

Functional Analyses of Melanocortin-4 Receptor Mutations Identified from Patients with Binge Eating Disorder and Nonobese or Obese Subjects

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Context: Whether mutations in the melanocortin-4 receptor (MC4R) are the cause of binge eating disorder was controversial. In addition, the penetrance of mutations in the MC4R in causing obesity was debated.

Objective: We investigated whether MC4R variants identified from obese patients with binge eating disorder (T11A, F51L, T112M, and M200V) and variants identified in nonobese (I102T, F202L, and N240S) or obese (I102S, A154D, and S295P) subjects cause loss-of-function and what are the defects.

Design: Variant or wild-type MC4Rs were expressed in HEK293 cells and examined for their pharmacological characteristics.

Setting: The study setting was *in vitro* bench-top laboratory experiments.

Main Outcome Measures: Ligand binding, signaling, and cell sur-

face expression of the variant MC4Rs were compared with wild-type MC4R.

Results: Our data clearly show a loss-of-function phenotype *in vitro* for I102T and N240S variants identified in nonobese individuals. Furthermore, not all MC4R variants identified in obese subjects exhibit a loss-of-function phenotype *in vitro*. Finally, the MC4R variants T11A, F51L, T112M, and M200V identified from patients with binge eating disorder displayed normal function with regards to the parameters measured in our study.

Conclusions: Patients harboring loss-of-function MC4R mutations do not always exhibit obesity. Novel MC4R variant identified from an obese patient cannot be assumed to be the cause of obesity without demonstrating a loss-of-function phenotype *in vitro* for the variant MC4R. Whether MC4R mutations are involved in the pathogenesis of binge eating disorder needs additional investigation. (*J Clin Endocrinol Metab* 90: 5632–5638, 2005)

THE MELANOCORTIN-4 RECEPTOR (MC4R) is a member of the G protein-coupled receptor (GPCR) superfamily. The topology of the MC4R is predicted to consist of seven transmembrane α -helices (TMs) connected by alternating extracellular and intracellular loops, with the N terminus lying on the outside of the cell and the C terminus lying on the inside of the cell. During hormone stimulation, MC4R activates adenylyl cyclase via the stimulatory G protein Gs to elevate intracellular cAMP levels. The endogenous ligands for the MC4R include the agonist α -MSH (derived from the posttranslational processing of proopiomelanocortin) and the antagonist agouti-related protein (AgRP). Pharmacological and mouse genetic studies established the MC4R as one of the critical factors involved in regulating energy homeostasis (reviewed in Ref. 1). Leptin, produced by adipocytes, binds to its receptor in the arcuate nucleus to stimulate α -MSH production and inhibit AgRP production, resulting in decreased food intake (2). Exogenous administration of α -MSH decreases food intake, whereas adminis-

tration of AgRP increases food intake (3–5). Disruption of the MC4R signaling pathway by deletion of MC4R or proopiomelanocortin or overexpression of AgRP leads to hyperphagia and obesity (6–9). These mouse genetic studies were replicated in human genetic studies. Frameshift mutations of the MC4R were first reported to be associated with early-onset severe obesity (10, 11). More than 70 different mutations have since been reported in various patient cohorts (for a recent review, see Ref. 12). Remarkably, in some patient cohorts, up to 6% of the morbidly obese patients with early onset harbor MC4R mutations (13). Detailed functional studies of the variant MC4Rs revealed that intracellular retention of the mutant receptor is the predominant cause of loss-of-function (14–17) (reviewed in Ref. 12).

Recently, Jacobson *et al.* (18) questioned the importance of MC4R mutations in causing human obesity. These investigators screened a total of 528 subjects for MC4R mutations by direct sequencing. They were unable to identify any variants of the MC4R that were associated with obesity. Interestingly, though, they identified three novel variants from normal-weight subjects: I102T, F202L, and N240S. However, they did not determine whether these variants cause functional impairments. This is important because, if any of the these variants results in functional defects, it will further strengthen the idea that MC4R mutations do not always cause obesity, *i.e.* the penetrance of MC4R mutations in causing obesity is not 100%.

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Abbreviations: AgRP, Agouti-related protein; GPCR, G protein-coupled receptor; MC4R, melanocortin-4 receptor; MCR, melanocortin receptor; NDP-MSH, [Nle⁴, D-Phe⁷]- α -MSH; TM, transmembrane α -helix; wt, wild type.

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The relevance of MC4R variants in eating disorders is not clear. Based on its functions in regulating food intake, it is expected that constitutively activating mutations of the MC4R would be involved in anorexia nervosa, whereas loss-of-function mutations of the MC4R might be involved in bulimia nervosa. So far, however, there are no reports of constitutively activating mutations in anorexia nervosa patients. The only naturally occurring constitutively activating mutation reported thus far (L250Q) was, paradoxically, identified from an obese patient (19). Recently, it was shown that this mutation has reduced cell surface expression, which might be the cause of obesity (14, 16). Although numerous reports of loss-of-function mutations in MC4R associated with obesity have been reported, the eating behavior of the obese patients with MC4R mutations have not been documented in detail in most of these studies. Obese patients with dominantly inherited MC4R mutations exhibit hyperphagia (20, 21), consistent with the observations in MC4R knockout mice (6). They may (22) or may not (20, 21) exhibit preference for macronutrients. With regards to binge eating disorder, previous studies by Hebebrand and colleagues (23, 24) identified rare cases of binge eating patients with functionally relevant MC4R mutations. Recently, Branson *et al.* (25) published a provocative and controversial article suggesting that all obese patients with MC4R “mutations” exhibited binge eating disorder. However, 75% of the subjects with the so-called MC4R mutations have V103I, T112M, or I251L substitutions. Extensive studies have shown normal functions for these three MC4R variants (19, 20, 26). In addition, these variants were found at similar frequencies in obese and nonobese subjects (reviewed in Ref. 21), again suggesting that they are not functionally relevant mutations. Using the criteria proposed by O’Rahilly and colleagues (27) that a MC4R mutation can be viewed as causative “only if it cosegregates with obesity in pedigrees, is absent in ethnically matched controls, and impairs the function of the encoded receptor,” these variants fail all three tests, casting serious doubt about the conclusion of the paper. Branson *et al.* (25) identified three novel variants, T11A, F51L, and M200V, that have not been reported in control subjects in their study or any other study. The functional characteristics of these novel variants were not known.

In addition, Cummings *et al.* (28) identified two novel variants of the MC4R (A154D and S295P) that cause partial hormone unresponsiveness. However, it is not known what underlies the loss-of-function (*i.e.* decreased cell surface expression, decreased ligand binding, and/or decreased agonist-stimulated activation of Gs). A154 is conserved in all melanocortin receptors (MCRs), whereas S295 is either Ser or Ala in other MCRs (available at <http://www.gpcr.org>, release 6.1). Because these two codons are highly conserved in all MCRs, it is reasonable to expect that these two residues might be important in receptor function.

In this study, we investigated the functional properties of the variants from these studies to address several questions. 1) How prevalent is intracellular retention in MC4R mutations that result in functional impairments? 2) Are MC4R variants identified from patients with binge eating disorder functionally impaired, therefore pathogenic? 3) Do all variants identified from obese patients cause a loss-of-function?

4) How penetrant are loss-of-function mutations of MC4R in causing obesity?

Materials and Methods

In vitro mutagenesis of MC4R variants

Wild-type (wt) MC4R with a myc epitope tag at the N terminus (after the initiating Met) has been described previously (17). Mutant MC4Rs were generated using this construct by QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as detailed previously (17, 29). Briefly, primers incorporating the desired mutations were synthesized (Integrated DNA Technologies, Coralville, IA) and used to replace the wt sequence by PCR using myc-MC4R in pBlueScript as the template and *pfu* Turbo DNA polymerase (the sequences of the primers are available on request from the corresponding author). The PCR cycles consisted of denaturation at 95 C for 30 sec and then 12 cycles of 95 C for 30 sec, 55 C for 30 sec, and 68 C for 12 min. *DpnI* was used to digest the parental methylated DNA in pBlueScript in the reaction product (37 C for 1 h), and the digested reaction product was transformed into the supercompetent XL1-Blue *Escherichia coli* cells (which repaired the nicked mutated plasmids). Individual colonies were grown and sequenced by automated DNA sequencing (performed by the DNA Core Facility of the University of Iowa Carver College of Medicine). The plasmids with the correct mutation and without any spurious mutation introduced during PCR were ligated back into pcDNA3.1. The final constructs in pcDNA3.1 were prepared with a Maxiprep kit (Qiagen, Valencia, CA) and were sequenced again before use in transfections.

Cells and transfections

HEK293 cells were maintained at 5% CO₂ 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 (Corning, Corning, NY). Calcium precipitation was used to transfect HEK293 cells, with 4 µg plasmid added to each 35-mm dish (30). Cells were used 48 h after transfection to measure ligand binding and hormone stimulation of cAMP generation. Nonclonal cells stably expressing MC4R were established by selecting cells in growth media containing 200 µg/ml geneticin for 2 wk after transfection. The cells were thereafter maintained in media containing geneticin.

[Nle⁴, D-Phe⁷]-α-MSH (NDP-MSH) binding to intact cells

The binding assay was described in detail previously (17). Briefly, 48 h 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 (hereafter referred to as Waymouth/BSA). Fresh Waymouth/BSA was added to each well, together with 100,000 cpm of [¹²⁵I]NDP-MSH (Perkin-Elmer Life and Analytical Sciences, Boston, MA) in 50 µl, with or without different concentrations of unlabeled NDP-MSH (Phoenix Pharmaceuticals, Belmont, CA), and incubated at 37 C for 1 h. Then cells were placed on ice and washed twice with cold Hank’s balanced salt solution containing 1 mg/ml BSA. The cells were then dissolved in 100 µl 0.5 N NaOH, collected using cotton swabs, and counted in a γ-counter. Binding capacity and IC₅₀ were calculated using DeltaGraph (Monterey, CA) software.

Signaling properties of the mutant MC4Rs

Signaling properties of the MC4Rs were assessed by measuring intracellular cAMP levels in response to NDP-MSH stimulation (17). Forty-eight hours after transfection, cells were washed twice with warm Waymouth/BSA, and 1 ml of fresh Waymouth/BSA containing 0.5 mM isobutyl methylxanthine was added to each well. After 15 min incubation, either buffer alone or different concentrations of NDP-MSH were added, and the cells were incubated for an additional 1 h at 37 C. Intracellular cAMP was extracted with 0.5 N perchloric acid containing 180 µg/ml theophylline and was measured by RIA. All determinations were done in triplicate. EC₅₀ values and maximal responses were calculated using DeltaGraph.

Confocal imaging of mutant MC4Rs stably expressed in HEK293 cells

HEK293 cells stably expressing MC4Rs were prepared as described above. The day before the experiment, HEK293 cells stably expressing wt or mutant MC4Rs were plated onto lysine-coated slides (Bioscoat Cellware; Falcon, Bedford, MA). On the day of the experiment, cells were washed with filtered PBS-IH [in mM: consisting of 137 NaCl, 2.7 KCl, 1.4 KH_2PO_4 , and 4.3 Na_2HPO_4 (pH 7.4)] and then incubated with blocking solution (5% BSA in PBS-IH) at room temperature for 1 h. After aspirating the blocking solution, the cells were incubated for 1 h at room temperature with fluorescein-conjugated monoclonal antibody 9E10 (Affinity Bioreagents, Golden, CO) diluted 1:100 in PBS-IH containing 1 mg/ml BSA. The cells were then washed five times with PBS-IH and sealed with Vectashield Mounting Media (Vector Laboratories, Burlingame, CA). Images were collected with a 1024 laser confocal microscope (Bio-Rad, Hercules, CA).

Results

In this study, we investigated the functional properties of 10 variants previously identified in several studies. These variants include the following: T11A in the extracellular domain, F51L in TM1, I102S and I102T in TM2, T112M in the first extracellular loop, A154D in the second intracellular loop, M200V and F202L in TM5, N240S in TM6, and S295P in TM7 (shown schematically in Fig. 1). These variants were identified from normal-weight subjects (I102T, T112M, F202L, and N240S), obese patients (I102S, A154D, and S295P), or obese patients with binge eating disorder (F51L, T112M, and M200V). The functional properties of these variants either were previously not investigated or conflicting results were reported. Therefore, we systematically studied the cell surface expression, ligand binding, and signaling properties of these variants.

Because previous studies showed that intracellular retention is the major defect underlying loss-of-function MC4R mutants, we studied the cellular localization of the MC4R variants. Cells stably expressing wt or mutant MC4Rs were immunostained with 9E10 monoclonal antibody against the

myc epitope and visualized by confocal microscopy. Although this method does not permit quantification of receptor expression, the results clearly show that all of the mutants are expressed on the cell surface (Fig. 2).

To study whether the MC4R variants expressed on the cell surface could bind agonist, whole-cell binding assays were performed. Of the 10 variants, I102S and I102T showed dramatic decreases in maximal binding (Fig. 3 and Table 1). I102S MC4R (identified from an obese patient) exhibited minimal binding, whereas I102T MC4R (identified from a normal-weight subject) retained 20% of wt binding capacity. N240S MC4R (identified from a nonobese subject) showed a partial decrease in maximal binding. The other seven variants had binding capacities and affinities similar to the wt MC4R (Fig. 3 and Table 1).

To investigate whether the variant MC4Rs retained normal signaling properties, cells transiently transfected with wt or mutant MC4Rs were stimulated with different concentrations of the superpotent agonist NDP-MSH, and intracellular accumulation of cAMP was measured. As shown in Fig. 4 and Table 1, all mutants except I102S (from an obese patient) and N240S (from a normal-weight subject) responded to NDP-MSH stimulation with similar maximal responses as the wt MC4R. N240S MC4R showed a small decrease in the maximal response. I102S MC4R had a maximal response equal to 50% of wt MC4R, and its EC_{50} was increased about 15-fold. The EC_{50} was increased by 4-fold in A154D MC4R (identified from an obese patient). The EC_{50} values for the other mutants were similar as the wt MC4R.

Srinivasan *et al.* (31) suggested recently that constitutive activity of the MC4R also plays a role in determining food intake. Therefore, we also compared the basal activities of the mutants. We found that I102S, I102T, A154D, F202L, and N240S have decreased basal activities, whereas T11A, F51L,

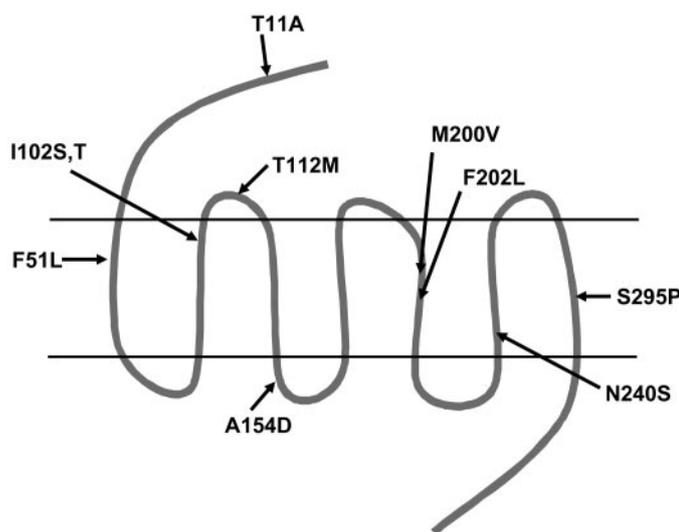


FIG. 1. Snake plot of human MC4R, depicting the locations of the variants studied in this study. These variants were identified from normal-weight subjects (I102T, T112M, F202L, and N240S), obese patients (I102S, A154D, and S295P), or obese patients with binge eating disorder (T11A, F51L, T112M, and M200V).

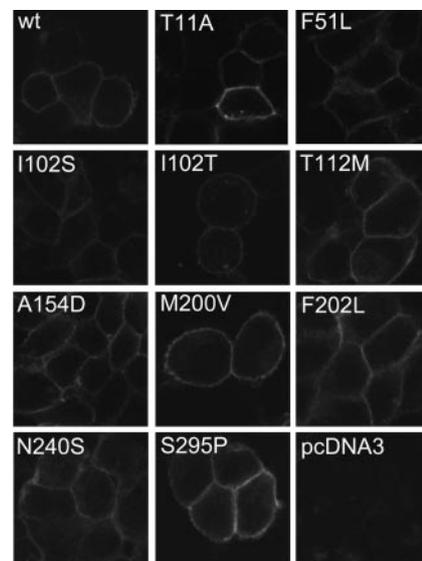
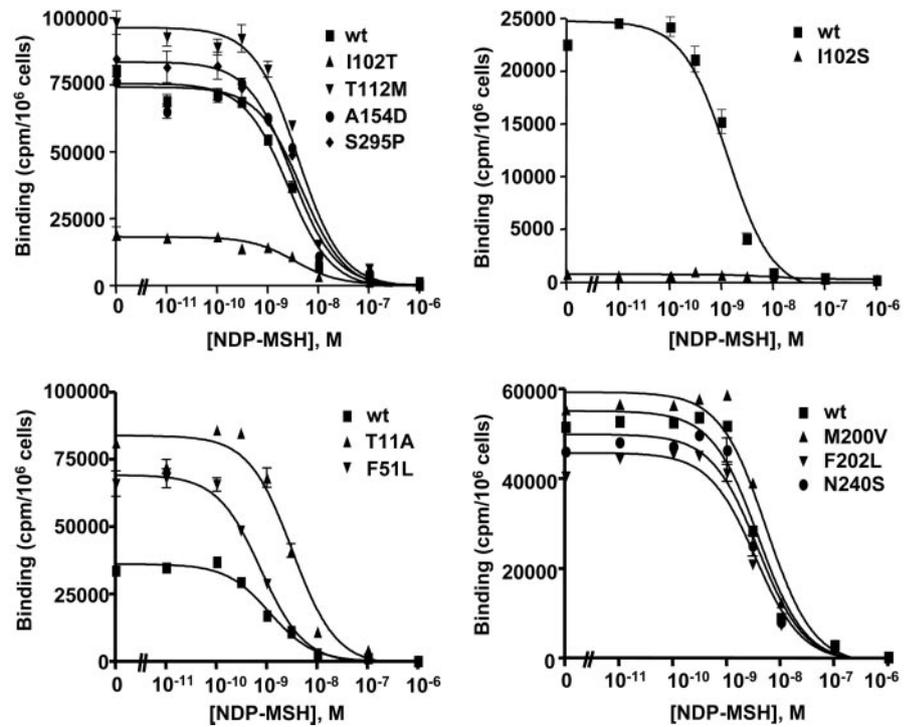


FIG. 2. Confocal imaging of wt and mutant myc-MC4Rs. Cells stably expressing wt or mutant myc-MC4Rs were fixed without permeabilization, stained with fluorescein-conjugated anti-myc monoclonal antibody 9E10, and imaged by confocal microscopy. The variants were from normal-weight subjects (I102T, T112M, F202L, and N240S), obese patients (I102S, A154D, and S295P), or obese patients with binge eating disorder (T11A, F51L, T112M, and M200V).

FIG. 3. Intact cell surface binding to NDP-MSH in HEK293 cells expressing wt or mutant MC4Rs. HEK293 cells were transiently transfected, and binding assays were performed as described in *Materials and Methods*. Different concentrations of unlabeled NDP-MSH were used to displace the binding of [¹²⁵I]NDP-MSH to wt or mutant MC4Rs on intact cells. Results shown are expressed as the mean ± range of duplicate determinations within one experiment. All experiments were performed at least two times, and similar results were obtained. The variants were from normal-weight subjects (I102T, T112M, F202L, and N240S), obese patients (I102S, A154D, and S295P), or obese patients with binge eating disorder (T11A, F51L, T112M, and M200V).



T112M, and S295P have similar basal activities compared with the wt MC4R (Fig. 5).

Discussion

Since the initial reports of MC4R mutations associated with early-onset severe obesity (10, 11), more than 70 variants have been reported from various patient cohorts (summarized in Ref. 12). Until recently, the functional consequences of the variants identified in many of these studies were not investigated. Therefore, only an association rather than a cause and effect relationship could be provided. Several

studies attempted to provide this missing link. These studies showed that the predominant cause of the loss-of-function phenotype exhibited by MC4R mutants is intracellular retention of the mutants, resulting in the absence or decreased cell surface expression of the mutant receptors (14–17). Very few mutant MC4Rs have been identified that are expressed on the cell surface but are defective in ligand binding or signaling (15, 22, 32) (reviewed in Ref. 12).

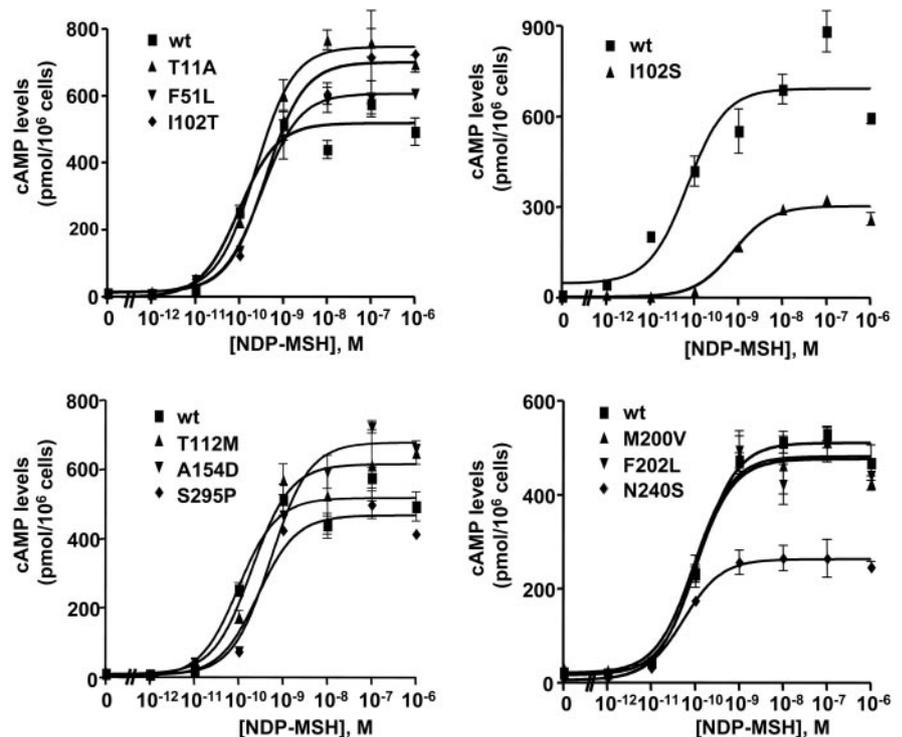
In this study, we performed detailed functional characterization of 10 variants identified previously from normal-weight subjects (I102T, T112M, F202L, and N240S), obese

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

| | | NDP-MSH binding | | NDP-MSH-stimulated cAMP | |
|-------------------------------------|---|-----------------------|-------------------------|-------------------------|-------------------------|
| | | IC ₅₀ (nM) | B _{max} (% wt) | EC ₅₀ (nM) | R _{max} (% wt) |
| wt | 6 | 5.41 ± 1.52 | 100 | 0.25 ± 0.08 | 100 |
| Normal weight subjects | | | | | |
| I102T | 4 | 8.67 ± 1.47 | 20 ± 5 | 0.59 ± 0.16 | 126 ± 33 |
| T112M | 3 | 8.05 ± 2.59 | 130 ± 21 | 0.18 ± 0.03 | 120 ± 33 |
| F202L | 3 | 5.75 ± 2.43 | 87 ± 2 | 0.27 ± 0.10 | 89 ± 10 |
| N240S | 3 | 5.92 ± 2.85 | 65 ± 17 | 0.12 ± 0.04 | 72 ± 11 |
| Obese patients | | | | | |
| I102S | 3 | 176.90 ± 63.40 | 2 ± 1 | 3.05 ± 2.08 | 51 ± 14 |
| A154D | 3 | 3.78 ± 0.98 | 126 ± 46 | 1.09 ± 0.31 | 126 ± 35 |
| S295P | 3 | 6.27 ± 2.36 | 129 ± 29 | 0.37 ± 0.14 | 112 ± 19 |
| Patients with binge eating disorder | | | | | |
| T11A | 3 | 9.72 ± 3.22 | 136 ± 46 | 0.12 ± 0.06 | 109 ± 38 |
| F51L | 3 | 3.34 ± 1.25 | 108 ± 40 | 0.36 ± 0.12 | 99 ± 13 |
| T112M | 3 | 8.05 ± 2.59 | 130 ± 21 | 0.18 ± 0.03 | 120 ± 33 |
| M200V | 2 | 8.77 ± 6.73 | 111 ± 3 | 0.33 ± 0.25 | 104 ± 10 |

Data shown are the mean ± SEM of the indicated number of experiments. The B_{max} (maximal binding) of cells expressing wt MC4R was 0.047 ± 0.005 nmol NDP-MSH bound/10⁶ cells, and the R_{max} (maximal response) was 490.0 ± 77.0 pmol cAMP/10⁶ cells (mean ± SEM of six experiments). IC₅₀ is the concentration of NDP-MSH that is needed to cause 50% inhibition in the binding assay. EC₅₀ is the concentration of NDP-MSH that results in 50% stimulation of the maximal response. The variants were from normal-weight subjects (I102T, T112M, F202L, and N240S), obese patients without binge eating disorder (I102S, A154D, and S295P), or obese patients with binge eating disorder (T11A, F51L, T112M, and M200V).

FIG. 4. Accumulation of intracellular cAMP in HEK293 cells expressing wt or mutant MC4Rs. HEK293 cells were transiently transfected and stimulated with various concentrations of NDP-MSH, and intracellular cAMP levels were measured as described in *Materials and Methods*. Results are expressed as the mean \pm SEM of triplicate determinations within one experiment. All experiments were performed at least two times, and similar results were obtained. The variants were from normal-weight subjects (I102T, T112M, F202L, and N240S), obese patients (I102S, A154D, and S295P), or obese patients with binge eating disorder (T11A, F51L, T112M, and M200V).



patients (I102S, A154D, and S295P), or obese patients with binge eating disorder (F51L, T112M, and M200V). Our results are summarized in Table 2. In contrast to previous studies on the MC4R mutants, the 10 MC4R variants studied here are all expressed on the cell surface (Fig. 2). Previously, using fluorescence activated cell sorting, Lubrano-Berthelie *et al.* (14) showed that I102S had decreased cell surface expression. Similarly, by confocal microscopy, we show that I102S MC4R is expressed on the cell surface, although with a lower intensity of staining compared with the wt MC4R. Interestingly, we observed minimal binding activity in cells expressing I102S, suggesting that this mutation may also be partially defective in ligand binding. Similarly, I102T also exhibits a decrease in binding activity greater than would be expected based on the cell surface immunolocalization of the mutant. The decreased cell surface expression and ligand-binding

defects in these mutants result in decreased hormone-stimulated signaling (Fig. 4 and Table 1).

It appears that there are spare receptors in the heterologous expression system used here. For example, I102S binding to NDP-MSH can barely be detected, but it retained 50% of maximal hormonal responsiveness compared with the wt MC4R. Similarly, I102T had only 20% binding capacity of wt MC4R, yet it had the same maximal response as the wt MC4R with only a small increase in EC_{50} (Table 1).

The relevance of MC4R mutations to binge eating disorder

The relevance of MC4R mutations in eating disorders is not known. No constitutively activating mutations have been

TABLE 2. Summary of the functional properties of the 10 variants studied herein

| MC4R | Surface expression ^a | Binding | Signaling | Class ^b |
|-------------------------------------|---------------------------------|---------|-----------|--------------------|
| Normal-weight subjects | | | | |
| I102T | + | ↓ | ↓ | III |
| T112M | + | + | + | V |
| F202L | + | + | + | V |
| N240S | + | ↓ | ↓ | II |
| Obese patients | | | | |
| I102S | + | ↓ | ↓ | III |
| A154D | + | + | + | V |
| S295P | + | + | + | V |
| Patients with binge eating disorder | | | | |
| T11A | + | + | + | V |
| F51L | + | + | + | V |
| T112M | + | + | + | V |
| M200V | + | + | + | V |

+, The particular function is normal.

^a Cell surface expression was assessed by confocal microscopy; therefore, it is not quantitative.

^b The classification of the MC4R mutants are based on the scheme we proposed previously (17).

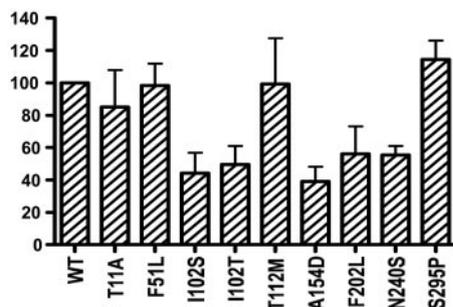


FIG. 5. Constitutive activities of wt and mutant MC4Rs. The basal activities of wt and mutant MC4Rs were assessed by measuring cAMP levels in cells expressing wt or mutant MC4Rs in the absence of NDP-MSH. The results are expressed as percentage of wt basal cAMP level. Shown are mean \pm SEM of three or more experiments. The basal cAMP level in wt MC4R was 14.65 ± 2.65 pmol/ 10^6 cells (mean \pm SEM of six experiments).

reported from anorexia nervosa patients. Although it is widely accepted that loss-of-function mutations in the MC4R gene are associated with obesity, the eating behaviors of the obese patients with MC4R mutations have not been documented in detail in most of these studies. Only a few studies reported hyperphagia either with a test meal (20, 21) or via medical history (22). With regards to binge eating disorder, 1 of 19 mutation carriers was found to have binge eating disorder in one study (23). A recent report suggesting that all obese patients harboring MC4R variants exhibited binge eating disorder (25) has aroused much interest and controversy. Three quarters of the patients in the study have variants V103I, T112M, or I251L, which had been shown previously to be polymorphisms with normal functions. Indeed, these variants have been found in normal-weight subjects with similar frequencies. Although T112M was reported to have a partial defect with decreased cell surface expression (16), our data are consistent with previous reports (20, 26) that showed that T112M has normal functions. These results cast serious doubt on the conclusions reached in the study of Branson *et al.* (25). The two novel variants we examined herein, F51L and M200V, also have normal functions compared with the wt MC4R (Figs. 2–4). Therefore, these variants cannot fulfill any of the criteria proposed by O’Rahilly and colleagues (27) to establish a cause and effect relationship for binge eating disorder. Recently, another study characterized the eating behaviors of carriers of functionally relevant MC4R gene mutation carriers and found no strong association of binge eating behavior with MC4R gene mutations (33). Whether MC4R mutations are involved in the pathogenesis of binge eating disorder remains to be investigated.

The expressivity of MC4R mutation in obesity

The penetrance of MC4R mutations in causing obesity is also controversial. Some investigators found that MC4R mutations are 100% in causing obesity, whereas other studies have reported lower levels of penetrance. One factor accounting for these apparent discrepancies could be different ethnic backgrounds of the patient cohorts studied. Another potential factor is that, in some studies, it was not ascertained whether the variants identified from the patients indeed cause a defect in the receptor’s function. A variant with normal pharmacological properties is not likely to result in obesity. Because a clear correlation has been observed between the severity of the defect and obesity (21), investigating the functional ramifications of the variants is of clinical importance. In the present study, of the three variants identified from obese patients, I102S, A154D, and S295P, only I102S results in a loss-of-function. I102S has been reported to have decreased cell surface expression compared with wt MC4R (14). The other two variants, A154D and S295P, behave similarly to the wt MC4R in cell surface expression, ligand binding, and signaling, except that A154D has a slightly decreased basal activity (Figs. 2–5). Therefore, whether these variants were the cause of obesity observed in the patients harboring the variants is not known. It was previously indicated that A154D and S295P are partially inactivating (28). We cannot determine the basis for the discrepancies between our results and the original report.

Another way to investigate the penetrance is to identify loss-of-function mutations from nonobese subjects. In a large screening study, Jacobson *et al.* (18) identified three variants, I102T, F202L, and N240S, from nonobese subjects. We showed here that I102T results in loss-of-function, whereas N240S results in a small decrease in both binding capacity and maximal response. Because the subjects harboring these variants were not obese, these results also suggest that MC4R mutations do not always result in obesity. Perhaps this also reflects the fact that obesity is a multifactorial disease, with both genetic and environmental components.

We recently proposed a classification scheme based on *in vitro* properties of the mutant receptors to catalog this ever-increasing battery of MC4R mutations. This classification scheme was modeled after the classification of low-density lipoprotein receptor mutations causing familial hypercholesterolemia (34) and cystic fibrosis transmembrane conductance regulator mutations causing cystic fibrosis (35). As such, class I are null mutants; class II are intracellularly trapped mutants; class III are binding-defective mutants; class IV are signaling-defective mutants; and class V are variants with apparently normal function (17). According to this scheme, all of the variants characterized here except I102S, I102T, and N240S would belong to class V. N240S may be classified as a class II mutant, with slightly decreased cell surface expression. Mutants I102S and I102T belong to class III. It should be emphasized that no direct relationship between the different categories of the mutations and severity of obesity can be assumed.

The importance of functional studies when new MC4R variants are identified

All of the studies on functional characterization of the MC4Rs used the artificial expression system in HEK293 cells. The only study that used a neuronal cell line showed that the same results were obtained when compared with the experiments using HEK293 cells (14). HEK293 cells are widely used for pharmacological studies because of its high expression of the downstream signaling molecules, including Gs and adenylyl cyclase. Together with its ease of transfection, these cells are ideal for studying Gs-coupled GPCRs. One validation of the system is that correlation of the severity of the defect *in vitro* with severity of obesity *in vivo* was observed (21). However, there is one obvious disadvantage of using this cell line, *i.e.* the presence of spare receptors. Therefore, although the expression is decreased for some mutants expressed in HEK293 cells, the signaling of those cells is similar to wt MC4R, presumably due to spare MC4Rs in the HEK293 cells. (Because loss-of-function MC4R mutations cause obesity by haploinsufficiency, no spare receptors are likely to be present *in vivo*.) It is important, therefore, that studies on the functional properties of MC4Rs variants in HEK293 cells examine cell surface receptor expression and ligand-binding properties in addition to hormone-stimulated cAMP because defects in receptor expression and/or ligand binding (which would have detrimental effects *in vivo*) may not be severe enough to permit detection of a loss of signaling in the HEK293 cells.

Of the about 70 variants that have been reported so far,

some variants are associated with dominantly inherited obesity (20, 21) with (32) or without (15) dominant-negative activity. In some reports, no appropriately genotyped controls or cosegregation data or functional studies were provided. The identification of a novel variant from an obese subject without functional data cannot be used to conclude that the novel variant is responsible for the obesity observed in the patient. The present study, together with our previous studies (17), showed that some variants identified from obese subjects do not cause functional defects as determined by the parameters measured. These results strongly emphasize the importance of comprehensive functional studies when new variants are identified from obese subjects. It is hoped that, in future studies on MC4R mutations and obesity, the criteria of O'Rahilly and colleagues (27) be used consistently.

In summary, the results presented here suggest that the expression of MC4R mutation in causing obesity is not 100%, because variants identified from obese patients have normal functions, and variants identified from nonobese subjects result in loss-of-function. Furthermore, the relevance of MC4R variants in binge eating disorder needs additional investigation.

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References

- Gantz I, Fong TM 2003 The melanocortin system. *Am J Physiol* 284:E468–E474
- Niswender KD, Schwartz MW 2003 Insulin and leptin revisited: adiposity signals with overlapping physiological and intracellular signaling capabilities. *Front Neuroendocrinol* 24:1–10
- Fan W, Boston BA, Kesterson RA, Hruby VJ, Cone RD 1997 Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature* 385:165–168
- Thiele TE, van Dijk G, Yagaloff KA, Fisher SL, Schwartz M, Burn P, Seeley RJ 1998 Central infusion of melanocortin agonist MTHI in rats: assessment of c-Fos expression and taste aversion. *Am J Physiol* 274:R248–R254
- Kim MS, Rossi M, Abusnana S, Sunter D, Morgan DG, Small CJ, Edwards CM, Heath MM, Stanley SA, Seal LJ, Bhatti JR, Smith DM, Ghatei MA, Bloom SR 2000 Hypothalamic localization of the feeding effect of agouti-related peptide and α -melanocyte-stimulating hormone. *Diabetes* 49:177–182
- Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berke-meier LR, Gu W, Kesterson RA, Boston BA, Cone RD, Smith FJ, Campfield LA, Burn P, Lee F 1997 Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* 88:131–141
- Yaswen L, Diehl N, Brennan MB, Hochgeschwender U 1999 Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. *Nat Med* 5:1066–1070
- Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I, Barsh GS 1997 Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. *Science* 278:135–138
- Graham M, Shutter JR, Sarmiento U, Sarosi I, Stark KL 1997 Overexpression of Agt leads to obesity in transgenic mice. *Nat Genet* 17:273–274
- Yeo GS, Farooqi IS, Aminian S, Halsall DJ, Stanhope RG, O'Rahilly S 1998 A frameshift mutation in MC4R associated with dominantly inherited human obesity. *Nat Genet* 20:111–112
- Vaisse C, Clement K, Guy-Grand B, Froguel P 1998 A frameshift mutation in human MC4R is associated with a dominant form of obesity. *Nat Genet* 20:113–114
- Tao YX 2005 Molecular mechanisms of the neural melanocortin receptor dysfunction in severe early-onset obesity. *Mol Cell Endocrinol* 239:1–14
- O'Rahilly S, Farooqi IS, Yeo GS, Challis BG 2003 Minireview: human obesity—lessons from monogenic disorders. *Endocrinology* 144:3757–3764
- Lubrano-Berthelie C, Durand E, Dubern B, Shapiro A, Dazin P, Weill J, Ferron C, Froguel P, Vaisse C 2003 Intracellular retention is a common characteristic of childhood obesity-associated MC4R mutations. *Hum Mol Genet* 12:145–153
- Yeo GS, Lank EJ, Farooqi IS, Keogh J, Challis BG, O'Rahilly S 2003 Mutations in the human melanocortin-4 receptor gene associated with severe familial obesity disrupts receptor function through multiple molecular mechanisms. *Hum Mol Genet* 12:561–574
- Nijenhuis WA, Garner KM, VanRozen RJ, Adan RA 2003 Poor cell surface expression of human melanocortin-4 receptor mutations associated with obesity. *J Biol Chem* 278:22939–22945
- Tao YX, Segaloff DL 2003 Functional characterization of melanocortin-4 receptor mutations associated with childhood obesity. *Endocrinology* 144:4544–4551
- Jacobson P, Ukkola O, Rankinen T, Snyder EE, Leon AS, Rao DC, Skinner JS, Wilmore JH, Lonn L, Cowan Jr GS, Sjostrom L, Bouchard C 2002 Melanocortin 4 receptor sequence variations are seldom a cause of human obesity: the Swedish Obese Subjects, the HERITAGE Family Study, and a Memphis cohort. *J Clin Endocrinol Metab* 87:4442–4446
- Vaisse C, Clement K, Durand E, Hercberg S, Guy-Grand B, Froguel P 2000 Melanocortin-4 receptor mutations are a frequent and heterogeneous cause of morbid obesity. *J Clin Invest* 106:253–262
- Farooqi IS, Yeo GS, Keogh JM, Aminian S, Jebb SA, Butler G, Cheetham T, O'Rahilly S 2000 Dominant and recessive inheritance of morbid obesity associated with melanocortin 4 receptor deficiency. *J Clin Invest* 106:271–279
- Farooqi IS, Keogh JM, Yeo GS, Lank EJ, Cheetham T, O'Rahilly S 2003 Clinical spectrum of obesity and mutations in the melanocortin 4 receptor gene. *N Engl J Med* 348:1085–1095
- Donohoue PA, Tao YX, Collins M, Yeo GSH, O'Rahilly S, Segaloff DL 2003 Deletion of codons 88–92 of the melanocortin-4 receptor gene: a novel deleterious mutation in an obese female. *J Clin Endocrinol Metab* 88:5841–5845
- Sina M, Hinney A, Ziegler A, Neupert T, Mayer H, Siegfried W, Blum WF, Remschmidt H, Hebebrand J 1999 Phenotypes in three pedigrees with autosomal dominant obesity caused by haploinsufficiency mutations in the melanocortin-4 receptor gene. *Am J Hum Genet* 65:1501–1507
- Hebebrand J, Fichter M, Gerber G, Gorg T, Hermann H, Geller F, Schafer H, Remschmidt H, Hinney A 2002 Genetic predisposition to obesity in bulimia nervosa: a mutation screen of the melanocortin-4 receptor gene. *Mol Psychiatry* 7:647–651
- Branson R, Potoczna N, Kral JG, Lentjes KU, Hoehe MR, Horber FF 2003 Binge eating as a major phenotype of melanocortin 4 receptor gene mutations. *N Engl J Med* 348:1096–1103
- Gu W, Tu Z, Kleyn PW, Kissebah A, Duprat L, Lee J, Chin W, Maruti S, Deng N, Fisher SL, Franco LS, Burn P, Yagaloff KA, Nathan J, Heymsfield S, Albu J, Pi-Sunyer FX, Allison DB 1999 Identification and functional analysis of novel human melanocortin-4 receptor variants. *Diabetes* 48:635–639
- Farooqi IS, Yeo GS, O'Rahilly S 2003 Binge eating as a phenotype of melanocortin 4 receptor gene mutations. *N Engl J Med* 349:606–609; author reply 606–609
- Cummings DE, Clement K, Purnell JQ, Vaisse C, Foster KE, Frayo RS, Schwartz MW, Basdevant A, Weigle DS 2002 Elevated plasma ghrelin levels in Prader Willi syndrome. *Nat Med* 8:643–644
- Tao YX, Segaloff DL 2004 Functional characterization of melanocortin-3 receptor variants identify a loss-of-function mutation involving an amino acid critical for G protein-coupled receptor activation. *J Clin Endocrinol Metab* 89:3936–3942
- Chen C, Okayama H 1987 High-efficiency transfection of mammalian cells by plasmid DNA. *Mol Cell Biol* 7:2745–2752
- Srinivasan S, Lubrano-Berthelie C, Govaerts C, Picard F, Santiago P, Conklin BR, Vaisse C 2004 Constitutive activity of the melanocortin-4 receptor is maintained by its N-terminal domain and plays a role in energy homeostasis in humans. *J Clin Invest* 114:1158–1164
- Biebermann H, Krude H, Elsner A, Chubanov V, Gudermann T, Gruters A 2003 Autosomal-dominant mode of inheritance of a melanocortin-4 receptor mutation in a patient with severe early-onset obesity is due to a dominant-negative effect caused by receptor dimerization. *Diabetes* 52:2984–2988
- Hebebrand J, Geller F, Dempfle A, Heinzl-Gutenbrunner M, Raab M, Gerber G, Wermter AK, Horro FF, Blundell J, Schafer H, Remschmidt H, Herpertz S, Hinney A 2004 Binge-eating episodes are not characteristic of carriers of melanocortin-4 receptor gene mutations. *Mol Psychiatry* 9:796–800
- Hobbs HH, Russell DW, Brown MS, Goldstein JL 1990 The LDL receptor locus in familial hypercholesterolemia: mutational analysis of a membrane protein. *Annu Rev Genet* 24:133–170
- Welsh MJ, Smith AE 1993 Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. *Cell* 73:1251–1254