# **Deletion of Codons 88–92 of the Melanocortin-4 Receptor Gene: A Novel Deleterious Mutation in an Obese Female**

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Genetic and pharmacological studies have shown that the melanocortin-4 receptor (MC4R) is an important regulator of food intake and energy homeostasis. Consistent with these studies, several mutations of the MC4R gene have been identified as being associated with early-onset severe obesity. We report here the first in-frame deletion mutation of the MC4R gene ( $\Delta$ 88–92) in an obese female patient with onset of obesity

MELANOCORTINS ARE PEPTIDES derived from the posttranslational processing of proopiomelanocortin. Neural melanocortin receptors, including the melanocortin-3 receptor and melanocortin-4 receptor (MC4R), are activated by  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH). On binding  $\alpha$ -MSH, the MC4R is activated and it catalyzes the exchange of GDP for GTP on  $Gs\alpha$ , resulting in activation of adenylyl cyclase and generation of intracellular cAMP. The cDNA for the MC4R was originally cloned by degenerate PCR and the expression of the MC4R was found to be localized to various brain regions (1, 2). In the past few years, pharmacological and genetic studies have uncovered important roles of MC4R in regulating food intake and energy homeostasis (reviewed in Ref. 3), one of which includes mediating the actions of leptin. The overall effects mediated by MC4R are stimulation of anorexigenic and inhibition of orexigenic neural pathways.

In 1998 two groups (4, 5) independently reported frameshift mutations in the MC4R gene in patients with early-onset severe obesity. Functional characterization of the mutant MC4Rs confirmed that these mutant receptors are not functional (6). Allelic variants of MC4R have since been reported (7–19), including frameshift, nonsense, and missense mutations scattered throughout the coding sequence of the MC4R gene. We report a novel in-frame deletion mutation ( $\Delta$ 88–92) identified from a female patient in Iowa. Functional studies demonstrated that this mutant receptor is expressed well on the cell surface; however, it cannot bind the hormone  $\alpha$ -MSH and therefore cannot elicit intracellular signaling. Whereas the majority of MC4R loss-of-function mutants that have been characterized thus far are inactivating because of their at less than 5 yr of age. Functional analysis revealed that the mutant receptor is expressed well on the cell surface but completely devoid of ligand binding and cAMP generation in response to agonist stimulation. We conclude that this novel mutation is the cause of obesity of this patient. (*J Clin Endocrinol Metab* 88: 5841–5845, 2003)

intracellular retention and reduced cell surface expression (17, 19–21), the  $\Delta$ 88–92 mutant is one of the few loss-of-function MC4R mutants that is expressed well on the cell surface but is impaired in its ability to bind agonist.

## **Subjects and Methods**

# Subjects

Subjects were recruited from the Bariatric Surgery Clinic at the University of Iowa Hospitals and Clinics, at which they were undergoing evaluation for surgical treatment of obesity. Written informed consent was obtained from all subjects, and the research protocol was approved by the Institutional Review Board of the Roy J. and Lucille A. Carver College of Medicine at the University of Iowa.

The subjects screened for MC4R mutations were a subset of a larger study population, currently consisting of approximately 600 obese adult probands, and from approximately 20% of the probands, we have recruited 460 relatives. The probands are undergoing screening for mutations and polymorphisms of candidate genes, and the data are analyzed in association studies. The families will be included in linkage studies. A total of 237 probands were screened for MC4R mutations, and the ethnic background of this group was 97% non-Hispanic White. There were 70 men [aged 17–59 yr, body mass index (BMI) range 31.59–87.85 kg/m<sup>2</sup>] and 167 women (aged 16–63 yr, BMI range 32.54–80.54 kg/m<sup>2</sup>).

# Mutation screening and identification

Genomic DNA samples were prepared from circulating leukocytes of markedly obese adults and screened for MC4R mutations using a method adapted from the one described by Hinney *et al.* (8). Briefly, the coding region of MC4R was amplified in two overlapping PCR segments of 615 (5') and 622 (3') bp. The 615-bp product was digested with the endonuclease *Bsu*36I giving pieces of 181 and 434 bp, and with *SspI* giving pieces of 396 and 219 bp. The 622-bp fragment was digested with *MspI*, giving pieces of 232, 222, and 168 bp. These digested fragments were subjected to single-strand conformational polymorphism (SSCP) analysis. For SSCP, digested PCR products were heat denatured at 95 C for 5 min and then separated on 4% polyacrylamide gels for 2.5 h at 20 W and detected by silver staining.

The 5' DNA region from position -36 to +579 of the MC4R gene from one subject gave rise to an abnormal conformer as detected by SSCP. This region was reamplified using a phosphorylated 5' primer and cloned into the unidirectional TA cloning vector pCR3.1Uni (Invitrogen, Carls-

Abbreviations: BMI, Body mass index; HEK, human embryonic kidney; MC4R, melanocortin-4 receptor;  $\alpha$ -MSH,  $\alpha$ -melanocyte stimulating hormone; NDP-MSH, [Nle<sup>4</sup>, p-Phe<sup>7</sup>]- $\alpha$ -MSH; SSCP, single-strand conformational polymorphism; Wa/BSA, Waymouth's MB752/1 containing BSA; wt, wild-type.

bad, CA). Inserts of two sizes were identified, and each was sequenced in both directions by automated sequencing performed by the DNA Core of the Diabetes and Endocrinology Research Center at the University of Iowa.

# Phenotype of subject with mutant MC4R

The subject was a 42-yr-old nondiabetic Caucasian female of Italian/ Irish descent who exhibited onset of obesity at less than 5 yr of age. Her height was 157.5 cm, weight was 126.4 kg, BMI was 51 kg/m<sup>2</sup>, and waist:hip ratio was 0.92. She reported failure of multiple weight loss therapies including vertical banded gastroplasty. She also reported that weight gain occurred with intake over 1200 kcal/d, even with an exercise regimen. She reported intense cravings for carbohydrate-rich foods. She had a past history of T<sub>4</sub> replacement therapy, cortisol replacement therapy, and evaluations for Cushing's syndrome. She had self-reported hirsutism, acanthosis nigricans, and menstrual irregularities. She was not available for further phenotypic characterization. Both of her parents were 160 cm tall with a history of obesity; her father had type 2 diabetes mellitus. Upon contacting this subject on several occasions to obtain additional information, there was no response. In keeping with institutional review board rules and regulations, no further contact was sought.

#### Construction of MC4R cDNA constructs

A cDNA construct encoding wild-type (wt) human MC4R in pcDNA3.1 was generously provided by Dr. I. Gantz (University of Michigan, Ann Arbor, MI). To aid in the localization of MC4R, a c-myc epitope tag was inserted at the N terminus and is referred to herein as myc-MC4R. This was accomplished using QuikChange site-directed mutagenesis kit (Strategene, La Jolla, CA). Preliminary experiments demonstrated that the addition of c-myc epitope tag at the N terminus does not affect the ligand binding or signaling properties (data not shown). The in-frame deletion  $\Delta 88-92$  was introduced into myc-MC4R by QuikChange site-directed mutagenesis kit as well. The entire coding region of each construct was sequenced to ensure that the mutation was correct and that no errors were inadvertently introduced.

# Cells and transfection

Human embryonic kidney (HEK)293 cells were cultured at 5% CO2 in DMEM containing 50  $\mu$ g/ml gentamicin, 10 mM HEPES, and 10% newborn calf serum. Cells were plated on gelatin-coated Corning plasticware. Transfections were performed using the calcium precipitation method (22) using 4  $\mu$ g plasmid in 2.0 ml per 35-mm dish. To establish stably transfected cells, cells were selected in medium containing 200  $\mu g/ml$  geneticin.

## Signaling properties of the mutant receptor

Two methods were used to assess the signaling properties of the mutant receptor. In one method, HEK293 cells were transiently transfected with wt or mutant MC4R in pcDNA3.1. The response to increasing concentrations of  $\alpha$ -MSH was assessed by cotransfection with a cAMPresponsive reporter plasmid and measuring luciferase activity (19). Data

FIG. 1. Identification of the  $\Delta 88-92$ MC4R mutation. The mutation in the MC4R gene of the subject was detected by SSCP gel analysis (A) and direct sequencing of the PCR fragment (B). In A, s refers to the 100-bp standard, u refers to the uncut PCR product, and m refers to the mutant pattern of a double band.



were normalized by cotransfection of an internal control plasmid, which constitutively expresses Renilla luciferase.

In another method, intracellular cAMP production in response to a superpotent agonist, [Nle<sup>4</sup>, D-Phe<sup>7</sup>]-α-MSH (NDP-MSH) (obtained from Phoenix Pharmaceuticals, Belmont, CA), was measured directly by RIA. Briefly, HEK293 cells were plated and transfected as described above. On the day of the experiment, cells were washed twice with warm Waymouth's MB752/1 containing 1 mg/ml BSA (Wa/BSA), and 1 ml of fresh Wa/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 another hour. Then the cells were placed on ice, media were aspirated, and 0.5 N perchloric acid containing 180  $\mu$ g/ml theophylline was added. Intracellular cAMP levels were measured using RIA. All determinations were performed in triplicate. EC<sub>50</sub> and maximal response values were calculated using DeltaGraph (Monterey, CA) software.

#### *Hormone binding studies*

Competitive binding assay was performed on intact cells. Iodinated NDP-MSH was obtained from Perkin-Elmer Life Sciences (Boston, MA). Cells were plated and transfected as described above. On the day of the experiment, cells were washed twice with warm Wa/BSA, and fresh Wa/BSA was added. Then 100,000 cpm of  $^{125}$ I-NDP-MSH in 50  $\mu l$  was added to each well, with either buffer alone or different concentrations of cold NDP-MSH, and incubated at 37 C for 1 h. Then cells were washed with cold Hank's balanced salt solution containing 1 mg/ml BSA twice and dissolved in 100 µl 0.5 N NaOH, collected using cotton swabs, and counted in a  $\gamma$ -counter. Binding capacity and IC<sub>50</sub> were calculated using DeltaGraph software.

### Confocal imaging of stably transfected cells

The day before the experiment, HEK293 cells stably expressing wt or  $\Delta 88-92$  myc-MC4R were plated onto lysine-coated slides (Biocoat cellware, Falcon, Bedford, MA). All solutions and procedures were at room temperature. On the day of the experiment, cells were washed three times with filtered PBS-IH (137 mм NaCl, 2.7 mм KCl, 1.4 mм KH<sub>2</sub>PO<sub>4</sub>, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4). Cells were then incubated with blocking solution (5% BSA in PBS-IH) for 1 h and incubated 1 h with fluorescinconjugated monoclonal antibody 9E10 (Affinity Bioreagents, Golden, CO) diluted 1:100 in PBS-IH containing 1 mg/ml BSA. The cells were washed five times with PBS-IH, allowed to dry, and covered with Vectashield Mounting Media (Vector Laboratories, Burlingame, CA) and a coverslip. Images were collected with a 1024 laser confocal microscope (Bio-Rad Laboratories, Hercules, CA).

## Results

Adults with early-onset severe obesity were screened for potential mutations of the MC4R gene. One subject whose MC4R gene exhibited an abnormal conformer on SSCP analysis, a double band derived from the 5' SspI fragment (Fig. 1A) was found. This segment was sequenced, and an inframe deletion of 15 bp was identified in the smaller of the



two cloned inserts (Fig. 1B). The deletion spanned codons 88–92 (Val-Ala-Asp-Met-Leu), which are predicted to lie within the second of the seven-transmembrane domains of the receptor. The larger insert had the normal wt sequence.

Initial functional characterization of the  $\Delta 88-92$  MC4R, using a reporter gene system, revealed a lack of signaling in response to  $\alpha$ -MSH, the native ligand (Fig. 2A). To explore this further, we examined whether the in-frame deletion mutant  $\Delta 88-92$  was responsive to the superpotent analog NDP-MSH stimulation with increased cAMP production. As shown in Fig. 2B, cells expressing wt *myc*-MC4R responded to NDP-MSH stimulation with a robust increase in cAMP production, with a maximal response 50- to 90-fold over basal levels and with an EC<sub>50</sub> of 0.2 ± 0.13 nM (mean ± sEM of three experiments). In contrast, cells transfected with  $\Delta 88-92$  MC4R MC4R did not respond with any detectable increases in intracellular cAMP to NDP-MSH at concentrations as high as 1  $\mu$ M.

The lack of response of the  $\Delta 88-92$  MC4R to agonist stimulation could reflect an intrinsic impairment in the ability of the mutant receptor to activate Gs in response to agonist binding. It is also possible, however, that the lack of response may be due to a defect in the ligand binding properties of the



FIG. 2. Signaling properties of wt and mutant MC4Rs. A, Functional analysis of wt and mutant MC4Rs expressed transiently in HEK293 cells using a cAMP-responsive luciferase assay. Data are expressed as the fold induction of luciferase activity in response to  $\alpha$ -MSH stimulation. Each point represents the mean  $\pm$  SEM of four independent experiments performed in quadruplicate. B, Accumulation of intracellular cAMP in HEK293 cells transfected, stimulated with wt and  $\Delta 88-92$  MC4Rs. Cells were transfected, stimulated with different concentrations of NDP-MSH, and intracellular cAMP measured as described in Subjects and Methods. Results are expressed as the mean  $\pm$  SEM of triplicate determinations of one experiment. All experiments were performed three times and similar results were obtained.



FIG. 3. Competitive binding of radiolabeled NDP-MSH to wt and mutant MC4Rs. HEK293 cells were transiently transfected and binding assays were performed as described in *Subjects and Methods*. Cells were incubated with a trace concentration of <sup>125</sup>I-NDP-MSH and increasing concentrations of unlabeled NDP-MSH. Results are expressed as the mean  $\pm$  SEM of triplicate determinations within one experiment. All experiments were performed three times and similar results were obtained.



FIG. 4. Confocal imaging of wt and mutant *myc*-MC4Rs. HEK293 cells stably expressing wt or  $\Delta 88-92$  *myc*-MC4Rs were stained with fluorescein-conjugated anti-*myc* monoclonal antibody and imaged by confocal microscopy.

mutant. Therefore, we performed competitive binding assays in intact cells to measure the number of cell surface MC4Rs and their affinity for NDP-MSH. As shown in Fig. 3, cells expressing wt MC4R bound NDP-MSH with an IC<sub>50</sub> of  $6.21 \pm 1.77$  nm (mean  $\pm$  sem of three independent experiments), whereas  $\Delta 88$ –92 MC4R MC4R displayed no specific binding of NDP-MSH.

To determine whether the inability of  $\Delta 88-92$  MC4R to bind NDP-MSH was due to a defect in the ligand binding properties of the mutant receptor or to the lack of expression of the mutant receptor at the cell surface, we performed confocal imaging of intact cells stably expressing the wt or  $\Delta 88-92$  myc-MC4Rs. The data presented in Fig. 4 show that both wt and  $\Delta 88-92$  myc-MC4Rs are expressed well on the cell surface, suggesting that the lack of hormone binding by the  $\Delta 88-92$  mutant MC4R is due to impairment in ligand binding *per se*.

# Discussion

In this study, we report a novel inactivating mutation  $(\Delta 88-92)$  of the MC4R gene in a female subject with earlyonset severe obesity. The patient's onset of obesity was before she was 5 yr old. She had hyperphagia, especially for carbohydrate-rich foods, and failed in multiple weight loss therapies. She was only one of 240 subjects in our study who had a mutation of MC4R using the SSCP method described above. This is a prevalence much lower than expected among obese Caucasians. This lower prevalence could be due to the specific genetic background of the population and/or the use of a screening method that lacked the sensitivity to detect some of the previously described single-base mutations of the gene.

This is the first in-frame deletion mutation reported for the MC4R gene. Extensive functional analyses were performed on the mutant MC4R expressed heterologously in HEK293 cells. We show that the mutant receptor is expressed well on the cell surface (Fig. 4), suggesting that the mutant receptor is transcribed, translated, and processed normally. Therefore, in the patient, who is heterozygous for this mutation, the mutant and wt alleles would be predicted to be expressed at equimolar concentrations. Hence, this genotype is likely to result in obesity because of haploinsufficiency, consistent with previous findings (23). However, we cannot rule out the possibility that the mutant receptor might exert dominant negative effect on the wt receptor. Because the major downstream effects of ligand binding to the central nervous system MC4R are activation of neural pathways that result in the inhibition of feeding, either haploinsufficiency of the normal receptor or a dominant negative effect of the mutant receptor would lead to increased feeding.

Although the mutant receptor is expressed well on the cell surface, it is completely devoid of ligand binding (Fig. 3) and agonist-stimulated signaling as measured using two different methods (Fig. 2). Recently several groups, including ours, have reported the functional characterizations of the known MC4R mutations associated with obesity (17, 19–21). From these studies, misfolding and intracellular trapping of the mutant receptors emerged as the primary defect of MC4R mutations associated with obesity. Only a few mutants have been shown to be expressed on the cell surface but defective in agonist binding. They include I137T (7), N97D, L106P, I125K (19), and the mutation reported here. Of particular relevance to the results of the naturally occurring  $\Delta 88-92$ MC4R mutant described herein is a MC4R mutant of laboratory design by Yang et al. (24) in which Asp90 was mutated to Ala. Analyses of the binding properties of the mutant showed there were 10- and 3-fold decreases in the binding affinities of NDP-MSH and  $\alpha$ -MSH, respectively, with no changes in the maximal binding of each. This mutant was also found to be unresponsive to  $\alpha$ -MSH or NDP-MSH stimulation in terms of cAMP accumulation. Whether the lack of response was due to the decreased binding affinity or impairment in agonist-stimulated Gs coupling is not clear. In either case, these studies show Asp90 to be important for agonist binding. Further studies will be needed to identify the exact roles of residues 88-92 in agonist binding and signaling.

Of the numerous naturally occurring mutations in G protein-coupled receptors that cause human diseases, in-frame deletion occurs rarely, compared with missense and frameshift mutations. Both activating and inactivating mutations caused by in-frame deletions have been reported. Examples of activating mutations include the calcium-sensing receptor (25) and the TSH receptor (26). Inactivating mutations that result from in-frame deletions include the V2 vasopressin receptor (27), the lutropin receptor (28), and rhodopsin (reviewed in Ref. 29). Some of these inactivating mutations result from misfolding, most likely because of disturbances in the packing of helices (30).

In summary, we have identified a novel in-frame deletion mutation ( $\Delta 88-92$ ) in the MC4R gene that results in a binding defect, causing early-onset severe obesity.

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