bourachot, B. & Yaniv, M. (1984) *Cell* **39**, 653–662
- Downes, C. P., Hawkins, P. T. & Irvine, R. F. (1986) *Biochem. J.* **238**, 501–506
- Bae, Y. S., Cantley, L. G., Chen, C. S., Kim, S. R., Kwon, K. S. & Rhee, S. G. (1998) *J. Biol. Chem.* **273**, 4465–4469
- Adan, R. A. & Gispen, W. H. (2000) *Eur. J. Pharmacol.* **405**, 13–24
- Raffin-Sanson, M. L. & Bertherat, J. (2001) *Eur. J. Endocrinol.* **144**, 207–208
- Cummings, D. E. & Schwartz, M. W. (2000) *Nat. Genet.* **26**, 8–9
- Strader, C. D., Fong, T. M., Graziano, M. P. & Tota, M. R. (1995) *FASEB J.* **9**, 745–754
- Ostrowski, J., Kjelsberg, M. A., Caron, M. G. & Lefkowitz, R. J. (1992) *Annu. Rev. Pharmacol. Toxicol.* **32**, 167–183
- Malmberg, A. & Strange, P. G. (2000) *J. Neurochem.* **75**, 1283–1293
- Hunyady, L., Zhang, M., Jagadeesh, G., Bor, M., Balla, T. & Catt, K. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 10040–10045
- Greasley, P. J., Fanelli, F., Scheer, A., Abuin, L., Nenniger-Tosato, M., DeBenedetti, P. G. & Cotecchia, S. (2001) *J. Biol. Chem.* **276**, 46485–46494
- Burstein, E. S., Spalding, T. A. & Brann, M. R. (1998) *Biochemistry* **37**, 4052–4058
- Konda, Y., Gantz, I., DelValle, J., Shimoto, Y., Miwa, H. & Yamada, T. (1994) *J. Biol. Chem.* **269**, 13162–13166
- Nijenhuis, W. A., Oosterom, J. & Adan, R. A. (2001) *Mol. Endocrinol.* **15**, 164–171

² C. S. Kim and J.-H. Baik, unpublished data.

Identification of Domains Directing Specificity of Coupling to G-proteins for the Melanocortin MC3 and MC4 Receptors

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