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

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Mutations in the melanocortin-4 receptor (MC4R) gene are the most frequent monogenic causes of severe obesity. Most mutations have been described as heterozygous with loss of function, suggesting that haploinsufficiency is the most likely mechanism of dominant inheritance. We detected a heterozygous mutation, D90N, in a patient with severe early-onset obesity. Functional characterization of the mutant receptor revealed normal cell-surface expression and binding properties but loss of signal transduction activity. In coexpression studies of wild-type (WT)-MC4R and D90N, the mutant receptor had a dominant-negative effect on WT-receptor function. Further investigation of this effect with sandwich enzyme-linked immunosorbent assays and fluorescence resonance energy transfer studies showed that the WT-MC4R and the D90N mutant form homodimers and heterodimers. We hypothesize that the dominant-negative effect of the D90N mutation is caused by a functionally altered WT-MC4R/D90N receptor heterodimer. These findings necessitate the reinvestigation of other heterozygous MC4R missense mutations to discriminate between haploinsufficiency and a dominant-negative effect. The finding of receptor dimerization highlights a more complex hypothalamic signaling network governing the regulation of body weight. Diabetes 52:2984–2988, 2003

RESEARCH DESIGN AND METHODS

Several genes that play a role in monogenic forms of obesity have been identified. In humans, mutations or disruptions of genes of the leptin-melanocortin pathway (1–4) were identified as rare causes for recessively inherited obesity. The gene for the melanocortin-4 receptor (MC4R) is an exception because mutations were detected in 3–5% of the studied population of severely obese patients (5,6). The MC4R belongs to the large superfamily of G protein–coupled receptors (GPCRs) (7) and is activated by proopiomelanocortin-derived peptides. The activated receptor couples to the Gs/adenyl cyclase system, resulting in decreased food intake and increased energy expenditure (8). Inactivating mutations of the MC4R gene were found to be mostly inherited in an autosomal-dominant manner. The molecular mechanism of this dominant manifestation of obesity as a result of heterozygous loss-of-function MC4R mutations is still unclear. However, a clear dosage effect on body weight has been shown in heterozygous versus homozygous MC4R knockout mice (9). Therefore, for the manifestation of an obese phenotype in heterozygous loss-of-function mutation carriers, haploinsufficiency is the widely accepted hypothesis (10,11). We report a heterozygous D90N mutation resulting in complete loss of function but normal cell-surface expression and ligand-binding affinity. Cotransfection studies revealed a dominant-negative effect on wild-type (WT)-receptor function, and further investigation demonstrated homomultimerization for the WT-MC4R and the mutant D90N receptor. Our findings on MC4R dimerization lay the foundation for a mechanistic understanding of a dominant-negative effect imparted by particular heterozygous MC4R mutations inherited in an autosomal-dominant manner.
levels in the oral glucose tolerance test, insulin secretion is inappropriately high resulting in an homeostasis model assessment resistance index of <0.3.

Mutational screening and functional characterization of WT and mutant MC4Rs. The entire coding region of the MC4R gene was amplified as one fragment and sequenced in three overlapping fragments. For investigating the frequency of the D90N mutation in normal-weight and obese populations, PCR products were generated with a degenerated primer introducing an EcoRV site in the absence of the mutation. Restricted PCR products were separated on a 2.5% agarose gel.

The WT-MC4R and the D90N mutation were amplified from the patient’s DNA and inserted into the expression vector. For functional studies, mutant and WT receptors were transiently transfected into COS-7 cells, and cAMP accumulation assays were performed as described previously (13).

For cell-surface binding studies, transfected cells were incubated overnight at 4°C with increasing amounts of NDP-α-melanocyte-stimulating hormone (MSH) and 125I-labeled NDP-α-MSH. After washing and solubilization, specifically bound 125I-NDP-α-MSH was measured. For investigating cell-surface expression, WT-MC4R and D90N were hemagglutinin (HA) tagged at the NH2-terminus, and cell-surface enzyme-linked immunosorbent assays (ELISAs) were performed as previously described (14).

**Dimerization studies**

**Sandwich ELISA.** For investigating receptor dimerization, a sandwich ELISA was performed with NH2-terminally tagged HA (N-HA) and COOH-terminal FLAG-tagged WT-MC4R (C-FLAG) and D90N-MC4R. As controls, the WT-MC4R-N-HA was transfected alone or after cotransfection with the thyrotropin receptor (TSHR). After transfection, cells were washed, harvested, and solubilized. After removal of cell debris, the supernatants were incubated with FLAG antibody–coated 96-well plates for 2 h. Detection of the HA epitope was performed in triplicate as previously described (14).

**Fluorescence resonance energy transfer.** The cDNAs of WT and mutant receptors were sub cloning into pcDNA3.1 containing cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP). The receptor-operated cation channel TRPC6 COOH-terminal fused to either CFP or YFP (15) served as a control. Experiments were carried out with a Polychrome IV monochromator and an IMAGO II pellicer-cooled charged-coupled device camera (TILL Photonics, Planegg, Germany) coupled to an inverted Olympus (New Hyde Park, NY) IX70 microscope as described recently (15).

**RESULTS**

**Functional characterization of WT-MC4R and D90N mutant.** We investigated the MC4R gene in a cohort of patients with early-onset obesity. In these patients, we detected a new heterozygous mutation in the MC4R gene resulting in an exchange of D90 to N located in the predicted transmembrane domain 2 that is highly conserved throughout the melanocortin receptor family (Table 1). The mutation was detected only in the patient; the lean mother did not carry the mutation. The father was obese, with a body weight of 120 kg and a height of 190 cm. He died of meningitis 8 years prior. A DNA sample from his chromosomes of obese patients were screened. The mutation was found in none of them, indicating that the D90N mutation is very rare.

For functional characterization, WT and mutant receptors were transiently expressed in COS-7 cells. Expression of the D90N-MC4R mutant did not result in measurable cAMP accumulation after stimulation with NDP-α-MSH (Fig. 1A). In cell-surface binding studies, the functionally inactive D90N mutant was shown to be expressed on the cell surface similarly to the WT receptor and displayed a high affinity for the agonist NDP-α-MSH indistinguishable from the WT receptor (Fig. 1B). To investigate agonist-independent cell-surface expression, we performed a cell-

**Table 1**

<table>
<thead>
<tr>
<th>Alignment of different melanocortin receptors and different MC4R species</th>
<th>TM2</th>
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<tbody>
<tr>
<td>hMC1R</td>
<td>CCLA1SDLLVS</td>
</tr>
<tr>
<td>hMC2R</td>
<td>CSLA1SDMLGS</td>
</tr>
<tr>
<td>hMC3R</td>
<td>CSVAADMVLVS</td>
</tr>
<tr>
<td>hMC4R</td>
<td><strong>CSLAADMVLVS</strong></td>
</tr>
<tr>
<td>hMC4R-D90N</td>
<td>CSVAADMVLVS</td>
</tr>
<tr>
<td>BovMC4R</td>
<td>CSVAADMVLVS</td>
</tr>
<tr>
<td>mMC4R</td>
<td>CSVAADMVLVS</td>
</tr>
<tr>
<td>ratMC4R</td>
<td>CSVAADMVLVS</td>
</tr>
<tr>
<td>susMC4R</td>
<td>CSVAADMVLVS</td>
</tr>
<tr>
<td>hMC5R</td>
<td><strong>CSLAvAdmLvS</strong></td>
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A part of the sequence transmembrane domain 2 was aligned for human melanocortin receptors 1–5, additionally for mouse MC4R (mMC4R), pig MC4R (susMC4R), and bovine MC4R (bovMC4R) and the mutant human MC4R-D90N with the program Omega. The sequence of the human MC4R is shown in bold; the patient’s mutation is shown in italics. The consensus sequence indicates a complete homology by capital letters and incomplete amino acid conservation by lowercase letters. The patient’s mutation was not taken into account for the consensus sequence.

**Cotransfection studies.** We asked whether the D90N mutant affected WT receptor function. Therefore, we performed cotransfection studies and measured agonist-induced cAMP accumulation. In COS-7 cells expressing D90N- and the WT-MC4R, we observed that EC50 values were shifted by nearly 2 orders of magnitude toward higher agonist concentrations as compared with cells solely transfected with WT-MC4R cDNA or to cells transfected with WT and the complete loss-of-function mutation Y35X as well as cells expressing the WT-MC4R and the TSHR (Fig. 1C). Furthermore, we tested whether the amount of transfected cDNA of the mutant receptor had an influence on WT function. Thus, we cotransfected WT-plasmid cDNA with increasing amounts of cDNA coding for the mutant receptor. Suppression of agonist-induced cAMP stimulation mediated by the WT-receptor was clearly dependent on the amount of mutant cDNA transfected (Fig. 1D). These results indicate an effect of the D90N mutant on WT receptor function that can be classified as dominant negative. Cotransfection studies for determination of cell-surface expression and binding properties revealed no differences between cells expressing the WT or the mutant receptor alone or coexpressing both receptors, indicating no effect of the mutant on WT cell-surface expression (Fig. 1B).

**Dimerization of melanocortin receptors.** To understand the molecular mechanism of the transdominant effect of the D90N mutant on WT receptor function, we speculated that the observed effect could be due to receptor dimerization. For a large variety of GPCRs, receptor di- or oligomerization has been reported (16), but to date, no information on melanocortin receptors is available. We performed a sandwich ELISA. In this assay, dimerization can be visualized if the C-FLAG tag receptor is fixed to the FLAG antibody–coated 96-well plate and is dimerized with an NH2-terminally tagged receptor that
was subsequently detected by an immunologic reaction. Cells cotransfected with MC4R-N-HA and MC4R-C-FLAG or with differentially tagged WT-MC4R and the D90N mutant as well as with D90N-HA and D90N-FLAG showed a significant increase in optical density compared with cells transfected with the WT-MC4R-N-HA alone or cells cotransfected with MC4R-N-HA and TSHR-C-FLAG. These findings demonstrate the formation of homodimers composed of WT-MC4R or D90N mutants and in addition of heterodimers consisting of WT-MC4R/D90N (Fig. 2).

To demonstrate direct protein-protein interactions of melanocortin receptors in living cells, we generated COOH-terminal fusion proteins of WT-MC4R and D90N-MC4R with CFP or YFP. Subsequently, by fluorescence resonance energy transfer (FRET), we assessed the proximity of MC4Rs coexpressed in HEK 293 cells and differentially tagged on their COOH-terminals. The quantitative FRET signal was measured under static conditions by recording the increase in donor (CFP) emission during selective photobleaching of the acceptor (YFP) at 512 nm (Fig. 3A). The percentage increase of donor emission at 480 nm after acceptor bleach was taken as a measure of steady-state FRET efficacy. The increase in donor fluorescence proved direct protein-protein interactions and thus the existence of monomeric receptor dimers consisting of MC4R/MC4R and D90N-MC4R/D90N-MC4R, as well as heterodimers composed of WT-MC4R and the loss-of-function D90N mutant. A summary of our experiments is shown in Fig. 3B. In addition to WT-MC4R and D90N-MC4R homomultimers, heteromultimer formation be-

FIG. 2. Receptor dimerization as assessed by sandwich ELISA. Cells cotransfected with differentially tagged MC4Rs were solubilized overnight and incubated in FLAG antibody–coated 96-well plates. In case of receptor dimerization, determination of the NH2-terminal HA epitope is indicated by an increase in optical density as shown for investigated cotransfection of N-HA-MC4R/C-FLAG-MC4R indicated as WT + WT, N-HA-MC4R/D90N-CFLAG (D90N + WT), N-HA-D90N/C-FLAG-MC4R (D90N + WT), and agonist-induced cAMP accumulation was determined in the presence of 10 mmol/l NDP-α-MSH (D). Statistical significance between EC50 values of WT-MC4R and WT-MC4R/TSHR against WT-MC4R/D90N was tested with the ANOVA test and resulted in a P < 0.05.
tween WT and mutant receptors was observed, thus further substantiating our conclusions drawn from the sandwich ELISA approach. The known ability of the receptor-activated cation channel TRPC6 to homomultimerize served as a positive control (Fig. 3B). However, no direct interaction was noted between the hexahelical cation channel and MC4Rs, highlighting that multimerization of MC4Rs is not an unspecific event taking place between unrelated membrane proteins.

**DISCUSSION**

In a patient with severe early-onset obesity, we detected a novel heterozygous D90N mutation. Functional characterization of the D90N-MC4R mutant resulted in a nearly complete loss of function in terms of Gs/adenylyl cyclase activation. It is interesting that the loss-of-function mutant is highly expressed at the cell surface and binds agonist with the same affinity as the WT receptor. High-affinity agonist binding of the D90N-MC4R mutant in conjunction with loss-of-functional activity may reduce the amount of agonist available for WT receptor function. Coexpression of the D90N mutant with the WT receptor gave rise to EC_{50} values for agonist-dependent cAMP accumulation that were shifted toward higher agonist concentrations than an expected factor of 2 if agonist capture was the underlying mechanism. So far, a dominant-negative effect was observed only in coexpression of WT GPCRs with inactivating mutants that led to truncation of the receptor, resulting in intracellular retention of the WT/mutant complex (17,18). During the past few years, the concept emerged that oligomerization is important for GPCR function (15,19,20).

Dimerization has not been demonstrated for the melanocortin receptor family. Therefore, we differentially tagged the WT-MC4R and the mutant and performed sandwich ELISA and FRET studies. In both systems, we were able to show that the WT-MC4R forms homodimers. Notably, the WT-MC4R and the mutant D90N receptor...
were also able to form heterodimers. Dimerization of MC4R and TSHR could not be shown, indicating that the interaction of WT-MC4R with the mutant receptor is a specific effect caused by protein-protein interaction that results in a dominant-negative effect. We conclude that dimerization of WT and mutant MC4Rs may be an underlying structural prerequisite to cause the profound shift of agonist potency observed in cotransfection studies.

In the present study, we propose a mechanistic explanation for why a particular heterozygous inactivating MC4R mutation led to the development of extreme obesity by a dominant-negative effect. So far, a dominant-negative effect was excluded at least for some loss-of-function MC4R mutations (5,6). However, we cannot exclude that other factors besides the mutant MC4R gene are responsible for the severe phenotype in the patient. Therefore, careful reinvestigation of other identified heterozygous MC4R missense mutations will help to assess the contribution of haploinsufficiency or dominant-negative suppression on the manifestation of the obese phenotype. This first report of receptor dimerization within the melanocortin system stresses an increasing complexity of signaling networks within the hypothalamic anorexigenic pathways.

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REFERENCES