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Functional Characterization of Melanocortin-3 Receptor Variants Identify a Loss-of-Function Mutation Involving an Amino Acid Critical for G Protein-Coupled Receptor Activation

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Although melanocortin-4 receptor mutations are the cause of the most common monogenic form of obesity, the involvement of the melanocortin-3 receptor (MC3R) in the pathogenesis of obesity is unknown. Earlier studies failed to identify any mutations in obese patients except for the identification of two variants (K6T and I81V) that likely represent polymorphisms. However, a potential mutation (I183N) was recently reported from patients having high-fat contents. We report here the functional characterization of these variants. We show that K6T and I81V have ligand binding and signaling properties similar to wild-type (wt) MC3R, indicating that they are indeed polymorphisms. However, the other variant, I183N, completely lacks signaling in response to agonist stimulation, although it binds ligand with normal affinity and with only slightly decreased capacity. Coexpression of the wt and I183N MC3Rs showed that I183N does not exert dominant-negative activity on wt MC3R. These results provide supporting evidence for the hypothesis proposed in the original case report that MC3R mutation might be a genetic factor that confers susceptibility to obesity, likely due to haploinsufficiency. Further mutations at I183 revealed a discrete requirement for I183 in agonist-induced MC3R activation. The corresponding residue is also important for agonist-induced human melanocortin-4 receptor and lutropin receptor activation. In summary, we identify a residue that is critical for activation of G protein-coupled receptors. (*J Clin Endocrinol Metab* 89: 3936–3942, 2004)

MELANOCORTINS ARE PEPTIDE hormones derived from the posttranslational processing of the precursor proopiomelanocortin (POMC). By tissue-specific processing, POMC gives rise to α-, β-, and γ-melanocyte stimulating hormone (MSH) and ACTH (1). The melanocortins bind to five known melanocortin receptors (MCRs), named MC1R to MC5R according to the sequence of their cloning (reviewed in Ref. 2). All five MCRs are members of the G protein-coupled receptor (GPCR) superfamily, coupling primarily to Gs and, therefore, increasing cAMP production upon agonist stimulation.

The MC3R was cloned by degenerate PCR, and initially its function was unknown (3, 4). *In situ* hybridization studies demonstrated that the MC3R is expressed in various brain regions including the cortex, thalamus, hippocampus, and hypothalamus (3, 4). It is also expressed in the placenta and gut (3). It has been localized to human chromosome 20q13 by florescence *in situ* hybridization (5).

Recently the physiological role of the MC3R was revealed by mouse gene targeting studies. These studies demonstrated nonredundant roles for the MC3R and MC4R in energy homeostasis. Whereas the MC4R primarily regulates food intake, the MC3R does not affect food intake or energy expenditure (6–8). Despite normal or decreased food intake and normal energy expenditure, MC3R knockout mice exhibited increased fat mass (approximately twice that of wild-type littermates) due to increased feed efficiency (7, 8). Mice lacking both the MC3R and MC4R showed exacerbated obesity, compared with MC3R or MC4R single gene knockout mice, again indicating that the two neural MCRs regulate different aspects of energy homeostasis (7).

Human genetic studies suggested that loci encompassing the MC3R gene on chromosome 20q are associated with obesity and non-insulin-dependent diabetes mellitus (NIDDM) (9–13). For example, a locus on 20q13 was associated with body fat including percent fat, fat mass, and sc fat (9). However, several screening studies have failed to identify any mutations in the MC3R gene from patients with morbid obesity and NIDDM except for two variants, K6T and I81V, which were also found in normal control subjects, and therefore likely represent polymorphisms (14–17). Very recently a group from Singapore (18) reported two patients (father and daughter) who each harbor a potential mutation in the MC3R gene resulting in an I183N substitution. However, no functional data were provided to verify whether this variant is indeed a pathogenic mutation. The present studies were undertaken, therefore, to functionally characterize the K6T, I81V, and I183N variants. Our studies confirm that the I183N variant does indeed represent a signaling defective mutant of the MC4R. Significantly, the mutated Ile represents a highly conserved residue in rhodopsin-like GPCRs that we

Abbreviations: CG, Chorionic gonadotropin; GPCR, G protein-coupled receptor; h, human; LHR, human lutropin receptor; MCR, melanocortin receptor; MSH, melanocyte stimulating hormone; NDP-MSH, [Nle^4,D-Phe^7]-αMSH; NIDDM, non-insulin-dependent diabetes mellitus; POMC, proopiomelanocortin; TM, transmembrane; wt, wild-type.

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show plays an essential role in agonist-stimulated GPCR activation.

Materials and Methods

Hormones and supplies

[Nle^4,D-Phe^7]-αMSH (NDP-MSH), a superpotent agonist of αMSH, was obtained from Phoenix Pharmaceuticals (Belmont, CA). Iodinated NDP-MSH was purchased from PerkinElmer Life Sciences (Boston, MA). Highly purified human (h) chorionic gonadotropin (hCG) was purchased from the National Institute of Diabetes and Digestive and Kidney Diseases (Dr. A. F. Parlow, National Hormone and Pituitary Program). hCG was iodinated as described (19). Crude hCG used for determining nonspecific binding was purchased from Sigma (St. Louis, MO). Tissue culture plasticwares were obtained from Corning (Corning, NY). Cell culture media and reagents were obtained from Invitrogen (Carlsbad, CA).

Site-directed mutagenesis of MC3R, MC4R, and human lutropin receptor (LHR)

Wild-type (wt) MC3R and MC4R were kindly provided by Dr. I. Gantz (University of Michigan, Ann Arbor, MI). Mutations were introduced into wt MC3R and MC4R by QuikChange site-directed mutagenesis kit (Strategene, La Jolla, CA) (20, 21). The LHR cDNA was obtained from Ares Advanced Technology (Ares-Serono Group, Randolph, MA), and the hLHR mutant was prepared by PCR overlap extension method (22, 23). The whole coding regions of the plasmids prepared using the Qiagen maxiprep kit (Qiagen, Valencia, CA) were sequenced by automated DNA sequencing (performed by the DNA Core Facility of the University of Iowa Carver College of Medicine) to verify that the mutation was correct and that there were no errors introduced during the PCR or ligation steps. All cDNAs were subcloned into pcDNA3.1/neo (Invitrogen).

Cells and transfections

HEK293 cells, obtained from American Type Culture Collection (Manassas, VA), 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, 6-well clusters. Cells were transfected using the calcium precipitation method (24). Four micrograms plasmid in 2 ml media were used per 35-mm dish. Cells were used 48 h after transfection for studies of ligand binding and hormone stimulation of cAMP generation.

NDP-MSH binding to intact cells

The methods for ligand binding to cells expressing the recombinant MCRs have been described previously (20). Briefly, 48 h after transfection, cells were washed twice with warm Waymouth's MB752/1 containing 1 mg/ml BSA (Waymouth/BSA) and incubated with 100,000 cpm [^{125}I]NDP-MSH with or without different concentrations of unlabeled NDP-MSH (from 10^{-12} to 10^{-6} M). After incubation at 37 C for 1 h, cells were placed on ice and washed twice with cold Hank's balanced salt solution containing 1 mg/ml BSA and 50 μg/ml gentamicin. Then 100 μl of 0.5 N NaOH were added to each well. Cells were collected using cotton swabs and counted in a γ-counter. Binding capacity and IC_{50} values were calculated using DeltaGraph software (Monterey, CA).

hCG binding assay

The levels of cell surface hLHR were determined by [^{125}I]hCG binding to intact cells. HEK293 cells transfected with the hLHR were washed twice with Waymouth/BSA modified to omit the sodium bicarbonate at room temperature and incubated with saturating concentration of [^{125}I]hCG (500 ng/ml final concentration) with or without an excess of unlabeled hCG (50 IU/ml final concentration). After a 1-h incubation at room temperature, cells were washed as described above for NDP-MSH binding and counted in a γ-counter.

Hormone 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 15 min incubation at 37 C, buffer alone, different concentrations of NDP-MSH or hCG 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 and EC_{50} values were calculated using DeltaGraph software.

Results

Two variants of the MC3R, K6T and I81V, function normally, whereas the I183N variant results in loss of function

Previously three MC3R variants were identified, including K6T in the extracellular N-terminal domain, I81V in transmembrane (TM) 1, and I183N at the second intracel-

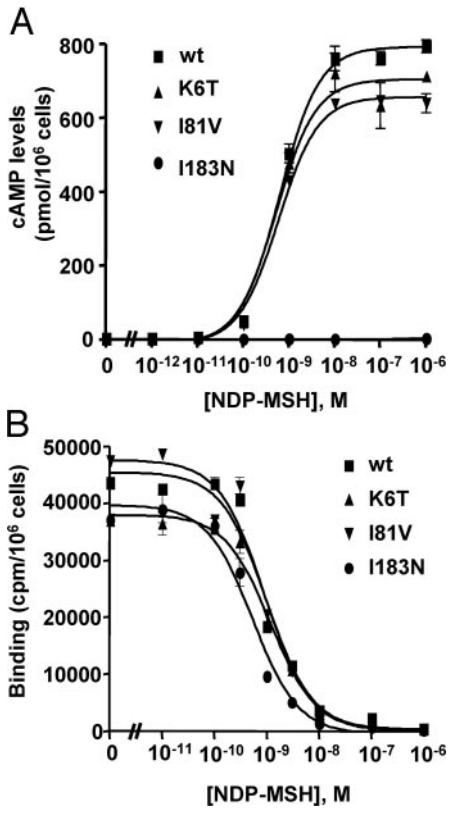

FIG. 1. Accumulation of intracellular cAMP and agonist binding in cells expressing wt or variant MC3Rs. A, HEK293 cells were transiently transfected, stimulated with various concentrations of NDP-MSH, and intracellular cAMP levels measured as described in *Materials and Methods*. Results are expressed as the mean ± SEM of triplicate determinations within one experiment. All experiments were performed at least three times, and similar results were obtained. B, 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 [^{125}I]NDP-MSH to wt or mutant MC3Rs on intact cells. Results shown are expressed as the mean ± range of duplicate determinations within one experiment. All experiments were performed at least three times, and similar results were obtained.

lular loop, although no functional studies had been performed. We generated these variants by site-directed mutagenesis and expressed them individually in HEK293 cells. Functional studies showed that two variants, K6T and I81V, exhibited normal cAMP production in response to NDP-MSH stimulation (Fig. 1A). The third variant, I183N, showed no response to NDP-MSH stimulation up to 10^{-6} M (Fig. 1A). Results from several experiments are summarized in Table 1.

To investigate whether these variants were expressed on the cell surface and bound NDP-MSH normally, we measured the ligand binding properties of intact cells expressing the wt MC3R or each of the variants. These results showed that all three variants bind NDP-MSH normally (Fig. 1B). Interestingly, cells expressing I183N MC3R bind NDP-MSH with a slightly higher affinity than cells expressing wt MC3R, although the maximal binding capacity to the I183N cells is slightly decreased (Table 1). We conclude, therefore, that all three variants are expressed on the cell surface and bind NDP-MSH normally. Importantly, our data show that the I183N variant is indeed a loss-of-function mutant in which agonist binding does not result in productive receptor activation.

Coexpression of wt and I183N MC3Rs demonstrates that I183N does not exert dominant-negative activity on wt MC3R

To examine whether I183N MC3R might cause obesity by haploinsufficiency or dominant-negative activity, we coexpressed the wt and I183N MC3R together. The results showed that I183N did not have dominant-negative activity on the coexpressed wt receptor in terms of signaling (Fig. 2A and Table 1) or ligand binding (Fig. 2B and Table 1).

Amino acid requirement at codon I183 required for MC3R activation

To gain further insight into what characteristics of the amino acids at codon 183 are required for MC3R activation, we generated additional substitutions of I183. These mutants included I183A, I183D, I183L, I183R, and I183V. Of the five mutants, only I183V signals normally compared with the wt

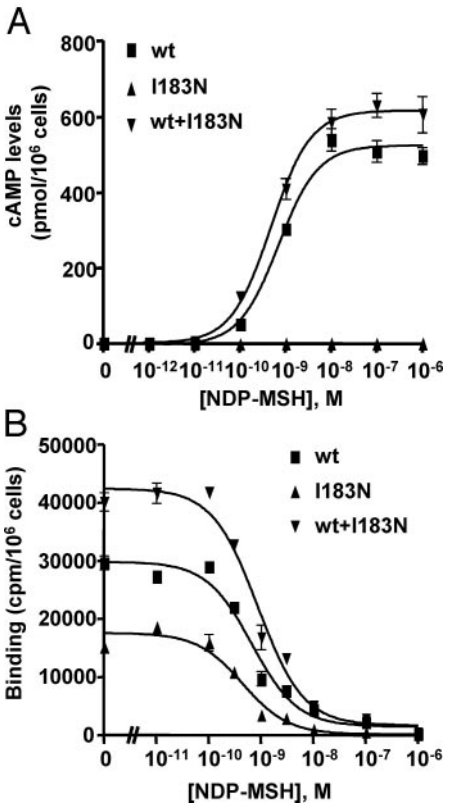

FIG. 2. Accumulation of intracellular cAMP and agonist binding in cells expressing wt and/or I183N MC3R. Cells were transiently transfected with the indicated MC3R constructs, and intracellular cAMP accumulation and binding assays were performed as described in *Materials and Methods*. Plasmid concentrations were kept constant by the addition of empty vector. A, HEK293 cells transiently transfected with the MC3R constructs were stimulated with various concentrations of NDP-MSH, and intracellular cAMP levels were measured. Results are expressed as the mean ± SEM of triplicate determinations within one experiment. All experiments were performed at least three times, and similar results were obtained. B, HEK293 cells transiently transfected with the MC3R constructs were used to measure ligand binding properties. Different concentrations of unlabeled NDP-MSH were used to displace the binding of [^{125}I]NDP-MSH to wt or mutant MC3Rs on intact cells. Results shown are expressed as the 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 MC3Rs

MC3R	n	NDP-MSH binding		NDP-MSH-stimulated cAMP	
		IC_{50} (nM)	B_{max} (% wt)	EC_{50} (nM)	R_{max} (% wt)
wt	8	3.30 ± 0.51	100	0.68 ± 0.13	100
K6T	3	3.10 ± 0.47	92 ± 7	0.60 ± 0.02	87 ± 2
I81V	3	3.65 ± 0.15	114 ± 16	0.52 ± 0.04	110 ± 17
I183N	5	1.47 ± 0.12	87 ± 11	ND[a]	ND[a]
wt+I183N	4	4.02 ± 1.74	99 ± 20	0.87 ± 0.13	102 ± 12
I183A	3	2.43 ± 0.27	82 ± 16	0.68 ± 0.47	5 ± 3
I183D	3	3.07 ± 0.50	73 ± 2	ND[a]	ND[a]
I183L	3	2.90 ± 1.57	62 ± 4	1.20 ± 0.47	18 ± 4
I183R	3	2.21 ± 0.75	29 ± 9	ND[a]	ND[a]
I183V	3	2.68 ± 0.43	123 ± 18	0.51 ± 0.13	107 ± 0

Data shown are the mean ± SEM of the indicated number of experiments. The B_{max} of cells expressing wt MC3R was 0.031 ± 0.003 nmol NDP-MSH bound/10^6 cells and the R_{max} was 609.2 ± 77.3 pmol cAMP/10^6 cells (mean ± SEM of eight experiments).

[a] ND indicates that the response to NDP-MSH stimulation (up to 1 μM) was less than 2-fold above basal cAMP levels and, therefore, the EC_{50} and R_{max} could not be calculated.

MC3R; all the other mutants exhibit either decreased (I183L and I183A) or no (I183D and I183R) detectable hormone-stimulated responses (Fig. 3A and Table 1). All mutants bound NDP-MSH with normal affinity (Fig. 3B and Table 1), demonstrating that the lack of signaling by I183 mutants substituted with residues other than Val is indeed due to functional impairment in the activation of the MC3R.

The corresponding residue is also important in agonist-induced receptor activation in the MC4R and hLHR

Interestingly, I183 is highly conserved within family A (rhodopsin-like) GPCRs, with 94% of these receptors containing an Ile or a Val at this position (25). The sequences of the TM3 regions of the MC3R, MC4R, and the hLHR, compared with TM3 of bovine rhodopsin are shown in Fig. 4. From this alignment, one observes that this Ile/Val lies in close proximity to a highly conserved D/E-R-Y/W motif near the cytoplasmic end of TM3. The conservation of the Ile/Val and its proximity to the D/E-R-Y/W motif suggested that it may play a general role in hormone-dependent GPCR activation. To examine this, we prepared mutations of the MC4R and the more distantly related hLHR in which the comparable Ile was substituted with Asn. As shown in Fig. 5, the MC4R harboring this mutation (I151N) displayed no detectable agonist-stimulated cAMP, although its binding properties were comparable with those of the wt MC4R. Similarly, the mutant hLHR (I468N) also bound hormone normally but displayed markedly reduced hormone-stimulated cAMP compared with cells expressing the same numbers of cell surface wt hLHR (Fig. 6). Over the course of three experiments, the response was reduced to 21.0 ± 3.7% of the maximal response observed in wt hLHR cells. Taken altogether, these data support a general role for the Ile/Val just downstream of the D/E-R-Y/W motif as being critical for agonist-stimulated GPCR activation.

Discussion

In the present study, we characterized three variants reported for the MC3R, K6T, I81V, and I183N. The K6T and I81V variants were identified in screenings for MC3R mutations from patients with obesity and NIDDM (14–17). However, in these studies, the variants were also found in normal subjects at a similar frequency, suggesting that they are not associated with obesity and NIDDM (14–17). Our studies show that the K6T and I81V variants are indeed polymorphisms with normal functions in terms of agonist binding and agonist-stimulated signaling (Fig. 1 and Table 1). In contrast, the I183N MC3R variant was identified from two patients with obesity and high percentage body fat and has not been found in normal subjects, suggesting that this

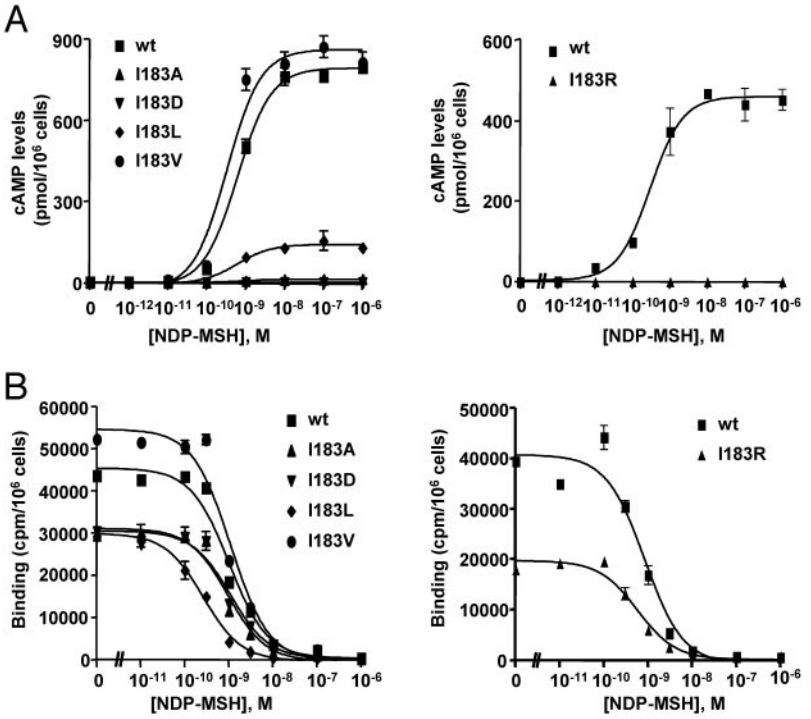

FIG. 3. Accumulation of intracellular cAMP and agonist binding in cells expressing wt or various MC3R mutants. Cells were transiently transfected with the indicated MC3R construct and intracellular cAMP accumulation and binding assays were performed as described in *Materials and Methods*. A, HEK293 cells transiently transfected with the MC3R constructs were stimulated with various concentrations of NDP-MSH, and intracellular cAMP levels were measured. Results are expressed as the mean ± SEM of triplicate determinations within one experiment. All experiments were performed three times, and similar results were obtained. B, HEK293 cells transiently transfected with the MC3R constructs were used for binding assays. Different concentrations of unlabeled NDP-MSH were used to displace the binding of [^{125}I]NDP-MSH to wt or mutant MC3Rs on intact cells. Results shown are expressed as the mean ± range of duplicate determinations within one experiment. All experiments were performed three times, and similar results were obtained.

FIG. 4. Alignments of TM3 and the second intracellular loop of the GPCRs studied herein with bovine rhodopsin. The demarcations of TM3 were based on the crystal structure of bovine rhodopsin (48). The D/E-R-Y/W motif is highlighted by an *asterisk* marking the end of TM3. The conserved Ile/Val residue that is mutated in the naturally occurring MC3R mutant and laboratory-designed mutants of the MC4R and hLHR is *shaded*.

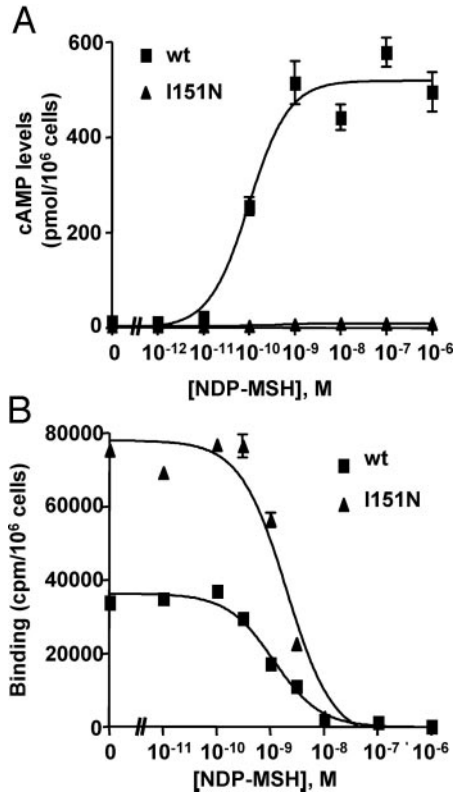

FIG. 5. Accumulation of intracellular cAMP and agonist binding in cells expressing wt or I151N MC4Rs. Cells were transiently transfected with either wt or I151N MC4R and intracellular cAMP accumulation and binding assay performed as described in *Materials and Methods*. A, HEK293 cells transiently transfected with wt or I151N MC4R were stimulated with various concentrations of NDP-MSH, and intracellular cAMP levels were measured. Results are expressed as the mean ± SEM of triplicate determinations within one experiment. All experiments were performed three times, and similar results were obtained. B, HEK293 cells transiently transfected with wt or I151N MC4R were used for binding assays. Different concentrations of unlabeled NDP-MSH were used to displace the binding of [^{125}I]NDP-MSH to wt or mutant MC4R on intact cells. Results shown are expressed as the mean ± range of duplicate determinations within one experiment. All experiments were performed three times, and similar results were obtained. The maximal binding capacity of cells expressing wt MC4R was 0.043 ± 0.017 nmol NDP-MSH bound/10^6 cells.

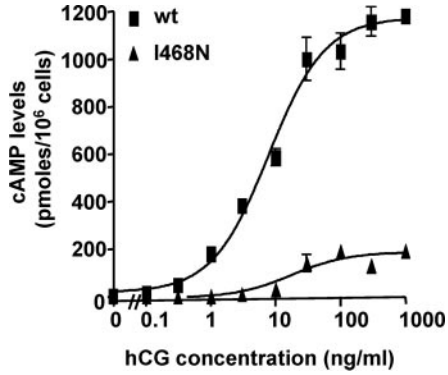

FIG. 6. Accumulation of intracellular cAMP in cells expressing wt or I468N hLHRs. Cells were transiently transfected with either wt or I468N hLHR, stimulated with various concentrations of hCG, and intracellular cAMP accumulation measured as described in *Materials and Methods*. Results are expressed as the mean ± SEM of triplicate determinations within one experiment. In this experiment, cells expressing wt hLHR had a binding capacity of 26.5 ng hCG/10^6 cells, and cells expressing I1468N hLHR had a binding capacity of 28.6 ng hCG/10^6 cells. All experiments were performed three times, and similar results were obtained.

is likely a causative mutation (18). The data presented here show that the I183N MC3R indeed represents a functionally impaired mutant of the MC3R in that it binds agonist normally but does not transduce the binding into G protein activation as measured by cAMP production (Fig. 1 and Table 1). These results provide supporting evidence that the I183N MC3R mutation might be a genetic factor predisposing the carriers to obesity (18). It should be pointed out that the other two family members who do not harbor the mutation were also obese. It is well accepted that obesity is a complex disease caused by multiple factors, including environmental, behavioral, and genetic factors. From epidemiological studies, it can be expected that the majority of obese patients do not harbor any mutations (new mutations cannot account for the dramatic increase in obesity rate during the past few decades). On the other hand, even in the patients harboring predisposing genetic factors, prudent dieting and exercise can overcome the genetic factors, as clearly demonstrated by the father in the case report (18). The original case report of the I183N mutation together with the pharmacological characterization presented herein prompt the tantalizing hypothesis that the MC3R might also be important for normal body weight regulation in humans.

That a defect in receptor activation is the basis for the loss of function of the I183N MC3R mutant is in sharp contrast to the closely related MC4R, in which numerous mutations have been reported to be associated with early-onset severe obesity and functional characterizations have revealed that a block in the trafficking of the MC4R mutants to the cell surface is the most common cause of the loss-of-function phenotype, and few mutants were identified to be defective in receptor activation *per se* (20, 26–28). Of the numerous GPCRs that naturally occurring mutations result in diseases, defects in receptor-G protein coupling and activation have been observed in rhodopsin (29), V2 receptor (30, 31), endothelin receptor subtype B (32, 33), thromboxane A_2 receptor (34), and MC1R (35, 36). However, as in MC4R, defects due to receptor/G protein coupling without affecting cell surface expression and/or ligand binding in naturally occurring disease-causing mutations in GPCRs are rare (37).

Cotransfection of the I183N MC3R mutant with the wt MC3R further showed that a heterozygous I183N MC3R mutation might be related to obesity due to haploinsufficiency rather than due to dominant-negative activity. Loss-of-function mutants of the MC4R have similarly been shown to result in obesity due to haploinsufficiency (27, 38).

Several monogenic forms of human obesity have been elucidated, including rare mutations in leptin, the leptin receptor, POMC, prohormone convertase 1, and the MC4R (reviewed in Ref. 39). The studies presented here suggest that inactivating mutations of the MC3R might be another genetic factor that is related to human obesity, notably with increased fat mass. Increased fat, especially abdominal visceral fat, is associated with a significantly increased risk of metabolic abnormalities such as glucose intolerance, insulin re-

sistance, dyslipidemia, and hypertension. Based on the mouse genetics studies, it has been argued that combined treatment with agonists for the MC3R and MC4R might result in greater weight loss than either agonist alone by combining decreased food intake (MC4R) and feed efficiency (MC3R) (40). The present study provides supporting evidence for this hypothesis.

The Ile that is the site of the I183N MC3R loss-of-function mutation is predicted to be within the second intracellular loop, just distal to the highly conserved D/E-R-Y/W motif at the cytoplasmic end of TM3 (see Fig. 4). An examination of the sequences of rhodopsin-like GPCRs reveals that this Ile or the closely related Val residue is present at this location in 94% of GPCRs (25). Although numerous studies suggest an important role in GPCR activation mediated by the D/E-R-Y/W motif (41), relatively few studies have examined the role of the conserved Ile/Val. Alanine scanning mutagenesis studies of the m1 muscarinic, α_{1b}-adrenergic, angiotensin II type 1, and gastrin-releasing peptide receptors suggested that the Ile/Val is important in hormone-stimulated GPCR activation (42–45). Our studies with the MC3R mutants show that residues placed in codon 183 other than Ile or Val caused a profound loss of agonist-stimulated cAMP production (see Fig. 5), further suggesting that an Ile or Val in this position is critical for GPCR activation. To explore this hypothesis further, mutants of the MC4R and the more distantly related hLHR were made in which the cognate Ile was substituted with Asn. Both mutant receptors exhibited a marked attenuation of hormone-stimulated cAMP production consistent with their also being functionally impaired in their coupling to Gs. It is relevant to point out that an exhaustive random saturation mutagenesis of the V2 vasopressin receptor, another Gs-coupled receptor, did not identify the corresponding Ile/Val as important for G protein coupling (46). It is not known whether mutation of Ile141 of the V2 receptor will result in defective adenylyl cyclase activation. To the best of our knowledge, our results are the first report of the conserved Ile/Val being important for Gs-coupled GPCR activation.

Further studies will be needed to identify the molecular mechanism underlying the critical role of this Ile/Val in GPCR activation. One possibility is that the mutation results in global conformational changes, disrupting critical interactions that are necessary for receptor activation. Although the crystal structure of rhodopsin, a prototypical GPCR, is available and homology modeling is used frequently to identify possible interactions, it is not applicable for modeling the intracellular loops, which are highly divergent. Another possibility is that this Ile/Val is directly involved in G protein coupling and activation. Due to the location of this residue, this is more likely. Previous studies have identified the second intracellular loop, together with the third intracellular loop and the carboxyl terminal tail, as molecular determinants for G protein coupling selectivity and activation (reviewed in Ref. 47). It is tempting to speculate that this hydrophobic Val/Leu interacts with the conserved hydrophobic residues at the extreme carboxyl terminus of Gα and contributes to the receptor activation after ligand binding (42). A recent study on the gastrin-releasing peptide receptor supported the hypothesis that the corresponding residue is involved in receptor-G protein coupling (45). The nonhydrolyzable guanine analog Gpp(NH)p inhibited ligand binding to the wt receptor, whereas it had significantly less effect on ligand binding of the mutant receptor with the conserved isoleucine mutated to alanine (45). Ongoing studies on the MCRs will try to differentiate between these possibilities.

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