Cell Surface Expression of the Melanocortin-4 Receptor Is Dependent on a C-terminal Di-isoleucine Sequence at Codons 316/317*

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Loss-of-function mutations in the human melanocortin-4 receptor (MC4R) are associated with obesity. Previous work has implicated a C-terminal di-isoleucine motif at residues 316/317 in MC4R cell surface targeting. It was therefore of interest to examine function and cell surface expression of an MC4R mutation found in an obese proband in which one of these isoleucines was substituted by threonine (I317T). Single mutant (I316T or I317T) and double mutant (I316T,I317T) forms of MC4R were constructed by oligonucleotide-directed mutagenesis and tested for function and cell surface expression in transfected cells. Function was assessed using assays for agonist, [Nle4-d-Phe7]α-melanocyte-stimulating hormone (NDP-α-MSH) or forskolin-stimulated cAMP accumulation. Cell surface expression was determined by whole-cell binding of [125I]NDP-α-MSH, fluorescence immunocytochemistry and fluorescence-activated cell sorting. Maximal cAMP generation of the single mutants was reduced by 40% of wild-type receptor; the double mutant further reduced function to 40% of control, effects that were mirrored by decreases in cell-surface expression. Quantitative RT-PCR showed that, relative to wild-type receptor, transcript levels for the mutated receptors were not reduced. The results further implicate the C-terminal di-isoleucines in cell surface expression of MC4R and suggest that mutations of residues 316 or 317 would predict MC4R hypofunction.

Monogenic forms of obesity have been identified in mouse and man (1–8). These genes are either expressed in brain or their products act on brain, indicating the prominent role of this tissue in energy homeostasis. Neurons that express these genes are found in the arcuate and paraventricular nuclei of the hypothalamus and form a neural circuit capable of sensing signals associated with metabolic state and coordinating behavioral and metabolic responses to the metabolic demands of the organism. This neural circuit is composed of at least three distinct neuronal subtypes. One subtype expresses the G protein-coupled melanocortin-4 receptor (MC4R). MC4R is a Gs-coupled receptor, and its stimulation leads to generation of intracellular cAMP. The other two subtypes provide the MC4R with ligands for MC4R. Activation of MC4R suppresses appetite, and disruption of the gene in mouse and man results in hyperphagia and obesity (11–13).

MC4R mutations are relatively common and have been estimated to account for as much as 5% of the obesity found within certain populations (3, 4, 14, 15). Frameshift mutations in the gene resulting in truncated MC4R protein can exhibit a dominant inheritance pattern, although one example of severe truncation demonstrates variable penetrance (15). Some of the truncated forms have been functionally evaluated, and they neither bind ligand nor become associated with the outer surface of the plasma membrane (16). The results from transfection experiments of cultured cells indicate that the truncated receptor forms do not appear to have a dominant negative function (16), supporting simple haploinsufficiency as an explanation for the dominant inheritance pattern when it occurs (12). MC4R haploinsufficiency is consistent with MC4R heterozygotic knockout mice, which exhibit obesity intermediate to wild-type and homozygotic knockout mice (11). MC4R haploinsufficiency is not supported in a report describing deletions of human chromosome 18q, which harbors MC4R, resulting in MC4R hemizygosity without obesity (17).

In the case of MC4R missense mutations, the impact on phenotype generally follows the severity of the impact on receptor function or expression. Notably, the receptor with the N62S mutation was shown to support a partial cAMP response to receptor stimulation and is associated with a recessive inheritance pattern of obesity within the pedigree (14). Results from these genetic analyses indicate little or no receptor reserve of MC4R in human or mouse brains given the sensitivity of the phenotype to reductions in MC4R receptor number or function.

The C termini of G protein-coupled receptors contain a conserved, dihydrophobic sequence composed of leucine, isoleu-
cine, phenylalanine, valine, or methionine and preceded by an acidic glutamate or aspartate (18). Mutation of the conserved diisoleucine motifs in the vasopressin V2 and lutropin/choriogonadotropin receptors have been shown to limit membrane targeting of these receptors (18–20). In the case of MC4R, the motif is composed of di-isoleucines at codons 316/317. We have shown that MC4R truncated proximal to the di-isoleucines is not expressed on the cell surface, whereas MC4R truncated two residues distal to this site is expressed (16). It was therefore of interest to note the report of a human mutation, I317T, in an obese proband (21). In the present report, we make the mutant MC4Rs containing threonine substitutions at either codon 316 or 317, or both, and test the mutants for cell surface expression and function.

**EXPERIMENTAL PROCEDURES**

**Materials—**[Nle$^{4}$-D-Phe$^{7}$]α-MSH (NDP-α-MSH) was purchased from Peninsula Laboratories, α-MSH and forskolin was purchased from Calbiochem. 3-Isobutyl-methylxanthine (IBMX) was from Sigma, and [125I]NDP-MSH was from Amersham Biosciences.

**Oligonucleotide-directed Mutagenesis—**To construct MC4R sequence variants, wild-type MC4R was modified by adding a Kozak sequence (GCCGCCGCC) and FLAG tag to the N termini of the MC4R coding sequence by conventional PCR-based techniques. PCR products were cloned into the vector pCRII.1 (Invitrogen). PCR products were treated with Tag polymerase (Invitrogen) at 72°C for 10 min to add 3′ overhanging Ts; subsequent cloning was carried out according to the manufacturer’s instructions. The modifications were confirmed by sequencing. Site-directed mutagenesis of the FLAG-MC4R plasmid was accomplished using the QuikChange™ site-directed mutagenesis kit (Stratagene). Primer pairs (5′-3′) were designed to introduce the desired mutations in the MC4R coding sequence: I316T upstream (CTT-CAAAGAGCCATCTGTGTG) and downstream (CAACAGATGCTCTTGGAG) primers; I317T upstream (ACCTCAGAGATACCCTGTTGCTATCCCTGTG) and downstream (CAGGGGATAGCAAAGGTCATCCTTTGAAGTTG) primers; and I316T/I317T upstream (CTTCAAGAGACCCACTTGTTG) and downstream (CAACAGGTTGCTCTTGGAG) primers.

Primer pairs (LifeTech) with the desired mutations were annealed to the wild-type expression construct. The plasmid-primer mixture was amplified in a PCR reaction with Pfu turbo DNA polymerase following the manufacturer’s recommendations. After amplification the products were treated with the restriction enzyme DpnI. The products of the restriction digest were used to transform competent Escherichia coli, strain XL1-Blue, following the procedure recommended by the manufacturer (Stratagene). Plasmids isolated from the resulting bacterial colonies were screened by DNA sequencing to identify clones with the desired mutations. The entire MC4R coding region of plasmids with mutations was sequenced to confirm 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; Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Cells were incubated at 37°C in humidified air containing 5% CO2. Cells were generally at 70–80% confluence on the day of transfection, and transfections of FLAG-tagged WT or mutant MC4R plasmids were carried out using LipofectAMINE reagent according to the manufacturer’s instruction (Invitrogen).

**Whole-cell Receptor Binding Assays—**Receptor binding assays were carried out on monolayers of 293T cells transiently transfected with MC4R mutants. HEK-293T cells were transfected in 150 x 25-mm dishes and allowed to incubate overnight at 37°C in humidified air containing 5% CO2. The cells were detached from plates with PBS, and function.

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**Pharmacological properties of MC4R WT and mutants**

Statistical significance between group means was determined by one-way analysis of variance followed by the Newman-Keuls test. Only the single mutant group means were not significantly different from each other (p < 0.01) for measures of maximal specific binding and cAMP accumulation. There were no significant differences between group means (p > 0.05) for measures of K and EC50.

|                      | WT | I316T | I317T | DMut |
|----------------------|----|-------|-------|------|
| Maximum specific binding, fmol of 125I-NDP-α-MSH/well | 0.66 ± 0.06 | 0.39 ± 0.04 | 0.44 ± 0.04 | 0.04 ± 0.006 |
| K, nM [NDP-α-MSH]; mean ± S.E. | 16 (7–39) | 13 (5–34) | 12 (7–22) | ND |
| Maximum cAMP accumulation, fold-stimulation; mean ± S.E. | 49 ± 1 | 35 ± 2 | 31 ± 2 | 15 ± 1 |
| EC50, nM [NDP-α-MSH]; mean ± 95% confidence interval | 0.9 (0.6–1.2) | 0.7 (0.2–2.1) | 1.0 (0.3–4.1) | 3.2 (0.5–18) |
0.02% EDTA and collected by centrifugation. The cells were plated into 24-well culture dishes at a density of $2.5 \times 10^5$ cells/well and allowed to attach overnight. The binding was carried out in a total volume of 200 
$\mu$l containing Dulbecco’s modified Eagle’s medium and 1 mg/ml BSA, 50 
$\mu$m [125I]NDP-a-MSH, and increasing concentrations of cold NDP-a-MSH. After a 90-min incubation, the medium was aspirated and the 
cells were washed three times with 0.5 ml/well of Dulbecco’s phosphate-buffered saline (DPBS, In vitrogen) containing 20 mM HEPES, pH 7.4, and 0.1 mg/ml BSA. 100 
$\mu$l of 0.1 N NaOH was added to the wells, and the cell lysates were transferred to 20-ml glass vials containing 15 
ml of scintillation mixture. The samples were counted in a Packard 
Tri-Carb liquid scintillation counter. Nonlinear regression analysis was 
performed using GraphPad Prism software (GraphPad Software, Inc., 
San Diego, CA).

cAMP Accumulation Assays—48 h after transfection, 293T cells were 
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 (In 
vitrogen) 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 ($\sim 5 \times 10^6$ cells/ml) was added to 0.1 ml of IBMX buffer 
containing various concentrations of agonists or 10 nM 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 cAMP125I scintillation proximity assay system (Amersham Biosciences). EC50 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).

Immunofluorescence Staining—COS-7 cells were chosen for this study as they were more adherent than 293T cells during the staining 
process. 24 h after transfection, COS-7 cells were seeded onto 25 mm 
circular microscope coverslips, size 1 (Fisher Scientific), in six-well 
culture plates (Corning-Costar) and incubated overnight. The next day, 
the cells were washed four times with DPBS 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 nonpermea-
blized staining or with DPBS containing 0.2% Triton X-100 for per-
meabilization. Following blocking for 30 min with DPBS containing 
10% fetal bovine serum and 2% BSA (blocking buffer), cells were incu-
bated for 1 h in blocking buffer containing 1 $\mu$g/ml anti-FLAG mono-
clonal antibodies M2 (Sigma) 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 Oregon Green 488 goat anti-
mouse IgG conjugate (highly cross-absorbed; Molecular Probes). After 
being washed twice for 10 min with DPBS, cells were mounted on glass 
slides using Aqua-PolyMount (Polysciences, Inc.) and viewed using a 
confocal laser scanning microscope (Fluoview, Olympus).

Flow Cytometry—48 h post-transfection, HEK-293 cell monolayers 
were washed with incubation buffer (DPBS containing 20 mM HEPES, 
pH 7.4, and 1 mg/ml BSA). The cells were detached from the plates by 
incubation in DPBS with 0.02% EDTA (Sigma, catalog No. E8008). The 
cells were collected by centrifugation, washed in incubation buffer, and 
then suspended to a concentration of $4.0 \times 10^6$ cells/ml in incubation 
buffer. 50 $\mu$l of cells were incubated with 50 $\mu$l of 10 $\mu$g/ml murine 
anti-FLAG M2 monoclonal antibody (Sigma, catalog No. 316) in incu-
bation buffer for 30 min at room temperature. The cells were collected 
by centrifugation and washed three times in incubation buffer. The cell 
pellets were suspended in 100 $\mu$l of incubation buffer containing 1 $\mu$g/ml 
Alexa Fluor® 488 goat anti-mouse IgG (H+L) (Molecular Probes, cata-
l og No. A-11001) and incubated at room temperature for 30 min. The 
cells were collected by centrifugation and washed three times with 
incubation buffer. The final cell pellets were suspended in incubation 
buffer to a concentration of $2.0 \times 10^6$ cells/ml. Propidium iodide was 
added to a final concentration of 1 $\mu$g/ml, and the cell suspensions were 
passaged through a 100-$\mu$m filter prior to flow cytometry. Flow cytometry 
was performed on a fluorescence-activated cell sorter (FACS Vantage 
S.E., BD Biosciences). Single live cells were gated by scatter and neg-
itive propidium iodide fluorescence, 488 nm excitation wavelength, 675 
nm emission wavelength. For each transfection, the fluorescence of 
25,000 single live cells was measured at 488 excitation wavelength, 530 
nm emission wavelength, to measure the expression of the FLAG anti-
gen on the surface of the cells.

Real-time Quantitative RT-PCR (TaqMan®)—48 h post-transfection, 
total RNA was isolated from cells using TriSol reagent (Invitrogen) 
following the manufacturer’s recommended protocol. RNA was reverse 
transcribed using TaqMan® reverse transcription reagents (Perkin-
Elmer Life Sciences). 400 ng of total RNA was transcribed in a 50-$\mu$l 
reaction. Reaction mixture contained 5.5 mM MgCl2, 500 $\mu$M each dNTP, 
2.5 $\mu$m random hexamers, 200 units of RNase inhibitor, and 62.5 units

![Figs. 3 and 4](http://www.jbc.org/Downloaded from http://www.jbc.org)
of MultiScribe reverse transcriptase. Reverse transcription reactions were incubated at 25 °C for 15 min, 48 °C for 30 min, and 95 °C for 5 min. Multiplex PCR reactions were run on reverse transcribed RNAs to quantify the relative abundance of MC4R RNA in the original RNA samples. Human MC4R primers and a FAM-Fluorescent probe to a region near the 3′/H11032-end of the coding region of the MC4R sequence, conserved in the wild-type and mutant sequences, were designed using the Primer Express software (PerkinElmer Life Sciences) and synthesized (PerkinElmer Life Sciences). The real-time quantitative RT-PCR, using 18 S ribosomal RNA (rRNA) for normalization, was performed using TaqMan® Universal PCR Master Mix reagents and TaqMan® Ribosomal RNA control reagents (VIC Dye) (PerkinElmer Life Sciences) essentially following the manufacturer’s protocol. Briefly, each reaction mixture contained 5.5 mM MgCl₂, 500 μM dNTP, 600 nM both forward and reverse 18 S rRNA primers, 200 nM 18 S rRNA-VIC probe, 600 nM both forward and reverse MC4R primers, 200 nM MC4R-FAM probe, and cDNA in a final volume of 25 μl. Reactions were run and analyzed using ABI PRISM 7700 sequence detection system (PerkinElmer Life Sciences). MC4R mRNA levels were normalized to 18 S rRNA, and the relative copy number was determined using the standard curve method for multiplex PCR as detailed in User Bulletin No. 2 for the ABI Prism 7700 sequence detection system (Applied Biosystems).

RESULTS

Substitutions I316T or I317T Decrease Cell Surface Expression of MC4R as Determined by Receptor Binding in Transfected HEK-293 Cells—To assess the impact of threonine substitution at isoleucines 316 and 317 on cell surface expression of MC4R, FLAG-tagged WT or mutated MC4Rs were transfected into HEK-293 cells and tested for whole-cell binding of 125I-

FIG. 5. Fluorescence-activated cell sorting of COS-7 cells transfected with N-terminal FLAG-tagged wild-type or mutated MC4Rs. Cells were transfected and fluorescence-activated cell sorting was performed as described under “Experimental Procedures.” The bar in each graph represents the gating set to exclude 95% of the background signal determined from cells transfected with wild-type MC4R without an N-terminal FLAG tag (blank histogram in top graph). Values above the gating bar represent percentage of 2 × 10⁵ cells expressing N-terminal FLAG-tagged WT or mutated MC4Rs having fluorescent intensity signals above the gate setting.

FIG. 6. Transcript levels of wild-type and mutated MC4Rs in transfected cells. Quantitative RT-PCR was performed on cells transfected with WT or mutated MC4R constructs, and the mRNA for WT or mutated MC4R was determined as described under “Experimental Procedures.” A, for each construct, RNA input levels of 2.5, 5, 10, and 20 ng were tested to show that the method had sufficient sensitivity to detect differences in transcript levels. A control plasmid carrying the cDNA for the 5-HT7 receptor was used as a negative control. n = 3 wells/condition. B, relative to wild-type levels, transcript copy level normalized to 18 S RNA was ~2-fold higher for the double mutant form. Bars represent means ± standard deviations of triplicate determinations. Statistical differences between means were determined by analysis of variance followed by the Newman-Keuls test (*, p < 0.01).

FIG. 7. Conservation of di-hydrophobic motif in human melanocortin receptors at positions 12 and 13 of the cytoplasmic tail.

Sequence of the Human MCRs Cytoplasmic Tail

| MCR | Sequence |
|-----|----------|
| MC1R | HSEQLRRTLVKEVLTCSW |
| MC2R | RSLERDAFKKMKFCSRYW |
| MC3R | RSLERLNFTREILCGCMNGMLG |
| MC4R | RSELRKKTFKELLCCYPLGGLCLDSR |
| MC5R | RSEQVRKTFFKELLCCRGFRACSFPRR |
labeled MC4R agonist. Compared with cells expressing FLAG-tagged wild-type MC4R, whole-cell binding of a subsaturating concentration (50 pM) of \( ^{[125]} \)NDP-\( \alpha \)-MSH was decreased in cells expressing either of the singly substituted mutants I316T or I317T by 41 and 34%, respectively (Fig. 1, Table I). Transfection of the double mutant (DMut), in which the isoleucines at both positions 316 and 317 were changed to threonines, resulted in no detectable specific binding (Fig. 1). The affinity (\( K_I \)) of MC4R for the agonist, NDP-\( \alpha \)-MSH, was not affected by either I316T or I317T (Table I). Specific whole-cell binding of the label to control HEK-293 cells transfected with an expression construct for green fluorescent protein (GFP) was undetectable and did not differ from untransfected cells (data not shown).

**Substitutions I316T or I317T Decrease Agonist-stimulated cAMP Accumulation in Transfected HEK-293 Cells**—Compared with HEK-293 cells transfected with FLAG-tagged WT MC4R, cells expressing the singly substituted receptor at I316T or I317T showed 29 and 37% reductions, respectively, in maximal agonist-stimulated cAMP accumulation (Fig. 2, Table I). In cells transfected with the double mutant, this measure was further reduced to 31% of WT values (Fig. 2 and Table I). Cells transfected with control GFP exhibited no agonist-stimulated cAMP accumulation (Fig. 2). The EC\(_{50}\) values for NDP-\( \alpha \)-MSH-dependent cAMP generation were not affected by threonine substitution of the isoleucines at positions 316 and 317 (Table I). Stimulation of HEK-293 cells by 10 \( \mu M \) forskolin increased cAMP accumulation by 5-fold, an effect that was greatly potentiated in cells transfected with WT MC4R (Fig. 3). The ability of MC4R receptor transfection to potentiate forskolin-stimulated cAMP generation appeared lower in the single mutants, although differences between the WT and single mutant group means were not statistically significant (Fig. 3). Transfection of the double mutant resulted in significantly lower forskolin-stimulated cAMP production than in the WT or single mutants and was not different from cells expressing control GFP (Fig. 3).

**Effect of I316T and I317T Mutations on Cell Surface Targeting of MC4R as Evaluated by Fluorescence Microscopy**—To visualize the cell surface expression of FLAG-tagged WT and mutated MC4R, COS-7 cells were transfected with the appropriate expression constructs and the cellular localization was analyzed by immunofluorescence microscopy. To distinguish receptors on the cell surface from intracellular receptors, a monoclonal anti-FLAG antibody was used with non-permeabilized and permeabilized cells, respectively. Threonine substitutions of the isoleucines at positions 316 and 317 had no effect on immunofluorescent labeling of receptor in permeabilized cells (Fig. 4). In non-permeabilized cells, it appeared that more WT receptors were targeted to the cell surface than either of the I316T or I317T mutants and that cell surface targeting was further reduced in the double mutant (Fig. 4).

**Effect of I316T and I317T Mutations on Cell Surface Targeting of MC4R as Evaluated by Fluorescence-activated Cell Sorting**—To quantitate immunofluorescent detection of FLAG-tagged WT or mutant MC4R, HEK-293 cells transiently expressing these receptor forms were detected and counted by fluorescence-activated cell sorting. Twenty-five thousand cells from each transfection were sorted, and the percentage of cells having a fluorescence intensity signal above a previously determined cutoff value was determined (Fig. 5). Forty-five percent of cells expressing the FLAG-tagged WT MC4R (mean fluorescence intensity signal = 61) met this criterion, whereas only 26% of cells expressing either I316T or I317T (mean fluorescence intensity signals = 31 and 32, respectively) and 6% of cells expressing the double mutant exhibited fluorescence intensity (mean fluorescence intensity signal = 14) above the cutoff value (Fig. 5).

**Deficient Cell Surface Expression of MC4R Mutants Is Not Due to Lower Transcript Levels**—To determine whether the decreased cell surface expression of MC4R mutants was due to decreased transcript levels mutant mRNA, because of either decreased transcription or stability, the transcript levels of WT and mutant receptor forms were measured by TaqMan® quantitative RT-PCR. Reversed transcribed RNA from each transfection was serially diluted and transcript level of MC4R determined as described under “Experimental Procedures.” Results obtained from the dilution experiments indicate that the technique is sufficiently sensitive to detect 2-fold differences in transcript level (Fig 6A). To show the specificity of the MC4R primers and probes, cells were transfected with a control expression construct for the 5-HT7 receptor and probed for MC4R transcript. RNA collected and reverse transcribed from these cells and probed for MC4R transcripts required many more amplification cycles to generate a signal (Fig 6A). Cycle threshold number for 5-HT7-transfected cells was equal to the background signal seen using herring sperm DNA as input (data not shown). Normalization of the data to 18S rRNA revealed that the transcript levels of wild-type and single mutant forms were not
different from each other (Fig 6B). However, the transcript level for the double mutant was greater. Relative to wild-type levels, double mutant transcript copy numbers were 2.2- and 2.4-fold greater at the 20 and 10 ng input levels, respectively (Fig. 6B).

DISCUSSION

Previous work has indicated that the di-isoleucine pair at amino acid residues 316 and 317 in the C terminus of the MC4R might be important for cell surface targeting (16). An MC4R transition mutation has been described in an obese human proband in which thymine is replaced by cytosine at base position 950, resulting in the substitution of threonine for the isoleucine at 317 (21). Results obtained from the present study indicate that these C-terminal isoleucines are important for normal cell surface expression of MC4R in transiently transfected cells and that substitution of isoleucine by threonine at position 316 or 317 lowers MC4R cell surface expression. Double substitutions by threonine are additive.

I316T or I317T each lowers the binding of the 125I-labeled MC4R agonist NDP-α-MSH to the cell surface but does not change receptor affinity for the ligand. The reduced binding is reflected in reduced agonist- as well as forskolin-stimulated cAMP accumulation. It is not clear whether the latter, agonist-independent activation of MC4R is due to a meaningful constitutive MC4R activity (22, 23) or whether it is an artifact of receptor overexpression. Regardless, it does appear to report changes in receptor cell surface expression. Qualitative imaging by immunofluorescence microscopy of changes in cell surface expression suggests reduced cell surface expression of the single mutants, although the differences in this measurement were more definitive using the quantitative approach afforded by fluorescence-activated cell sorting.

This work establishes the importance of the C-terminal di-isoleucine pair for normal cell surface expression of MC4R. Previous work (18) has indicated that the di-leucine motif in the C terminus of the vasopressin V2 receptor is important in the exiting of the receptor from the endoplasmatic reticulum. In that study, single substitutions of hydrophobic leucines by hydrophilic isoleucines had no effect on V2 receptor cell surface expression (18); however, substitution of either leucine by the polar threonine decreased cell surface expression, and the double substitution completely suppressed it.

An examination of the C terminus of the family of melanocortin receptors indicates high sequence conservation only through the first 15 residues (Fig. 7). The dihydrophobic motif, at C-terminal residues 12 and 13, is completely conserved in this receptor family, although it is composed of various hydrophobic amino acids (Fig. 7). A survey of C termini from 384 G protein-coupled receptors (GPCRs) from the Swiss Protein Database (European Molecular Biology Laboratory, Heidelberg, Germany) showed that 180 GPCRs contained a C-terminal dihydrophobic pair consisting of II, LL, IL, or LI within the first 45 residues and that the most frequently occurring starting position of these pairs was position 12 (Fig. 8).

Results from the present work do not indicate how the di-isoleucine pair functions to regulate cell surface expression of MC4R or even whether the motif operates by affecting transit to or from the plasma membrane. Acidic dihydrophobic motifs, in particular di-leucine pairs preceded by a cluster of acidic amino acids, have been shown to be sorting receptor binding sites for the GGA (Golgi-localizing, 2-adaptin ear homology domain, ARF-interacting) proteins required for the transport of protein cargo from the trans-Golgi network to endosomes (24, 25). The C-terminal dihydrophobic motif that appears in GPCRs is preceded, in many GPCRs, by a conserved acidic amino acid (18). Moreover, substitution of this residue (glutamate) by glutamine in the vasopressin V2 receptor also blocks cell surface expression of the receptor (18). It is possible that the GPCR dihydrophobic motif serves as an intramolecular binding site necessary for protein folding or an intermolecular binding site for an adaptor-related protein required for appropriate membrane trafficking.

On the basis of results from the present study, we would predict that the MC4R mutation I317T would decrease MC4R cell surface expression and function. The MC4R haplinsufficiency seen in human carriers of truncation mutations (3, 4) and in heterozygotic MC4R knockout mice (11) suggest that a 50% decrease in receptor number might support an obesity phenotype. Extrapolating from the present cell culture data, a heterozygotic carrier of the I317T mutation might be expected to have at least 80% normal MC4R expression. The reported obese proband was heterozygotic for I317T and was extremely obese (above the 99th percentile, body mass index = 36.5 kg/m2). If this were the sole obesity-related mutation for the proband, it would suggest an extreme sensitivity to the loss of MC4R. Further genetic investigation of this pedigree and of others with mutations of the C-terminal di-isoleucines 316/317 is required.

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