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Functional Characterization of Mutations in Melanocortin-4 Receptor Associated with Human Obesity*

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Melanocortin-4 receptor (MC4R) is a G protein-coupled receptor implicated in the regulation of body weight. Genetic studies in humans have identified two frameshift mutations of MC4R associated with a dominantly inherited form of obesity. We have generated and expressed the corresponding MC4R mutants in 293T cells and found that cells transfected with the truncation mutants failed to exhibit agonist binding or responsiveness despite retention of structural motifs potentially sufficient for binding and signaling. Immunofluorescence studies showed that the mutant proteins were expressed and localized in the intracellular compartment but absent from the plasma membrane, suggesting that these mutations disrupted the proper cellular transport of MC4R. Further studies identified a sequence in the cytoplasmic tail of MC4R necessary for the cell surface targeting. We further investigated a possible dominant-negative activity of the mutants on wild-type receptor function. Co-transfection studies showed that the mutants affected neither signaling nor cell surface expression of wild-type MC4R. We also characterized three human sequence variants of MC4R, but these exhibited identical affinities for peptide ligands and identical agonist responsiveness. Thus, unlike the obesity-associated MC4R truncation mutants, the polymorphisms of MC4R are unlikely to be contributors to human obesity.

Melanocortin receptors belong to the superfamily of G protein-coupled receptors (GPCRs).[1] Five melanocortin receptor subtypes have been identified thus far. They differ in their tissue distribution as well as physiological functions. Melanocortin-1 receptor (MC1R) is expressed in melanocytes where it regulates pigmentation (1). MC2R is expressed in adrenal cortex, where it binds ACTH and mediates glucocorticoneogenesis (2). The MC5R is wildly expressed in peripheral tissues (3–5) and is implicated in the regulation of exocrine gland function (6). Both MC3R and MC4R are found primarily in the brain (7, 8), and their role in the central regulation of weight homeostasis has been the subject of intense investigation.

Several lines of evidence indicate that the central melanocortin receptors are involved in feeding behavior in rodents. First, MC3R and MC4R are expressed in the hypothalamic paraventricular nucleus, which has been strongly implicated in the central control of food intake and energy balance (9). Pharmacological studies show that intracerebroventricular injection of melanocortin peptide α-melanocyte-stimulating hormone (αMSH), the putative physiological agonist of MC3R and MC4R, inhibits feeding in mice, and this effect is completely blocked by co-administration of the melanocortin receptor antagonist SHU9119. In addition, administration of SHU9119 alone augments nocturnal feeding in mice (10). These observations led to the proposal that the central melanocortin receptors exert tonic inhibition on feeding. Consistent with this concept, overexpression of agouti-related protein (AGRP), a neuropeptide that antagonizes both MC3R and MC4R, results in mice that are hyperphagic, hyperinsulinemic, and obese (11, 12). Because these phenotypes are recapitulated in mice with the MC4R gene deletion (13), the specific role of MC4R in the development of obese phenotypes is established. In addition, reduction of food intake by central administration of leptin can be blocked by co-administration with the melanocortin receptor antagonist SHU9119 (14, 15), thereby implicating the melanocortin system in leptin signaling.

Recent genetic evidence has correlated the melanocortin system with the development of human obesity. αMSH is derived from the precursor proopiomelanocortin via posttranslational modification. Patients with compound heterozygous or homozygous mutations in the proopiomelanocortin gene resulting in the absence of αMSH production are obese (16). In addition, a major quantitative trait locus for obesity on human chromosome 2 contains the proopiomelanocortin gene (17). Obesity has also been associated with two different MC4R frameshift mutations involving a four-base pair deletion at codon 211 and a four-base pair insertion at codon 244, respectively (18, 19). Both mutations encode a truncated MC4R consisting of five transmembrane domains (TMs). The insertion mutant also contains the third cytoplasmic loop, thought to be the chief signaling domain of GPCRs. Interestingly, differing from other known monogenic changes that are associated with the development of obesity (16, 20–23), these obese individuals are heterozygous for the MC4R mutations. Since GPCRs generally function as monomers, the obese phenotype was speculated to be a result of haplo-insufficiency. However, accumulating evidence suggests that GPCRs can form dimers and oligomers (24, 25), raising the possibility that the mutant MC4R may exert a dominant negative effect on the function of wild-type receptors. To distinguish between these two possibilities as well as to establish the cellular mechanism underlying the pathogenesis of obesity, we expressed the two MC4R mutants *in vitro* and assessed their functions.

In this study, we also examined the contribution of MC4R polymorphisms to the development of obesity. Three sequence

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[1] The abbreviations used are: GPCR, G protein-coupled receptor; MC1R, MC2R, MC3R, MC4R, and MC5R, melanocortin 1, 2, 3, 4, and 5 receptor, respectively; αMSH, α-melanocyte-stimulating hormone; AGRP, agouti-related protein; NDP-MSH, [Nle^4-D-Phe^7]MSH; IBMX, 3-isobutylmethyxanthine; G_s, stimulatory G protein; TM, transmembrane domain; PCR, polymerase chain reaction; DPBS, Dulbecco's phosphate-buffered saline.

variants of MC4R had been identified when we began the study. The predominant form (96%) in a British population carries valine at codon 103 and isoleucine at codon 169 in both alleles (26). Another 4% in the population is heterozygous for valine to isoleucine substitution at codon 103 (8, 26). One sequence variant was found in the published data base (2, 8). This variant carries an isoleucine to valine substitution at codon 103 and a serine to isoleucine substitution at codon 169. These three variants were expressed and characterized functionally *in vitro*.

EXPERIMENTAL PROCEDURES

Materials—[Nle^4-D-Phe^7]MSH (NDP-MSH) and AGRP (amino acid residues 83–132) were purchased from Peninsula Laboratories and Phoenix Pharmaceuticals, respectively. αMSH and forskolin were purchased from Calbiochem. 3-Isobutylmethylxanthine (IBMX) was from Sigma, and ^{125}I-labeled NDP-MSH was from Amersham Pharmacia Biotech.

Oligonucleotide-directed Mutagenesis—For constructing MC4R sequence variants, wild-type MC4R was cloned in vector pALTER-II, and the point mutations were introduced according to the instructions of the Altered Sites II *in vitro* mutagenesis systems (Promega). For constructing frameshift mutations, a two-stage PCR technique was used (27). For constructing C-terminal truncation mutations and adding Kozak sequences (GCCGCCGCC) and FLAG tags to the N termini of all MC4R variants, the conventional PCR-based technique was employed. All PCRs were carried out with PfuTurbo DNA polymerase (Stratagene) to minimize undesired mutations. To aid cloning into the TA cloning vector pCR3.1 (Invitrogen), PCR products were treated with *Taq* polymerase (Life Technologies, Inc.) at 72 °C for 10 min to add 3′-overhanging A nucleotides, and subsequent cloning was carried out according to the manufacturer's instructions. All PCR products were sequenced to confirm the presence of the desired mutations and the absence of unwanted mutations that might have been introduced during the PCR.

Cell Culture and Transfection—293T and COS-7 cells were maintained in Dulbecco's modified Eagle's medium (with glutamine; Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). Cells were incubated at 37 °C in humidified air containing 5% CO_2. Cells were generally at 70–80% confluence on the day of transfection, and transfections were carried out using Lipofectamine reagent according to the manufacturer's instructions (Life Technologies).

Adenylate Cyclase Assays—48 h after transfection, 293T cells were washed once with PBS and then detached from the plate with PBS containing 0.02% EDTA (Sigma). The detached cells were harvested by centrifugation and resuspended in Hanks' balanced salt solution (Life Technologies) containing 0.5 mM IBMX, 2 mM HEPES, pH 7.5 (IBMX buffer). After incubation at 37 °C for 15 min to allow for IBMX uptake, 0.4 ml of cell suspension ($\sim 5 \times 10^5$ cells/ml) were added to 0.1 ml of IBMX buffer containing various concentrations of agonists or 10 μM forskolin. The cells were subsequently incubated at 37 °C for 15 min to allow for cAMP accumulation. The activity was terminated by adding 0.5 ml of 5% trichloroacetic acid, and cAMP released from lysed cells was assayed by the cAMP ^{125}I scintillation proximity assay system (Amersham Pharmacia Biotech). EC_{50} values were calculated with a 95% confidence interval using GraphPad Prism software (using nonlinear regression analysis fitted with a sigmoidal dose-response curve with variable slope).

Receptor Binding Assays—Receptor binding assays were carried out with crude membrane preparations prepared as follows. Two days after transfection, 293T cells were detached from plates with PBS, 0.02% EDTA and collected by centrifugation. Cells were then resuspended in ice-cold TEM buffer (25 mM Tris, 6 mM $MgCl_2$, 5 mM EDTA, 1 μM phenylmethylsulfonyl fluoride, pH 7.4) followed by sonication. The membrane was collected by centrifugation at 17,000 rpm for 15 min in a Sorval rotor at 4 °C and resuspended in TEM buffer for subsequent binding assays or for storage at −80 °C for future uses. The binding was carried out in a total volume of 200 μl containing 50 mM Tris, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, 0.1% bovine serum albumin, 0.25 mg of wheat germ agglutinin SPA beads (Amersham Pharmacia Biotech), 2.5–5 μg of membranes, and various concentrations of ^{125}I-labeled NDP-MSH and competitors as specified in the legend to Fig. 2. Nonspecific binding was determined in the presence of a 1000-fold molar excess of cold NDP-MSH. After gentle rocking overnight at room temperature, radioactivity associated with SPA beads was counted in a Parkard Cobra Microbeta Counter (Wallac). K_d and K_i values were calculated with a 95% confidence interval using GraphPad Prism software with model equations specified in the figure legend.

Immunofluorescence Detection—COS-7 cells were chosen for this study, since they were more adherent than 293T cells during the staining process. 24 h after transfection, COS-7 cells were seeded onto two-well chamber slides (Nunc, Inc.) and incubated overnight. The next day, the cells were washed four times with Dulbecco's phosphate-buffered saline (DPBS; Life Technologies) and fixed for 20 min in 4% paraformaldehyde in DPBS followed by four washes with DPBS. Cells were then incubated for 5 min either with DPBS alone for nonpermeabilized staining or with DPBS containing 0.2% Triton X-100 for permeabilization. Following blocking for 30 min with DPBS containing 10% fetal bovine serum and 2% bovine serum albumin (blocking buffer), cells were incubated for 1 h in blocking buffer containing 1 μg/ml of anti-FLAG monoclonal antibodies (M2; Sigma), which was followed by three washes each for 10 min with blocking buffer. Cells were then incubated for 30 min in blocking buffer containing a 1:1000 dilution of the Oregon Green 488 rat anti-mouse IgG conjugate (highly cross-absorbed; Molecular Probes, Inc., Eugene, OR). After washing twice each for 10 min with DPBS, cells were mounted with coverslips using Aqua-polyMount (Polysciences, Inc.) and viewed using a confocal laser scanning microscope (Fluoview, Olympus).

RESULTS

Expression of Frameshift Mutants of MC4R Fails to Mediate Agonist-stimulated Intracellular cAMP Accumulation—All melanocortin receptors belong to the family of seven-transmembrane G protein-coupled receptors, and upon stimulation all subtypes activate adenylate cyclase via G_s and elevate intracellular 3′,5′-adenosine monophosphate. One frameshift mutation of MC4R occurs at codon 211 as a result of a four-base pair deletion, which introduces five aberrant amino acids before termination (18). This frameshift mutation is predicted to code for a truncated protein (D4) that contains the first five TMs and lacks the third cytoplasmic loop, the last two TMs, and the cytoplasmic tail (Fig. 1). Another frameshift mutation occurs at codon 244, as a result of a four-base pair insertion, which introduces 40 aberrant amino acids before termination (19). This frameshift mutation encodes a truncated protein (I4) consisting of the first five TMs in addition to an intact third intracellular loop (Fig. 1). We constructed the D4 and I4 mutants according to the genetic abnormalities. The two mutant constructs were transiently expressed in 293T cells and subjected to functional analysis. As shown in Fig. 2*A*, cells transfected with either I4 or D4 did not exhibit cAMP accumulation upon stimulation by the high affinity agonist NDP-MSH, whereas under the same conditions cells transfected with the wild-type MC4R displayed significant levels of cAMP production. In parallel experiments in which transfected cells were treated with forskolin, a direct activator of adenylate cyclase, both wild type and mutant-transfected cells showed high levels of cAMP generation (Fig. 2*A*). The data suggest that cells transfected with the mutants are viable and competent and that the failed cAMP production is probably due to either a failed coupling to G_s or an altered affinity for ligands. To distinguish these two possibilities, we performed binding assays. As shown in Fig. 2*B*, when assayed for binding to ^{125}I-labeled NDP-MSH, crude membranes isolated from D4- or I4-transfected cells displayed no detectable binding as compared with the wild type (Fig. 2*B*). To assess whether the mutant proteins were expressed, both mutant and wild-type MC4Rs were tagged at the N terminus with the FLAG epitope (DYKDDDDK). Western blot analysis with anti-FLAG monoclonal antibody on cell lysates as well as membranes from transfected cells indicated that the mutant proteins were produced at similar levels to that of wild type (data not shown). The FLAG tag did not affect either binding or signaling of the wild-type MC4R (data not shown).

The Mutant MC4Rs Fail to Reach to the Plasma Mem-

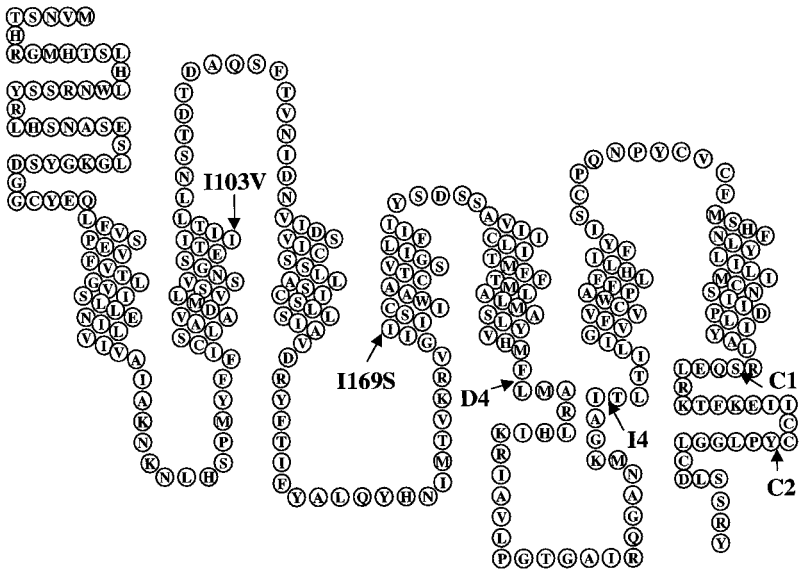

FIG. 1. **A schematic representation of the wild-type MC4R.** Wild-type MC4R consists of an extracellular N terminus, seven TMs, three intracellular and three extracellular loops, and a cytoplasmic tail. Sites at which truncation mutants I4, D4, C1, and C2 occurred are indicated. Isoleucine 103 to valine (I103V) and isoleucine 169 to serine (I169S) substitutions in the MC4R sequence variants analyzed in this study are also indicated.

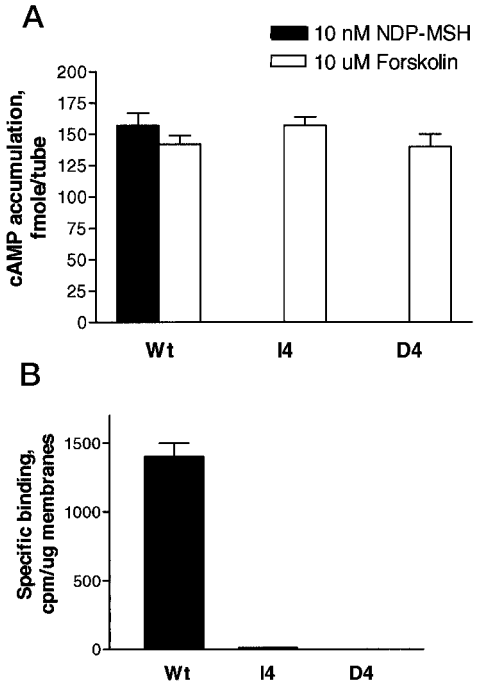

FIG. 2. **The frameshift mutants of MC4R are nonfunctional.** *A*, cAMP production in 293T cells transfected with wild-type and mutant MC4Rs. Two days after transfection, cells (2.5×10^5) were treated with either 10 nM NDP-MSH or 10 μM forskolin. The cAMP production was measured following incubation at 37 °C for 15 min and calculated by subtracting basal levels from levels obtained in the presence of drug. Forskolin treatment was used as an internal control. *B*, binding of ^{125}I-labeled NDP-MSH to membranes obtained from 293T cells transfected with wild-type and mutant MC4Rs. Crude membranes isolated from cells transiently transfected with MC4Rs were incubated with a subsaturating amount of ^{125}I-labeled NDP-MSH (200 pM) in the conditions described under "Experimental Procedures." The specific binding was derived from the total binding minus nonspecific binding. The *numbers* represent means and S.E. of two or more independent experiments, and each was performed in triplicate.

brane—To assess whether the mutant I4 and D4 MC4R receptors were properly localized on the plasma membrane, the N-terminal FLAG-tagged MC4Rs were transiently expressed in COS-7 cells, and their cellular localization was analyzed by immunofluorescent microscopy. To detect cell surface-bound and intracellular receptors, monoclonal anti-FLAG antibodies were utilized with nonpermeabilized and permeabilized cells, respectively. For cells expressing wild-type receptors, a fluorescent signal was detected at the plasma membrane of intact cells and in the intracellular membrane compartments of permeabilized cells (Fig. 3), indicating that wild-type receptors are transported to the cell surface. However, for cells transfected with mutant I4 or D4, immunofluorescence labeling was observed exclusively inside permeabilized cells (Fig. 3). Despite of the relatively strong intracellular signal seen in permeabilized cells (transfected with either wild type or mutant receptors) due to overexpression, there was no cell surface staining with I4- and D4-transfected cells in any fields examined. These data indicate that the mutant MC4Rs are expressed as abundantly as the wild-type protein in transfected cells but are unable to reach to the plasma membrane.

Amino Acid Residues at the Cytoplasmic Tail of MC4R Are Essential for Its Cell Surface Localization—Little is known regarding the structural motif of GPCRs necessary for their proper cell surface delivery. As an initial attempt to delineate the sequence crucial for MC4R cell surface targeting, we truncated the last 27 amino acid residues from the cytoplasmic tail (a total of 28 amino acid residues long) of MC4R and analyzed the function of the tailless protein (C1; Fig. 1). To our surprise, truncation of the tail obliterated the ability of MC4R to signal in response to NDP-MSH challenge (Fig. 4*A*). Because C1 was incapable of binding to ^{125}I-labeled NDP-MSH (Fig. 4*B*), it was further analyzed by immunofluorescence studies. Cells transfected with C1 showed no cell surface staining while exhibiting intense intracellular signals (Fig. 5), a staining pattern reminiscent of I4- and D4-transfected cells. Inspection of the truncated sequence revealed that the N-terminal 14 amino acids of the cytoplasmic tail (SQELRKTFKEIICC) of MC4R are conserved among the five melanocortin receptors, whereas the C-terminal 13 amino acid residues (YPLGGLCDLSSRY) bear few sequence similarities. To assess whether the conserved sequence in the cytoplasmic tail is crucial for MC4R trafficking, we generated another truncation mutant of MC4R that retained the N-terminal 14 but lacked the C-terminal 13 amino acid residues (C2; Fig. 1). C2 showed the same capacity as wild-type receptors in binding to ^{125}I-labeled NDP-MSH and mediating cAMP generation upon NDP-MSH challenge (Fig. 4). Correspondingly, C2-transfected cells displayed the cell surface immunofluorescence similar to that of wild type-transfected cells (Fig. 5). These data indicated that the amino acid residues at the N-terminal half of the cytoplasmic tail of MC4R are involved in the plasma membrane targeting. Since this stretch

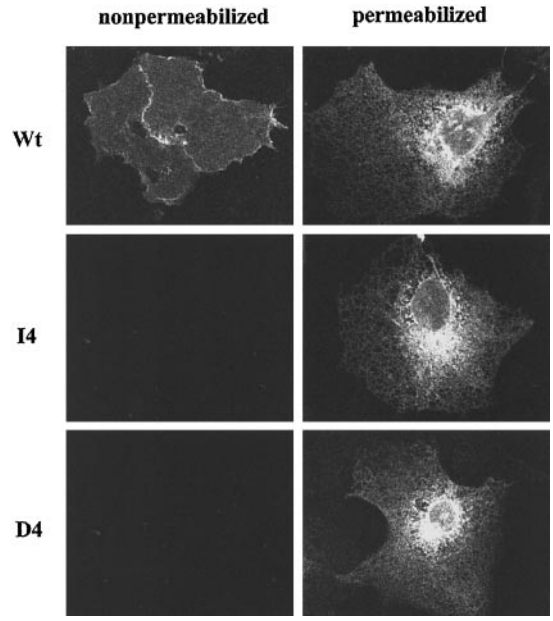

FIG. 3. **Immunofluorescence studies with COS-7 cells transiently transfected with N-terminal FLAG-tagged wild-type, I4, and D4 MC4Rs.** 48 h after transfection, cells grown in chamber slides were treated with or without permeabilizing agent and stained with monoclonal anti-FLAG antibodies (M2) and Oregon Green 488-conjugated anti-mouse IgG. Immunofluorescent signals were analyzed by confocal microscopy. Vector-transfected cells showed negative staining (not shown). Each photograph shows a representative *xy* scan.

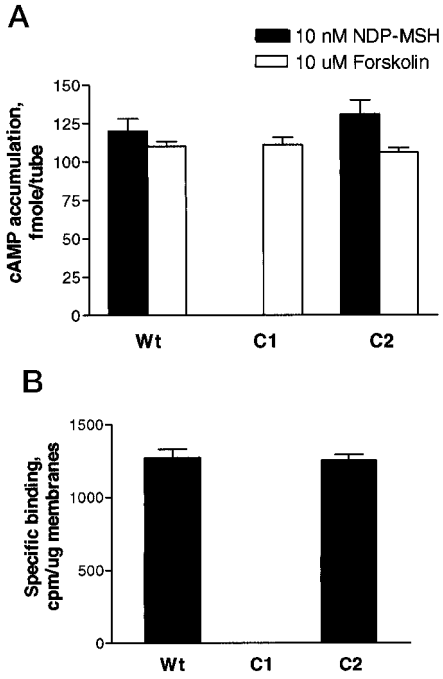

FIG. 4. **The carboxyl terminus of MC4R is necessary for its function.** *A*, cAMP generation in 293T cells transfected with C1 and C2 mutants. Forskolin treatment was used here as an internal control. *B*, binding of ^{125}I-labeled DNP-MSH to membranes from 293T cells transfected with C1 and C2. The experiments were carried out in the same manner as those described in the legend to Fig. 2. The *numbers* represent mean and range of two independent experiments, and each was performed in triplicate.

of residues is conserved among other members of melanocortin receptors, it may be necessary for the proper trafficking of other family members.

Mutant MC4Rs Do Not Exert Dominant Negative Effect on the Function of Wild-type Receptors—Accumulating evidence

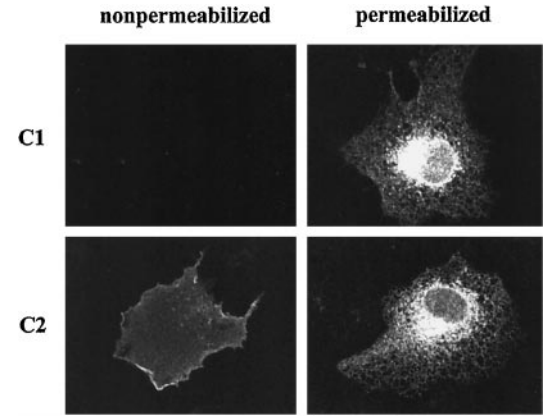

FIG. 5. **Immunofluorescence studies with COS-7 cells transiently transfected with N-terminal FLAG-tagged C1 and C2 mutants.** The experiment was carried in an identical manner as that described in Fig. 3.

suggests that GPCRs can form dimeric and oligomeric arrays (24, 25). It is also shown via *in vitro* co-expression studies that the vasopressin V2 receptor containing three or more TMs strongly inhibits the function and cell surface trafficking of the coexpressed full-length V2 receptor (28). The frameshift mutants I4 and D4 contain five TMs. It was therefore tempting to know whether a single allele carrying the truncated MC4Rs seen in obese patients would result in a simple haplo-insufficiency or, alternatively, whether the mutant copy would exert a dominant-negative effect on the function of the wild-type receptor. To address this question, the wild-type MC4R was co-expressed with either mutant I4 or D4 or control constructs, and the functional properties of wild-type receptors were examined. When co-transfected into 293T cells at a 1:1 ratio (total of 8 μg of DNA) of the wild-type receptor to vector, cells expressed a binding activity of 2240 cpm/μg of membrane proteins (Fig. 6*A*). Under the same conditions, cells co-expressing either I4 or D4 showed a binding activity of ~1680 cpm/mg of membrane proteins (Fig. 6*A*). However, a similar degree of reduction in binding was seen with cells co-transfected with RGS2 (Fig. 6*A*), a cytoplasmic protein that does not interact with GPCRs (29). Similar results were found when co-transfected with the cytoplasmic green fluorescent protein. These data suggest that the reduced binding of the wild-type MC4R upon co-expression with mutants is not an effect of negative activity of the mutants. Rather, it probably results from nonspecific competition for the protein synthesis machinery between the wild-type receptor and co-transfected proteins. In order to accentuate a possible dominant negative effect that might not be detectable at low mutant/wild type ratios, cells were cotransfected at a ratio of 1:10 of the wild-type to mutant receptors while maintaining a constant total DNA amount (8 μg). As shown in Fig. 6*A*, with a decreased amount of wild-type MC4R DNA and increased amount of vector used in the co-transfection, a parallel reduction of the binding activity to 1190 cpm/μg of membrane proteins from co-transfected cells was observed. Although the binding activity of wild-type receptors was further reduced to ~360 cpm/μg of membrane proteins in the presence of a higher amount of mutant DNA used in the co-transfection, the same magnitude of decrease was displayed by co-expressing with RGS2 (Fig. 6*A*). The data suggest that I4 and D4 do not specifically affect the expression of wild-type MC4R on the cell surface. To ascertain that I4 and D4 did not affect the signaling of wild-type MC4R, cells co-transfected at a 1:10 ratio of wild type to mutants or controls were subjected to cAMP assays upon NDP-MSH challenge. As shown in Fig. 6*B*, cells co-transfected with I4 or D4 showed the similar level of

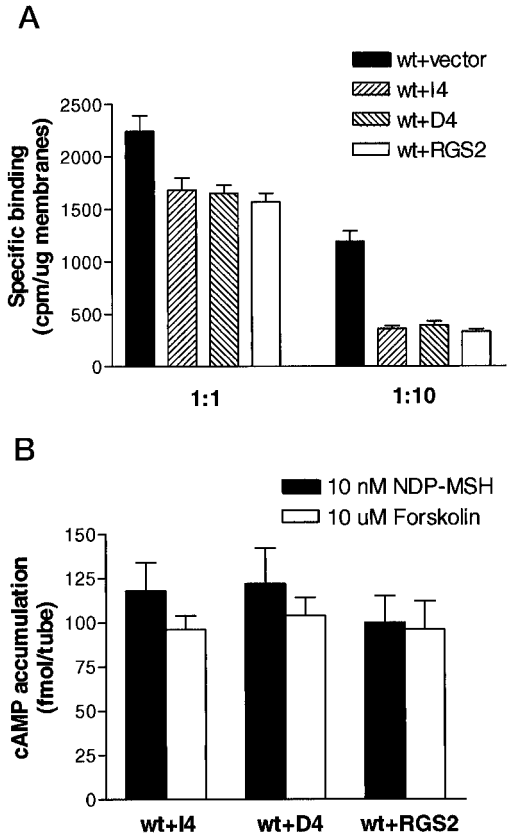

FIG. 6. **Mutants I4 and D4 do not affect the function of wild-type MC4R.** *A*, binding of ^{125}I-labeled NDP-MSH to the membrane isolated from 293T cells co-transfected at a ratio of 1:1 or 1:10 of wild-type to mutant I4 or D4 or the control plasmids. Co-transfection with RGS2 was used as an appropriate control. *B*, cAMP generation in 293T cells co-transfected at a ratio of 1:10 of wild-type MC4R to mutant I4 or D4 or control plasmids. Forskolin treatment was used as an internal control. Binding and cAMP assays were performed as described in Fig. 2. The *numbers* represent mean and S.E. of triplicate values from one experiment. The experiments were performed two or three times, each showing the same pattern as shown above.

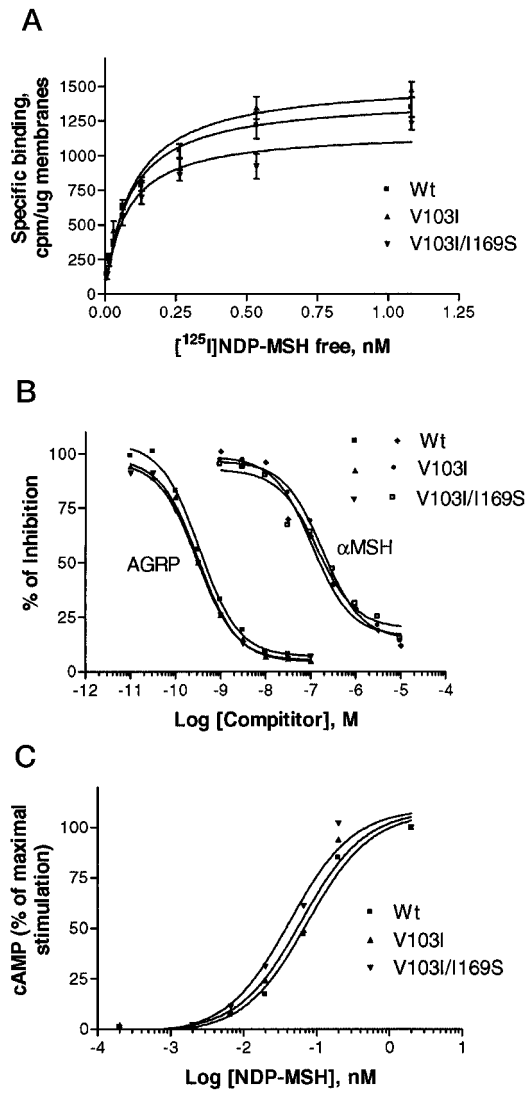

FIG. 7. **Pharmacological properties of MC4R sequence variants.** *A*, saturation binding. Membranes isolated from 293T cells transiently transfected with the sequence variants were incubated with the indicated concentrations of ^{125}I-labeled NDP-MSH and assayed as described under "Experimental Procedures." Curves are fitted with a nonlinear regression and a one-site binding model (GraphPad Prism). *B*, competition binding. Membranes from transfected cells were incubated with 200 pM ^{125}I-labeled NDP-MSH in the presence of increasing concentrations of either the carboxyl-terminal fragment of AGRP (amino acid residues 83–132, which is as potent as the full-length form in binding to MC4R) or αMSH as indicated. The *ordinate* is expressed as a percentage of the total specific binding (B_{max}). Curves are fitted using a nonlinear regression analysis and a one-site competition model (GraphPad Prism). *C*, functional coupling of MC4R variants in 293T cells. Cells transiently transfected with each variant were treated with various concentrations of NDP-MSH and cAMP production assayed. The *ordinate* is expressed as a percentage of the maximal cAMP level obtained for each curve. Curves are fitted with sigmoidal dose-response model with variable slope (GraphPad Prism). All curves are representative of three or four independent experiments, and each point is the mean of triplicate values.

intracellular cAMP accumulation as those co-transfected with RGS2. The data suggest that the MC4R mutants affect neither binding nor the signaling of wild-type MC4R in transfected cells.

Pharmacological Characterization of Three Missense Variants of MC4R Identified in Humans—There were three sequence variants of MC4R identified in humans when we started this work. Among a British population screened for MC4R gene polymorphism, 96% carry valine at codon 103 and isoleucine at condon 169 on both alleles. This genotype was referred to as wild type and utilized for constructing the above MC4R mutants. Another 4% of the population are heterozygous for valine 103, which is replaced with isoleucine (8, 26). In the originally published sequence data, MC4R sequence differs from the wild type in that it carries isoleucine at codon 103 and serine at codon 169 (2, 8). We assigned the three sequence variants as wild-type, V103I, and V103I/I169S in this study (Fig. 1). To examine whether the polymorphism of MC4R contributed to functional differences, they were transiently expressed in 293T cells and subjected to pharmacological assessment. When assayed for the affinity for ^{125}I-labeled NDP-MSH, the three variants exhibited similar saturation binding curves with K_d values of ~0.1 nM (Fig. 7*A* and Table I). The maximal binding from four sets of independent saturation binding experiments was 1281 ± 98, 1579 ± 81, and 1441 ± 129 cpm/μg of membrane proteins for wild type, V103I, and V103I/I169S, respectively, suggesting that the three sequence variants do not differ in their expression on the cell surface either. Examination via competition binding assays of the affinity for AGRP, the physiological antagonist of MC4R (11, 12), showed a similar K_i value of ~0.4 nM for the three variants (Fig. 7*B* and Table I). The similar K_i value of ~100 nM for αMSH was also obtained for the three mutants (Fig. 7*B* and Table I). To investigate a possible alteration of the three variants in signaling, transfected cells were assessed for the EC_{50} in response to NDP-

TABLE I
Pharmacological properties of MC4R variants

The K_d, K_i, and EC_{50} values were calculated using GraphPad Prism software with model equations used in Fig. 7. Values represent means and S.E. of indicated numbers (n) of independent experiments.

Variant	NDP-αMSH		K_i	
	K_d	EC_{50}	AGRP-(83–132)	αMSH
	nM		nM	
Wild type	0.09 ± 0.02 (4)	0.07 ± 0.01 (2)	0.39 ± 0.10 (4)	62 ± 16 (3)
V103I	0.10 ± 0.02 (4)	0.05 ± 0.01 (2)	0.44 ± 0.12 (4)	95 ± 21 (3)
V103I/I169S	0.11 ± 0.01 (4)	0.04 ± 0.01 (2)	0.39 ± 0.10 (4)	60 ± 18 (3)

MSH challenge. As shown in Fig. 7*C* and Table I, cells transfected with the three variants showed a similar dose-response pattern with EC_{50} values of ~0.05 nM and the maximal activation of 134 ± 8, 141 ± 8, and 134 ± 15 fmol/2.5 × 10^5 cells for wild type, V103I, and V103I/I169S, respectively. These data indicate that the polymorphisms of MC4R do not differ in the pharmacological properties examined and, therefore, are not likely to contribute differentially to the development of human obesity.

DISCUSSION

It is generally thought that the N terminus and the first two extracellular loops are crucial for peptide ligand/GPCR interactions (30). Mutagenesis studies show that residues in the TMII and TMIII of MC1R, whose residues are also conserved among all five melanocortin receptors, are crucial for NDP-MSH binding (31). However in our binding studies, MC4R mutants containing five or even seven (in the case of C2) TMs are incapable of binding to NDP-MSH. Of note, all of our binding assays were performed on the crude membrane prepared from cells disrupted by sonication such that the preparation comprises plasma membranes as well as those from intracellular organelles. Since all nonbinding mutants trapped in the endoplasmic reticulum and/or Golgi thus are not fully glycosylated, it is possible that binding to NDP-MSH may require a matured MC4R (*i.e.* the fully glycosylated plasma membrane form). In fact, MC4R carries several potential *N*-glycosylation sites in the extracellular domains, and the protein is highly heterogeneous when run on SDS gels.[2] It is also possible that MC4R located on the membrane of endoplasmic reticulum and Golgi (prior to reaching the final destiny) may not have assumed the proper folding state and therefore would be incapable of binding. Moreover, incorrect folding might be responsible for intracellular retention of the MC4R mutants, since it is known that misfolded proteins are often retained in the endoplasmic reticulum due to the quality control system in that organelle (32). Further mutagenesis studies may be necessary to sort out these possibilities.

Limited data regarding the intracellular transport of GPCRs appear to suggest that diverse structural motifs are required for trafficking of different receptors. For the rat m3 muscarinic receptor, immunofluorescence studies show that the receptor fragment containing only the N-terminal two TMs are capable of being transported to the plasma membrane (33). Chemokine receptors containing five TMs are not only stably expressed on the cell surface but fully functional (34). In contrast, rat glucagon and rhodopsin receptors containing five amino-terminal TMs are transport-deficient (35, 36). Here we show that MC4Rs containing five TMs are incapable of being transported to the plasma membrane. Further analysis identified a stretch of amino acid residues in the cytoplasmic tail of MC4R that is necessary for the cell surface localization. This sequence (SQELRKTFKEIICC) contains two putative palmitoylation cysteine residues and 12 amino acid residues N-terminal to them. Mutations in the cysteine residues of the vasopressin V2 receptor reduce its transport (37) and mutation of a dileucine sequence N-terminal to the cysteine residues, and an upstream glutamate residue of the vasopressin V2 receptor abolishes its cell surface transport (38). The MC4R contains two isoleucine and two glutamate residues in the above C-terminal sequence. Whether those residues are involved in MC4R targeting awaits further analysis. Because the proximal half of the cytoplasmic tail of MC4R is conserved among the members of the melanocortin receptor family, it may have an important function, and our data suggest that this might involve plasma membrane targeting.

GPCRs are classically viewed as monomers, but the concept that GPCRs can exist as oligomers is supported by several recent studies examining receptor expression via immunological techniques (39–42). For instance, using differential epitope tagging, it was demonstrated that β_2-adrenergic receptors form SDS-resistant homodimers (39). A functional importance of the dimerization is supported by the observation that a peptide corresponding to the sixth TM of the receptor inhibits dimerization as well as β-adrenergic agonist-promoted stimulation of adenylyl cyclase activity (39). Very recently, the GABAB receptor was shown to be a heterodimer. This receptor consists of two subunits, GBR1 and GBR2, sharing sequence similarity. Neither of these subunits gave rise to a functional receptor when expressed alone, but co-expression of both subunits gave rise to a γ-aminobutyric acid type B receptor efficiently coupled to G proteins (43–46). In addition, opioid receptors κ and δ can heterodimerize and assume functional properties that are distinct from those of either receptor (47). Since the obesity associated with the I4 and D4 mutants is dominantly inherited, and based on the compelling evidence that GPCR can dimerize, we examine whether the mutant MC4R could interact with the wild-type receptor and exert negative activity on its function. We show, via *in vitro* co-expression studies, that even when co-transfected at high mutant/wild type ratios (10:1), these mutants affected neither the signaling (as determined by adenylyl cyclase activities) nor the cell surface expression (as determined by radioligand binding) of the wild-type receptors in co-transfected cells. Our *in vitro* data may suggest that the obese phenotype seen with patients heterozygous for MC4R mutations is more likely an effect of haplo-insufficiency, which is supported by the observation that mice heterozygous for MC4R gene deletion are obese (13). Moreover, results from a recent study involving the screening of the MC4R coding region of obese and normal humans support the haplo-insufficiency model for the truncation mutants we examined here. This study found an additional nonsense mutation (Tyr-35-stop) that leads to a truncated MC4R containing only 35 amino acid residues at the N-terminal extracellular domain (48). This truncation mutant might be less likely to exert a dominant negative effect on the function of wild-type receptors, but as for the other truncation mutants, heterozygocity for this mutation is associated with obesity (48). Based on their genetic screening data, the authors estimated that haplo-insufficiency of the MC4R might occur in ~1% of individuals with extreme obesity (48).

Screening for MC4R polymorphism in a British population shows that 96% of the screened population is homozygous for valine at codon 103 and 4% have an isoleucine substitution in one allele. Although there was no apparent association of the sequence variant with obesity or with serum glucose or insulin levels (26), this substitution occurs at TMII of MC4R, and

[2] G. Ho and R. G. MacKenzie, unpublished observations.

amino acid substitution close to this site in TMII of MC1R results in constitutive activation (49). In order to detect functional alterations in the variant that might be recessive to the wild-type allele, we set out to characterize its pharmacological properties *in vitro*. We have also analyzed another sequence variant, V103I/I169S (with amino acid substitutions in TMII and TMIV), that exists in the published data base (2, 8). We show that these two variants do not differ from the wild type in their affinity for agonists αMSH and NDP-MSH or the antagonist AGRP and are equally capable of being activated by agonist NDP-MSH. While this manuscript was in preparation, a study on the polymorphism of MC4R was published, in which one of the three variants examined was V103I. In that study, the EC_{50} of V103I for αMSH in cAMP assays also showed no difference from that of the wild-type receptor (50). Thus, the three sequence variants of MC4R do not differ pharmacologically in the *in vitro* studies.

In this study, we have characterized the frameshift mutations of MC4R that are associated with the development of human obesity. The mutants are nonfunctional and retained intracellularly. We further identified a sequence in the cytoplasmic tail that is necessary for the targeting of MC4R to the plasma membrane. The mutants, however, do not exert a dominant negative activity on the function of wild-type receptors in coexpression studies. Our data establish the mechanism underlying the pathogenesis of obesity in patients with the MC4R mutations and lend support to the speculation that patients heterozygous for the truncation mutations are a result of haploinsufficiency at the MC4R locus. Further, our data support the notion that variations in human obesity are not explained by certain MC4R polymorphisms, since these polymorphic receptors do not vary in the manner in which they bind and respond to MC4R ligands. Nevertheless, based on the profound defects in trafficking and function that we demonstrated in the MC4R frameshift mutations, we believe that with more MC4R sequence variants identified, it might be possible to establish a genetic and functional link between certain MC4R polymorphisms and increased risk of developing obesity.

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REFERENCES

1. Mountjoy, K. G., Robbins, L. S., Mortrud, M. T., and Cone, R. G. (1992) *Science* **257,** 1248–1251
2. Mountjoy, K. G., Mortrud, M. T., Low, M. J., Simerly, R. B., and Cone, R. D. (1994) *Mol. Endocrinol.* **8,** 1298–1308
3. Labbé, O., Desarnaud, F., Eggerickx, D, Vassart, G., and Parmentier, M. (1994) *Biochemistry* **33,** 4543–4549
4. Gantz, I., Shimoto, Y., Konda, Y., Miwa, H., Dickinson, C. J., and Yamada, T. (1994) *Biochem. Biophys. Res. Commun.* **200,** 1214–1220
5. Griffon, N., Mignon, V., Facchinetti, P., Diaz, J., Schwartz, J. C., Sokoloff, P. (1994) *Biochem. Biophys. Res. Commun.* **200,** 1007–1014
6. Chen, W., Kelly, M. A., Opitz-Araya, X., Thomas, R. E., Low, M. J., and Cone, R. D. (1997) *Cell* **91,** 789–798
7. Gantz, I., Konda, Y., Tashiro, T., Shimoto, Y., Miwa, H., Munzert, G., Watson, S. J., DelValle, J., and Yamada, T. (1993) *J. Biol. Chem.* **268,** 8246–8250
8. Gantz, I., Miwa, H., Konda, Y., Shimoto, Y., Tashiro, T., Watson, S. J., DelValle, J., and Yamada, T. (1993) *J. Biol. Chem.* **268,** 15174–1517
9. Woods, S. C., Seeley, R. J., Porte, D., Jr., and Schwartz, M. W. (1998) *Science* **280,** 1378–1383
10. Fan, W., Boston, B. A., Kesterson, R. A., Hruby, V. J., and Cone, R. D. (1997) *Nature* **385,** 165–168
11. Ollmann, M. M., Wilson, B. D., Yang, Y., Kerns, J. A., Chen, Y., Gantz, I., and Barsh, G. S. (1997) *Science* **278,** 135–138
12. Shutter, J. R., Graha, M., Kinsey, A. C., Scully, S., Lüthy, R., and Stark, K. L. (1997) *Genes Dev.* **11,** 593–602
13. Huszar, D., Lynch, C. A., Fairchild-Huntress, V., Dunmore, J. H., Fang, Q., Berkemeier, L. R., Gu, W., Kesterson, R. A., Boston, B. A., Cone, R. D., Smith, F. J., Campfield, L. A., Burn, P., and Lee, F. (1997) *Cell* **88,** 131–141
14. Seeley, R. J., Yagaloff, K. A., Fisher, S. L., Burn, P., Thiele, T. E., Van Dijk, G., Baskin, D. G., and Schwartz, M. W. (1997) *Nature* **390,** 349
15. Kask, A., Rago, L., Wikberg, J. E. S., Yeo, G. S. H., Farooqi, I. S., Aminian, S., Halsall, D. J., Stanhope, R. G., and Schioth, H. B. (1998) *Eur. J. Pharmacol.* **360,** 15–19
16. Krude, H., Biebermann, H., Luck, W., Horn, R., Braband, G., and Grüters, A. (1997) *Nat. Genet.* **19,** 155–157
17. Comuzzie, A. G., Hixson, J. E., Almasy, L., Mitchell, B. D., Mahaney, M. C., Dyer, T. D., Stern, M. P., MacCluer, J. W., and Blangero, J. (1997) *Nat. Genet.* **15,** 273–276
18. Yeo, G. S. H., Farooqi, I. S., Aminian, S., Halsall, D. J., Stanhope, R. G., and O'Rahilly, S. (1998) *Nat. Genet.* **20,** 111–112
19. Vaisse, C., Clement, K., Guy-Grand, B., and Froguel, P. (1998) *Nat. Genet.* **20,** 113–114
20. Jackson, R. S., Creemers, J. W., Ohagi, S., Raffin-Sanson, M. L., Sanders, L., Montague, C. T., Hutton, J. C., and O'Rahilly, S. (1997) *Nat. Genet.* **16,** 218–220
21. Montague, C. T., Farooqi, I. S., Whitehead, J. P., Soos, M. A., Rau, H., Wareham, N. J., Sewter, C. P., Digbe, J. E., Mohammed, S. N., Hurst, J. A., Cheetham, C. H., Earley, A. R., Barnett, A. H., Prins, J. B., and O'Rahilly, S. (1997) *Nature* **387,** 903–908
22. Strobel, A., Issad, T., Camoin, L., Ozata, M., and Strosberg, A. D. (1998) *Nat. Genet.* **18,** 213–215
23. Clement, K., Vaisse, C., Lahlou, N., Cabrol, S., Pelloux, V., Cassuto, D., Gourmelen, M., Dina, C., Chambaz, J., Lacorte, J. M., Basdevant, A., Bougneres, P., Lebouc, Y., Froguel, P., and Guy-Grand, B. (1998) *Nature* **392,** 398–401
24. Hebert, T. E., and Bouvier, M. (1998) *Cell Biol.* **76,** 1–11
25. Bockaert, J., and Pin, J. P. (1999) *EMBO J.* **18,** 1723–1729
26. Gotoda, T., Scott, J., and Aitman, T. J. (1997) *Diabetologia* **40,** 976–979
27. Ho, G., Chen, T. L., and Chisholm, R. L. (1995) *J. Biol. Chem.* **270,** 27977–27981
28. Zhu, X., and Wess, J. (1998) *Biochemistry* **37,** 15773–15784
29. Dohlman, H. G., and Thorner, J. (1997) *J. Biol. Chem.* **272,** 3871–3874
30. Ji, T. H., Grossmann, M., and Ji, I. (1998) *J. Biol. Chem.* **273,** 17299–17302
31. Yang, Y., Dickinson, C., Haskell-Luevano, C., and Gantz, I. (1997) *J. Biol. Chem.* **272,** 23000–23010
32. Xiong, X., Hammond, C., and Helenius, A. (1995) *Curr. Opin. Cell Biol.* **7,** 523–529
33. Schöneberg, T., Lui, J., and Wess, J. (1995) *J. Biol. Chem.* **270,** 18000–18006
34. Ling, K., Wang, P., Zhao, J., Wu, Y., Cheng, Z., Wu, G., Hu, W., Ma, L., and Pei, G. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 7922–7927
35. Unson, C. G., Cypess, A. M., Kim, H. N., Goldsmith, P. K., Carruthers, C. J., Merrifield, R. B., and Sakmar, T. P. (1995) *J. Biol. Chem.* **270,** 27720–27721
36. Heymann, J. A., and Subramaniam, S. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 4966–4971
37. Schulein, R., Liebenhoff, U., Muller, H., Birnbaumer, M., and Rosenthal, W. (1996) *Biochem J.* **313,** 611–616
38. Schulein, R., Hermosilla, R., Oksche, A., Dehe, M., Wiesner, B., Krause, G., and Rosenthal, W. (1998) *Mol. Pharmacol.* **54,** 525–535
39. Hebert, T. E., Moffett, S., Morello, J., Loisel, T. P., Bichet, D. G., Barret, C., and Bouvier, M. (1996) *J. Biol. Chem.* **271,** 16384–16392
40. Romano, C., Yang, W., and O'Malley, K. L. (1996) *J. Biol. Chem.* **271,** 28612–28616
41. Cevejic, S., and Devi, L. A. (1997) *J. Biol. Chem.* **272,** 26959–26964
42. Nimchinsky, E. A., Hof, P. R., Janssen, W. G., Morrison, J. H., and Schmauss, C. (1997) *J. Biol. Chem.* **272,** 29229–29237
43. Kuner, R., Kohr, G., Grumewald, S., Gisela, E., Bach, A., and Kornau, H. (1999) *Science* **283,** 74–77
44. Jones, K. A., Borowsky, B., Tamm, J. A., Craig, D. A., Durkin, M. M., Dai, M., Yao, W., Johnson, M., Gunwaldsen, C., Huang, L., Tang, C., Shen, Q., Salon, J. A., Morse, K., Laz, T., Smith, K. E., Branchek, T. A., and Gerald, C. (1999) *Nature* **396,** 674–679
45. White, J. H., Wise, A., Main, M. J., Green, A., Fraser, N. J., Disney, G. H., Barnes, A. A., Emson, P., Foord, S. M., and Marshall, F. H. (1999) *Nature* **396,** 679–682
46. Kaupmann, K., Malitschek, B., Schuler, V., Heid, J., Froestl, W., Beck, P., Mosbacher, J., Bischoff, S., Kulik, A., Shigemoto, R., Karshin, A., and Bettle, B. (1999) *Nature* **396,** 683–687
47. Jordan, B. A., and Devi, L. A. (1999) *Nature* **399,** 697–700
48. Hinney, A., Schmidt, A., Nottebom, K., Heibult, O., Becker, I., Ziegler, A., Gerber, G., Sina, M., Gorg, T., Mayer, H., Siegfried, W., Fichter, M., Remschmidt, H., and Hebebrand, J. (1999) *J. Clin. Endocrinol. Metabol.* **84,** 1485–1486
49. Robbins, L. S., Nadeau, J. H., Johnson, K. R., Kelly, M. A., Roselli-Rehfuss, L., Baack, E., Mountjoy, K. G., and Cone, R. D. (1993) *Cell* **72,** 827–834
50. Gu, W., Tu, Z., Kleyn, P. W., Kissebah, A., Duprat, L., Lee, J., Chin, W., Maruti, S., Deng, N., Fisher, S. L., Franco, L. S., Burn, P., Yagaloff, K. A., Nathan, J., Heymsfield, S., Albu, J., Pi-Sunyer, F. X., and Allison, D. B. (1999) *Diabetes* **48,** 635–639