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Functional Characterization of Melanocortin-4 Receptor Mutations Associated with Childhood Obesity

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The melanocortin-4 receptor (MC4R) is a member of the rhodopsin-like G protein-coupled receptor family. The binding of α-MSH to the MC4R leads to increased cAMP production. Recent pharmacological and genetic studies have provided compelling evidence that MC4R is an important regulator of food intake and energy homeostasis. Allelic variants of MC4R were reported in some children with early-onset severe obesity. However, few studies have been performed to confirm that these allelic variants result in an impairment of the receptor's function. In this study, we expressed wild-type and variant MC4Rs in HEK293 cells and systematically studied ligand binding, agonist-stimulated cAMP, and cell surface expression. Six of the 11 mutants examined had either decreased (S58C, N62S, Y157S, C271Y) or no (P78L, G98R) ligand binding, with proportional impairments in [Nle^4, D-Phe^7]-α-MSH-stimulated cAMP production. Confocal microscopy confirmed that the observed decreases in hormone binding by these mutants are associated with decreased cell surface expression due to intracellular retention of the mutants. The other five allelic variants (D37V, P48S, V50M, I170V, N274S) were found to be expressed at the cell surface and to bind agonist and respond with increased cAMP production normally. The data on these latter five variants raise the question as to whether they are indeed causative of the obesity or not and, if so, by what mechanism. Our data, therefore, stress the importance of characterizing the properties of MC4R variants associated with early-onset severe obesity. We further propose a classification scheme for mutant MC4Rs based upon their properties. (*Endocrinology* 144: 4544–4551, 2003)

G PROTEIN-COUPLED RECEPTORS (GPCRs) comprise one of the largest families of cell surface proteins (1). A large variety of stimuli such as photons, odorants, amines, ions, peptides, and large glycoprotein hormones rely on GPCRs to relay information to the inside of the cell by activating G proteins and downstream effector molecules. Based on sequence homologies, the superfamily of GPCRs can be divided into at least six families, the largest of which is the rhodopsin-like (family A) of GPCRs (1). The numerous human diseases caused by mutations of GPCRs highlights the importance of GPCRs. Activating and inactivating mutations of GPCRs have been associated with, among many other diseases, retinitis pigmentosa, night blindness, nephrogenic diabetes insipidus, dwarfism, familial glucocorticoid deficiency, and infertility (2). Gain-of-function mutations are thought to result in constitutive activation of a GPCR by disrupting interhelical interactions that maintain the receptor in the ground state (3, 4) or impairing desensitization (5). Functional characterization of GPCR mutants that result in loss-of-function have identified several defects, including defects in expression due to either reduced biosynthesis and/or accelerated degradation (6), defects in trafficking of intracellular receptor to the plasma membrane (6–9), defects in ligand binding (6, 10), and defects in G protein coupling (11), or a combination thereof (12, 13).

Melanocortin receptors (MCRs) are members of family A, rhodopsin-like GPCRs. They have been named MC1R to MC5R according to the sequence of their cloning. The ligands for the MCRs are derived from posttranslational processing of the pre-prohormone proopiomelanocortin (POMC) (14). MC1R is the classical receptor for α-MSH present in melanocytes that regulates the production of pigments (15, 16). MC2R is the classical receptor present in the adrenal cortex that binds ACTH and is involved in the regulation of adrenal steroidogenesis and growth (16). MC3R and MC4R, the neural melanocortin receptors, are expressed in the brain (17–20). MC3R is highly expressed in the hypothalamus and in a few regions of the brainstem (17, 18), whereas MC4R is expressed more widely in the brain, including the cortex, thalamus, hypothalamus, brainstem, and spinal cord (20). MC5R, expressed in various peripheral tissues, is involved in the regulation of exocrine gland functions (21–23). All five MCRs couple primarily to Gs and, therefore, increase cAMP production upon agonist stimulation.

Recent studies using transgenic and knockout mice have unraveled important functions for MC3R and MC4R. Mice lacking POMC or MC4R expression were obese (24, 25). Mice overexpressing the endogenous antagonist of MC3R and MC4R, Agouti-related protein (AgRP), were also obese (26, 27). Yellow agouti mice are obese because of the ubiquitous expression of Agouti, which is also an antagonist for MC4R (28). Pharmacological evidence further links the MC3R and MC4R to food intake. Thus, injection of melanotan II, an agonist of MC3R and MC4R, into the third ventricle decreased food intake (29, 30), whereas injection of the C-terminal peptide of AgRP, an antagonist of these receptors, increased food intake (31). More recently, mutations in MC4R and POMC have provided human genetic evidence supporting a role of MC4R in energy homeostasis. A nonsense mutation in POMC was found to result in obesity, in addition to red hair, fair skin (due to lack of MC1R function)

Abbreviations: AgRP, Agouti-related protein; ER, endoplasmic reticulum; GPCR, G protein-coupled receptor; MCR, melanocortin receptor (MC1R–MC5R); NDP-MSH, [Nle^4, D-Phe^7]-α-MSH; POMC, proopiomelanocortin; R_{max}, maximal response; TM, transmembrane; wt, wild-type.

and adrenal failure (due to lack of MC2R function) (32). Nonsense mutations in MC4R were found in early-onset severe obesity (33, 34). Since these initial reports, numerous mutations were reported in different patient cohorts (35–47). However, in many of the earlier reports, the functions of the variant MC4Rs were not studied, and therefore only an association rather than a cause/effect link of the mutation with the physical phenotype was provided. We set out to provide this missing link by functionally characterizing several mutant MC4Rs. Indeed, our data strongly argue that variant DNA does not necessarily mean that it is the cause of obesity in the patient.

Materials and Methods

In vitro mutagenesis of MC4R

A c-myc epitope tag was inserted at the N terminus of the wild-type (wt) MC4R (kindly provided by Dr. I. Gantz, University of Michigan, Ann Arbor, MI) by QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Briefly, oligonucleotide primers including the sequences encoding the c-myc epitope (EQKLISEEDL) displaced wt sequences in the double-strand plasmid prepared in pBlueScript vector using *pfu* Turbo DNA polymerase. The PCRs consisted of initial denaturation at 95 C for 30 sec, followed by 18 cycles of 95 C for 30 sec, 55 C for 30 sec, and 68 C for 12 min. Then the amplicon was digested with *Dpn*I, which digested the methylated parental DNA from pBlueScript, and left the nicked mutated plasmids intact. Repair of nicked plasmids was achieved during transformation using supercompetent XL1-Blue *Escherichia coli* cells. After verifying that the intended mutation was achieved by automated DNA sequencing (performed by the DNA Core Facility of the University of Iowa Diabetes and Endocrinology Research Center), the insert containing the whole coding region was inserted back into pcDNA3.1 (Invitrogen, Carlsbad, CA). The entire coding region of the plasmid in pcDNA3.1 was sequenced to verify that the mutation was correct and there were no unintended errors during the PCR or ligation steps.

After verifying that the inserted c-myc tag did not affect ligand binding or hormone stimulation of cAMP production (data not shown), eleven point mutations were introduced into the wt MC4R containing the c-myc epitope tag (herein referred as myc-MC4R) using the same approach as described above except that the PCR consisted of 12 cycles instead of 18 cycles. The mutants created were D37V in extracellular domain (38), P48S in transmembrane (TM) I (43), V50M in TM I (41), S58C in TM I (41), N62S in TM I (39), P78L in TM II (38), G98R in TM II (42), Y157S in the second intracellular loop (48), I170V in TM IV (41), C271Y in the third extracellular loop (39), and N274S in the third extracellular loop (35).

Cells and transfection

HEK293 cells were obtained from American Type Culture Collection (Manassas, VA). These cells were maintained at 5% CO_2 in DMEM containing 50 μg/ml gentamicin, 10 mM HEPES, and 10% newborn calf serum. Cells were plated on gelatin-coated 35-mm six-well clusters (obtained from Corning, Corning, NY). Transfection was performed using the calcium precipitation method except that the overnight incubation was performed at 5% CO_2 (49). Four-microgram plasmid in 2 ml media was used per 35-mm dish. Cells were used 48 h after transfection for studies of ligand binding and hormone stimulation of cAMP generation.

To establish nonclonal cells stably expressing MC4R, cells were selected for two weeks in growth media containing 200 μg/ml geneticin. The cells were thereafter maintained in media containing geneticin.

[Nle^4, D-Phe^7]-α-MSH (NDP-MSH) binding to intact cells

Iodinated NDP-MSH, a superpotent agonist for the MC4R (50), was purchased from Perkin-Elmer Life Sciences (Boston, MA). Forty-eight hours after transfection, cells were washed twice with warm Waymouth's MB752/1 media modified to contain 1 mg/ml BSA and 50 μg/ml gentamicin (referred to as Waymouth/BSA). One milliliter of fresh Waymouth/BSA was added to each well, and then 100,000 cpm of ^{125}I-NDP-MSH (50 μl) was added to each well, with or without different concentrations of unlabeled NDP-MSH (Phoenix Pharmaceuticals, Belmont, CA). The final concentration of unlabeled NDP-MSH ranged from 10^{-12} to 10^{-6} M.

After incubation at 37 C for 1 h, cells were placed on ice, washed twice with cold Hanks' balanced salt solution modified to contain 1 mg/ml BSA and 50 μg/ml gentamicin. Then 100 μl of 0.5 N NaOH was added to each well. Cells were collected using cotton swabs, and counted in γ counter. Binding capacity and IC_{50} values were calculated using DeltaGraph software (Monterey, CA).

NDP-MSH stimulation of intracellular cAMP production

HEK293 cells were plated and transfected as described above. Forty-eight hours after transfection, cells were washed twice with warm Waymouth/BSA. Then 1 ml of fresh Waymouth/BSA containing 0.5 mM isobutylmethylxanthine (Sigma) was added to each well. After a 15-min incubation at 37 C, either buffer alone or different concentrations of NDP-MSH were added and the incubation was continued for another hour. The cells were then placed on ice, media were aspirated, and intracellular cAMP were extracted by the addition of 0.5 N percholoric acid containing 180 μg/ml theophylline, and measured using RIA. All determinations were performed in triplicate. Maximal responses (R_{max}) and EC_{50} values were calculated using DeltaGraph software.

Imaging of cells stably expressing MC4R by confocal microscopy

HEK293 cells stably expressing wt or mutant myc-MC4R were established as described above. The day before the experiment, HEK293 cells stably expressing wt or mutant MC4Rs were plated onto lysine-coated slides (Biocoat cellware purchased from Falcon). All solutions and procedures were at room temperature. On the day of the experiment, cells were washed three times with filtered PBS for immunohistochemistry [PBS-IH, 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4, 4.3 mM Na_2HPO_4 (pH 7.4)]. Cells were then fixed with 4% paraformaldehyde in PBS-IH for 30 min. After a further wash of three times with PBS-IH, cells were permeabilized with 1% Triton X-100 in PBS-IH for 5 min. For cell surface staining, this step was omitted. Then the cells were incubated with blocking solution (5% BSA in PBS-IH) for 1 h, and incubated 1 h with fluorescein-conjugated monoclonal antibody 9E10 (Affinity Bioreagents, Golden, CO) diluted 1:100 in PBS-IH containing 1 mg/ml BSA. Cells were then washed five times with PBS-IH, allowed to dry, and covered with Vectashield Mounting Media (Vector Laboratories, Burlingame, CA) and a glass coverslip. Images were collected with a Bio-Rad 1024 laser scanning confocal microscope.

Results

In the present study, 11 different mutations previously identified in obese patients were functionally characterized in HEK293 cells. These cells have been used extensively in previous studies on the functional characterization of mutant MC4Rs associated with obesity (see Refs. 36 and 51 for examples). All mutants, as well as wt MC4R, were transfected at maximal plasmid concentrations (4 μg per 35-mm dish in 2 ml of media). In the same experiment, both competitive binding assays and dose-dependent hormone stimulation of cAMP production were measured. From the competitive binding assay, the IC_{50} (concentration of cold NDP-MSH that resulted in 50% inhibition) as well as B_{max} (binding capacity) were calculated. From the dose-dependent NDP-MSH stimulation of intracellular cAMP production, the R_{max} as well as EC_{50} (concentration of NDP-MSH that cause 50% maximal cAMP production) were determined. From the results of at least three independent experiments, it became clear that some mutants behave like wt MC4R in that all the parameters

were not significantly different from wt MC4R. These mutants include D37V, P48S, V50M, I170V, and N274S (Figs. 1 and 2 and Table 1). Therefore, it remains to be determined whether these variants are responsible for the obesity observed in the patients and, if so, by what mechanism.

The other six mutants were found to have either decreased (S58C, N62S, Y157S, and C271Y) or no (P78L and G98R) ligand binding, with proportional impairments in NDP-MSH stimulated cAMP production (Figs. 1 and 2 and Table 1). It should be pointed out that the G98R MC4R mutant had been carefully characterized in the original report by Kobayashi *et al.* (42). It had been shown that this mutant receptor is completely devoid of ligand binding and cAMP generation in response to NDP-MSH stimulation. Our data are in agreement with this report (data not shown). We included this mutant in our study, though, so that we could further determine its cellular localization (see below). It is interesting to note that mutant S58C had the same R_{max} as wt MC4R, even though it exhibited greatly diminished ligand binding, suggesting the presence of spare receptors in this heterologous expression system. Impaired binding of agonist to the Y157S and C271Y mutants were reflected in a rightward shift of their dose-response curves, resulting in increased EC_{50} (EC_{50} for Y157S was increased 9-fold compared with wt, and EC_{50} was increased 66.5 fold for C271Y, Table 1).

To ascertain the cellular localization of the mutant MC4Rs, cells stably expressing wt or mutant MC4R were stained with fluorescein-conjugated monoclonal antibody for c-myc

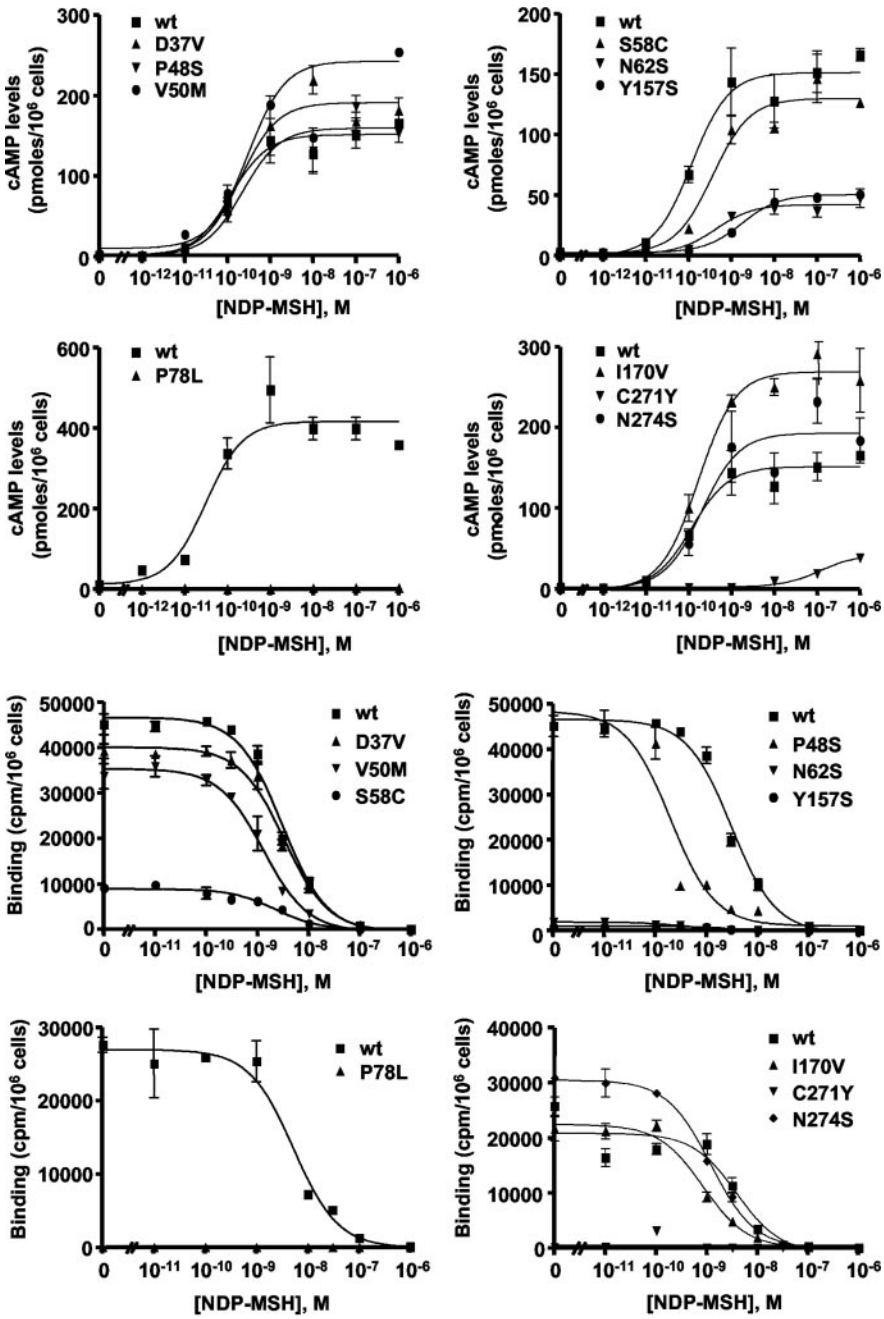

FIG. 1. Accumulation of intracellular cAMP in cells transiently transfected with wt and mutant MC4Rs. Cells were transfected, stimulated with different concentrations of NDP-MSH, and intracellular cAMP measured as described in *Materials and Methods*. Results are expressed as mean ± SEM of triplicate determinations within one experiment. All experiments were performed at least three times and similar results were obtained.

FIG. 2. Competitive binding of radiolabeled NDP-MSH in cells transiently transfected with wt and mutant MC4Rs. Cells were transfected and binding assays were performed as described in *Materials and Methods*. Different concentrations of unlabeled NDP-MSH were used to inhibit the binding of ^{125}I-NDP-MSH to wt and mutant. Results shown are mean ± range of duplicate determinations within one experiment. All experiments were performed at least three times and similar results were obtained.

TABLE 1. Ligand binding and agonist-stimulated cAMP production of wt and mutant MC4Rs

MC4R	n	Binding		NDP-MSH-stimulated cAMP	
		IC_{50} (nM)	B_{max} (% wt)	EC_{50} (nM)	R_{max} (% wt)
wt	5	4.36 ± 1.69	100	0.24 ± 0.13	100
Class II mutants					
S58C	3	9.17 ± 5.29	14 ± 7	0.47 ± 0.12	98 ± 25
N62S	4	0.82 ± 0.71	5 ± 1	0.39 ± 0.07	23 ± 2
P78L	3	ND	ND	ND	ND
Y157S	4	2.13 ± 1.14	5 ± 2	2.17 ± 0.53	25 ± 7
C271Y	5	ND	ND	15.97 ± 7.95	20 ± 6
Class V mutants					
D37V	3	2.75 ± 0.92	129 ± 35	0.19 ± 0.04	217 ± 76
P48S	5	2.10 ± 0.71	79 ± 21	0.20 ± 0.03	101 ± 14
V50M	4	1.65 ± 0.85	74 ± 19	0.71 ± 0.35	123 ± 29
I170V	3	2.30 ± 0.98	108 ± 40	0.17 ± 0.03	147 ± 25
N274S	3	0.62 ± 0.27	144 ± 7	0.18 ± 0.06	136 ± 27

Data shown are the mean ± SEM of the indicated number of experiments. ND denotes that the experimental parameters could not be determined due to lack of cell surface expression of the mutant. For ligand binding studies, ND means the total cpm bound to cells expressing the mutant were no greater than nonspecific cpm bound to cells expressing the wt MC4R. The B_{max} of cells expressing wt MC4R was 0.030 ± 0.08 nmoles NDP-MSH bound/10^6 cells (mean ± SEM of five experiments). For cAMP studies, ND means the response to NDP-MSH stimulation (up to 1 μM) was less than 2-fold above basal cAMP levels.

epitope, and images were obtained from a laser confocal microscope. Cells that were not subjected to permeabilization revealed cell surface receptor expression, whereas cells that were first permeabilized revealed both cell surface and intracellular receptor expression. The wt MC4R was easily detected on the cell surface of nonpermeabilized cells (Fig. 3). Permeabilization revealed that a significant amount of wt MC4R was also located intracellularly (Fig. 3). As expected, the mutants that showed normal binding, including D37V, P48S, I170V, and N274S, were expressed well on the cell surface (Fig. 3). Those mutants with diminished binding, including S58C, N62S, P78L, G98R, Y157S, and C271Y, showed a lack of cell surface expression. Staining of permeabilized cells showed that these mutants were expressed, but were retained intracellularly (Fig. 3). These results suggest that defective trafficking is the major cause of obesity caused by loss-of-function MC4R mutations.

Discussion

There are two major findings from our study of variant MC4Rs. First, we determined that not all variant MC4Rs with amino acid changes result in a loss-of-function when tested in a heterologous expression system. In fact, 5 of 11 mutants we studied exhibited apparently normal functions in terms of cell surface expression, ligand binding and second messenger generation (Figs. 1–3 and Table 1). It remains to be determined whether these amino acid changes affect the functions of the MC4R with respect to the endogenous antagonist, AgRP. In addition, these mutants could potentially affect other aspects of MC4R function, such as desensitization, which has been reported in V_2 vasopressin receptor mutations (52). One must also consider that these variants might affect other signaling systems such as mobilization of intracellular free calcium (53). Finally, it is pertinent to emphasize that obesity is a multi-factorial disease. Therefore, these variants might, when coexpressed with other polymorphisms, lead to an increased susceptibility to obesity. For example, Liggett and colleagues (54) recently demonstrated synergism between β_1- and α_{2C}-adrenergic receptors polymorphisms. In any case, our data emphasize the importance of functional studies when new DNA allelic variants of MC4R are reported.

Another important finding from this study is that intracellular trapping of the mutated MC4R is the major defect of the MC4R when early-onset severe obesity is associated with a loss-of-function of the MC4R mutant. Confocal laser scanning microscopy revealed that all six mutants that exhibit a loss-of-function phenotype are trapped intracellularly (Fig. 3). Functional characterization of two frameshift mutations of the MC4R also revealed that these two mutants were trapped intracellularly (51). Because the MC4R, like other GPCRs, is an integral membrane protein that transverses the membrane seven times, its folding is understandably complicated. Any perturbations in the folding process will result in a receptor that is misfolded and will be recognized by the cell's quality control mechanisms within the endoplasmic reticulum (ER), thereby prevented it from exiting the ER. Indeed, transit out of the ER is the rate-limiting step in expression of human δ-opioid receptor at the cell surface (55). In general, intracellular retention of misfolded mutant receptors is one of the most common reasons for loss-of-function phenotypes in GPCRs. Such observations have been found for, among others, rhodopsin (7, 8), the vasopressin receptor (reviewed in Ref. 56), the endothelin B receptor (57), the calcium-sensing receptor (58), the GnRH receptor (59), the LH receptor (9), and the FSH receptor (60).

While our studies were being prepared for publication, three papers were published that also examined the functional properties of several different MC4R variants (47, 61, 62). Our study, however, is not redundant in that it is the only one to functionally characterize the MC4R variants D37V, P48S, Y157S, and N274S. Furthermore, our studies shows that the G98R mutant, previously shown to have a loss-of-function phenotype (42), is due to a lack of cell surface expression of this mutant receptor. Importantly, the data from our study as well as the others point to decreased cell surface expression as being the most prevalent defect in loss-of-

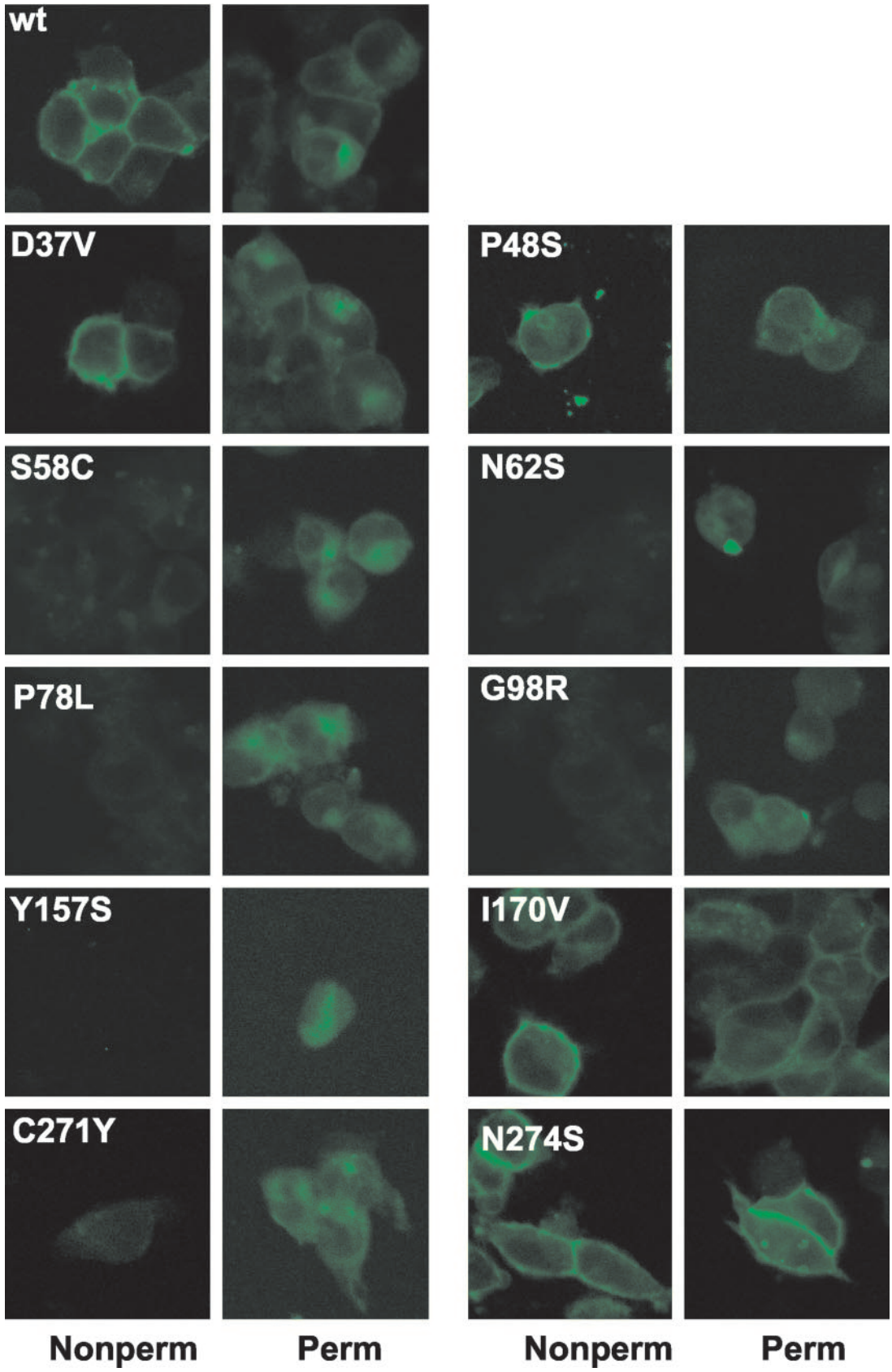

FIG. 3. Confocal imaging of wt and mutant myc-MC4Rs. Cells stably expressing wt and mutant myc-MC4Rs were permeabilized or left intact as indicated and then stained with florescein-conjugated anti-myc monoclonal antibody and imaged by confocal microscopy.

function MC4R mutants associated with early-onset severe obesity.

Of the MC4R variants that were examined both in our study as well other recent studies, there are both confirmatory as well as dissimilar observations. Similar results were obtained with the mutant P78L, which was consistently shown to lack signaling and cell surface expression (present study as well as Refs. 61 and 62). Our results on N62S are also in agreement with Yeo *et al.* (47) in that this mutant appears to retain partial signaling capabilities. Finally, our results on the MC4R mutant V50M are generally in agreement with the studies of Vaisse and colleagues (61). Both studies show that that the EC_{50} and R_{max} are not significantly different between cells expressing the wt *vs.* V50M mutant. However, we observed only a 3-fold increase in the EC_{50} with S58C mutant, whereas they reported a 15-fold increase. This may be due to either or both of two technical differences between the studies. First, different methods were used to measure signaling by the MC4R. In their study, a luciferase reporter gene assay was used, which is less sensitive than the direct measurement of intracellular cAMP. Furthermore, the native ligand α-MSH was used in their study, while we used the superpotent analog NDP-MSH. With regards to the C271Y mutant, Yeo *et al.* (47) were unable to observe any signaling, whereas we detected some signaling at high concentrations of NDP-MSH (Fig. 1). Again, these different observations may be due to the different methods used to measure cAMP and/or the agonist used. There are also some discrepancies with regards to I170V. Whereas our data show that I170V is normal with respect to cell surface expression, ligand binding, as well as signaling (Figs. 1–3 and Table 1), Vaisse and colleagues (61) suggested that this mutant exhibited a loss-of-function due to decreased cell surface expression. However, their data showed only a 16% reduction in maximal binding capacity of the I170V mutant and the signaling properties were not examined. The study by Adan and colleagues (62) also suggested that I170V exhibited a loss-of-function phenotype with the cell surface expression level 45% of wt and the R_{max} 47% of wt (as determined by measuring adenylyl cyclase activity). The reasons for these apparent discrepancies are not clear.

With the data from the present study, as well as the recently published data about the functional defects of MC4R variants associated with childhood obesity, and following the model of LDL receptor mutations (63), we propose the following scheme for classifying MC4R mutations:

Class I: null mutations

This class of mutants produces no receptor protein due to defective synthesis and/or accelerated degradation. Although there are no studies performed yet to directly verify this, W16X (45), Y35X (38, 64) and L64X (44) are proposed to belong to this class.

Class II: intracellularly trapped mutants

This class comprises the largest set of MC4R mutations reported to date, including the frameshift mutations ΔCTCT at codon 211 (51) and the TGAT insertion at codon 244 (51), S58C (present study and Ref. 61), N62S (present study and Ref. 47), P78L (present study and Refs. 61 and 62), N97D (47), G98R (present study), I102S (61), L106P (47), I125K (47), R165Q (62), R165W (61, 62), L250Q (61), Y287X (47), C271Y (present study and Ref. 47), P299H (61), I316S (47), and I317T (61, 65).

Class III: binding defective mutants

These mutant MC4Rs display relatively normal expression on the cell surface, but ligand binding is impaired in terms of decreased maximal binding and/or binding affinity, therefore causing impairments in hormone-stimulated signaling. These mutants include I137T (36), N97D, L106P, I125K, I316S (47) and Δ88–92 (Donohoue, P. A., Y.-X. Tao, M. Collins, G. S. H. Yeo, S. O'Rahilly, and D. L. Segaloff, manuscript submitted for publication). It is of particular interest that O'Rahilly and colleagues identified a MC4R mutation (I316S) that alters the relative affinities of the receptor for its endogenous agonist (α-MSH) and antagonist (AgRP) (47). Therefore, this mutant can be classified as a subset within this class.

Class IV: signaling defective mutants

These mutant receptors are expressed on the cell surface and bind ligand relatively normally, but agonist-stimulated signaling is impaired. Mutants I137T (36), A175T and V253I may belong to this class (47), although in one report, V253I was found to have a relatively normal R_{max}, albeit the EC_{50} was increased 3-fold (61). In fact, this variant was found recently in one normal weight control subject (45), suggesting that this variant has relatively normal functions.

Class V: variants with unknown defects

These variants behave similarly to the wt MC4R in heterologous expression systems in the parameters studied. Some of the variants, such as D37V, P48S, V50M, I170V, and N274S (present study), exhibit normal cell surface expression, ligand binding, and agonist-stimulated cAMP. Whether and how these variants cause energy imbalance and therefore obesity is unclear. D37V is present as a double mutation Y35X/D37V (38, 64). We would argue that the phenotype is most likely the result of the Y35X nonsense mutation. The difference between these variants and common polymorphisms such as V103I and I251L is that these variants are not assumed to be present in normal subjects. However, as mentioned above, a variant (V253I) previously identified as a missense mutation associated with obesity was later found in a normal weight subject (45). Other MC4R mutants that would fall within this category are I102T, F202L and N240S, which were found only in normal weight subjects but not obese subjects (44). Finally, it is unclear whether T112M falls into this category or not. Whereas it was originally identified in obese subjects (38, 39) and recent studies suggest that it causes a loss-of-function phenotype (62), earlier functional studies showed it to have normal functions (36, 39) and it has since been reported in one control subject (44).

In summary, our results stress the importance of analyzing the functional properties of variant MC4Rs identified in obese subjects to more accurately determine if the phenotype

of the variant is indeed consistent with the clinical phenotype. Moreover, we anticipate that a classification system such as one proposed herein will be beneficial in managing the increasingly large array of MC4R mutations associated with early childhood obesity.

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