

Intracellular retention is a common characteristic of childhood obesity-associated MC4R mutations

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Heterozygous mutations in the coding region of the serpentine Melanocortin 4 receptor are the most common genetic cause of human obesity described to date. There are still conflicting data regarding the overall prevalence of such mutations in obesity and limited information is available on the functional defects caused by most obesity-associated MC4R mutations. We report here the screening for mutations in the coding region of the MC4R of a new cohort of 172 patients presenting with severe childhood obesity and a family history of obesity. Three heterozygous MC4R mutations (Ser127Leu, Ala244Glu and Pro299His) were found in three patients of this cohort (1.74%), confirming that such mutations are implicated in a significant number of childhood obesity cases. A functional analysis of these mutant receptors, in addition to 11 other childhood obesity-associated MC4R mutations, indicates that they all alter the activation of the receptor by the endogenous agonist α -MSH. To further examine the functional defects caused by childhood obesity-associated MC4R mutations, we developed a novel, sensitive technique to quantitatively analyze the effect of a mutation on MC4R cell surface expression. Using this method we analyzed the cell surface expression of all the 14 described childhood obesity-associated MC4R missense mutations. We demonstrate that 81.3% of childhood obesity-associated heterozygous MC4R mutations lead to intracellular retention of the receptor. This result has implications for the potential pharmacologic rescue of childhood obesity-associated MC4R mutations and for the treatment of patients presenting with this condition.

INTRODUCTION

The prevalence of childhood obesity, a multifactorial disease caused by the interaction of genetic and environmental factors (1,2), is increasing at an alarming rate in industrialized countries. Since it resists currently available treatments, the elucidation of the pathophysiologic mechanisms underlying childhood obesity is a necessary prerequisite to the identification of new therapeutic strategies for this condition. The recent description of at least six different monogenic forms of childhood obesity (3–15) has confirmed the implication of genetic factors in the development of human obesity, particularly in its early onset form. Most of these genetic forms of obesity are rare recessive disorders associated with multiple endocrine abnormalities (3–7). On the contrary, mutations in the MC4R gene are a cause of dominant and

recessive obesity, accounting for up to 6% of all cases of severe obesity in some studies.

MC4R is a 332 amino acid protein encoded by a single exon gene localized on chromosome 18q22 (16,17). MC4R belongs to the family of seven transmembrane G-protein coupled receptors (GPCR) and transduces signal by coupling to the heterotrimeric G_s protein and activating adenylate cyclase (16). The expression of MC4R is restricted to the brain, where it is found in hypothalamic nuclei involved in food intake regulation (18). MC4R regulates food intake by integrating an agonist (satiety) signal provided by Alpha-Melanocyte Stimulating Hormone (α -MSH) (19) and an antagonist (orexigenic) signal provided by the Agouti-Related Protein (AGRP) (20). In addition, recent data suggest that MC4R exhibits a constitutive, food intake inhibiting, activity on which AGRP acts as an invert agonist (21,22). The critical role of

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MC4R in the long-term regulation of body weight has been well demonstrated in mice (23). Mice lacking both alleles of MC4R (MC4R^{-/-} mice) develop a severe obesity syndrome while mice heterozygous for an MC4R deletion show an average weight that is intermediate between that of wild-type and MC4R^{-/-} mice.

We and others have reported that multiple rare mutations in MC4R cause a common non-syndromic form of obesity (8–14). Over 15 dominant alleles have been identified in cohorts of obese children, most of which are missense mutations. While the demonstration of a functional defect of the mutated gene product is an important argument for the pathogenic role of a rare mutation, this information is limited and only available for a few of the childhood obesity-associated MC4R mutations.

In this study, we report the prevalence of MC4R mutations in a new cohort of obese children selected for a family history of obesity and demonstrate that all described childhood obesity-associated MC4R mutations impair the function of the protein. Using a novel, sensitive approach to quantify cell surface expression of MC4R, we demonstrate that intracellular retention is a common functional defect of childhood obesity-associated MC4R mutants.

RESULTS

Detection of novel MC4R mutations in a cohort of obese children

In addition to the known polymorphism Ile103Val, three different heterozygous point mutations, Ser127Leu (C380T), Ala244Glu (C731A) and Pro299His (C896A), were detected in three children of our cohort of 172 obese children (1.74%) by systematic sequencing of the MC4R gene. These mutations had not previously been described. The locations of all the newly detected mutations on the primary structure of the MC4R as well as the other childhood obesity-associated mutations are shown in Figure 1. Transmission of these obesity-associated MC4R mutations in the family of the probands (Fig. 2) was tested by direct sequencing of the MC4R gene of the available family members of the three probands. All the carriers of the mutations were overweight or obese according to WHO criteria. The degree and precocity of obesity matched that of the probands only in a family member of the carrier of mutation Ser127Leu. This confirms the previously observed partial penetrance of heterozygous MC4R mutations.

α -MSH activation of MC4R is impaired by childhood obesity-associated MC4R mutations

An important argument in favor of the pathogenic role of a rare mutation is the demonstration of a functional defect of the mutated gene product. We first investigated the pathogenic role of the missense mutations found in our cohort by comparing the α -MSH induction of cAMP production by the different mutated MC4Rs to that of the wild-type receptor. In addition, we also investigated the α -MSH activation of all the described childhood obesity-associated MC4R mutations for which this information was not available: Val50Met (13), Ser58Cys (13), Ile102Ser (13), Ser30Phe/Gly252Ser (10), Pro78Leu (10),

Ile317Thr (10) and Val253Ile (24). To determine their relative effect, the mutations Ser30Phe and Gly252Ser, found on a single allele in one patient (10), were studied both separately and on the same allele. The wild-type and all mutated receptors were cloned, transiently expressed in HEK 293 cells stably expressing a cAMP-dependent luciferase reporter gene and compared for their ability to be activated by α -MSH. In this assay, as previously described for mutations Arg165Trp (12), Ile170Val (12) and Cys271Tyr (24), all childhood obesity-associated MC4R mutants tested demonstrate an impaired response to α -MSH (Fig. 3). α -MSH activation of MC4R is differentially affected and is only totally abolished in the case of the Pro299His, Pro78Leu and Ile102Ser mutations. The extent of the decrease in α -MSH activation does not seem to correlate with the severity of obesity in the mutation carriers nor with the age at onset of obesity (Table 1 and Fig. 2).

Cell surface expression of MC4R assayed by immunofluorescence and FACS analysis

Intracellular retention of mutated proteins is a common disease-causing defect. To allow for the rapid evaluation of cell surface expression of MC4R relative to total expression of the receptor in individual transiently transfected cells, we developed a method based on immunostaining and fluorescence detection by flow cytometry. We constructed a chimeric receptor containing a C-terminal intracellular green fluorescence protein (GFP) and an N-terminal extracellular Flag epitope. This chimeric construct remains responsive to the natural agonist α -MSH with the same EC₅₀ as the native receptor (Fig. 4A). The GFP fluorescence emitted by cells expressing the chimera can be detected by FACS, allowing for the detection and analysis of cells expressing a similar level of receptor for further analysis. Immunostaining of the transfected cells with a mouse anti-Flag primary antibody and a phycoerythrin (PE) conjugated anti-mouse secondary antibody in the absence of detergent allows for the detection of cell surface expression of the transfected receptor. Concurrent FACS analysis of both GFP and PE fluorescence for each individual cell therefore allows quantification of cell surface expression relative to total expression for each selected cell (Fig. 4B).

Most childhood obesity-associated MC4R mutations impair cell surface expression of the receptor

While obesity-associated MC4R frameshift mutations have been found to impair cell surface expression of human MC4R (25), and while mutation-induced misfolding and intracellular retention by the endoplasmic reticulum (ER) quality control system is a common occurrence for diseases caused by mutations in GPCR and other membrane proteins (26), the effects of missense mutations on MC4R cell surface expression have never been assessed. Using the assay described above, we evaluated the effect of all 14 described childhood obesity-associated MC4R mutants on the membrane expression of the receptor in both HEK 293 and Neuro 2A cells. Following FACS analysis of transiently transfected, immunostained cells, we compared the membrane expression of the mutated MC4Rs to that of the wild-type receptor for cells expressing low levels of the receptor.

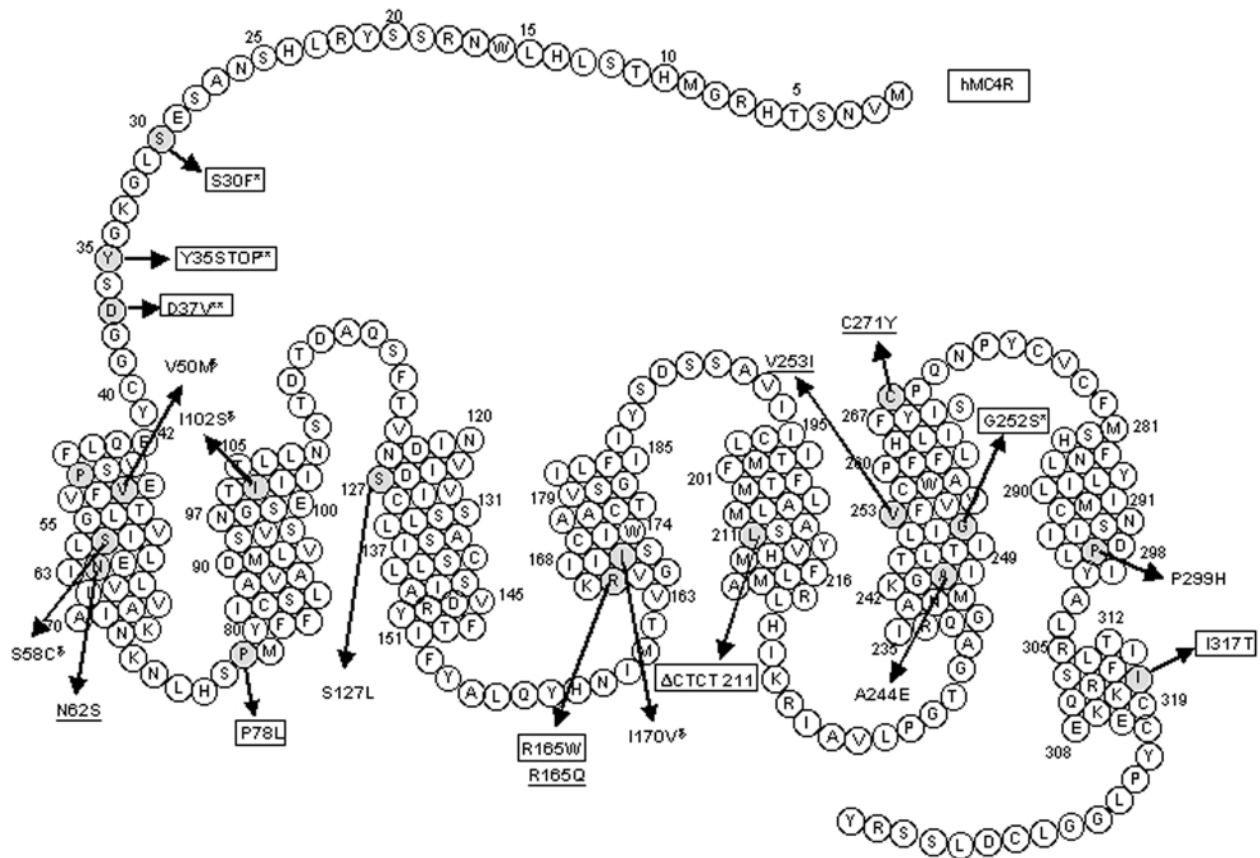


Figure 1. Schematic representation of MC4R and the sequence variants detected in childhood obesity. The positions of the sequence variants described in Table 1 are indicated on the secondary structure of MC4R. Amino acids are indicated as circles in single-letter code. Mutations indicated in bold are from the present study; mutations in a square are from Hinney *et al.* (10); underlined mutations are from Farooqi *et al.* (24); mutations indicated with ^s are from Dubern *et al.* (13). S30F*/G252S* and Y35STOP**/D37V** are double mutants.

All but three (Ser127Leu, Ala244Glu and Val253Ile) childhood obesity-associated MC4R mutations (Table 1 and Fig. 5) decrease cell surface expression of the receptor. The intracellular retention of the mutants was variable but closely correlated with the alteration in the α -MSH activation of the receptor (Table 1) except for Ile102Ser Ser127Leu, Ala244Glu and Val253Ile. In addition, the decrease in membrane expression seems to correlate with the age at onset of obesity (Table 1). Interestingly, the allele Ser30Phe/Gly252Ser had an altered membrane expression while both mutations taken separately did not (Fig. 5). The results obtained for all the mutants were not cell line-specific as the behavior of the mutated receptors versus the wild-type MC4R was similar in Neuro 2A cells (data not shown).

Membrane expression of the constitutively active, obesity-associated, Leu250Gln mutant

In addition to the membrane expression of MC4R mutants detected in childhood obesity, we studied the membrane expression of the mutant Leu250Gln. This mutant is of particular interest since it was found in an obese adult patient but has been demonstrated to unexpectedly display a constitutive activity (12). In our cell surface localization assay, we demonstrate that the

membrane expression of the Leu250Gln mutant is significantly decreased compared with that of the wild-type MC4R (Fig. 6).

DISCUSSION

The recent description of multiple rare obesity-causing mutations in at least six different genes suggests that the genetic predisposition to severe obesity in a given environment depends upon multiple rare penetrant mutations in a large set of genes, rather than the additive and interactive effects of a limited number of common genetic variants (3–15). By showing a significant association with severe obesity, and by accounting for a significant percentage of early and/or severe obesity cases, mutations of the MC4R gene are currently the best model for such a genetic predisposition to obesity (8–14). By systematically screening a total of 172 children with severe obesity, we detected three new heterozygous mutations in the MC4R (1.74%). In the first four studies on childhood obesity, 16 MC4R mutation carriers were found in a total of 675 obese children representing 2.37% CI (1.22–3.52) of the studied populations (Table 1, Fig. 1) (twelve missense, two frameshift and one non-sense mutations). Despite specifically selecting patients on the basis of a family history of obesity, the frequency of mutations in our cohort does not significantly differ from that

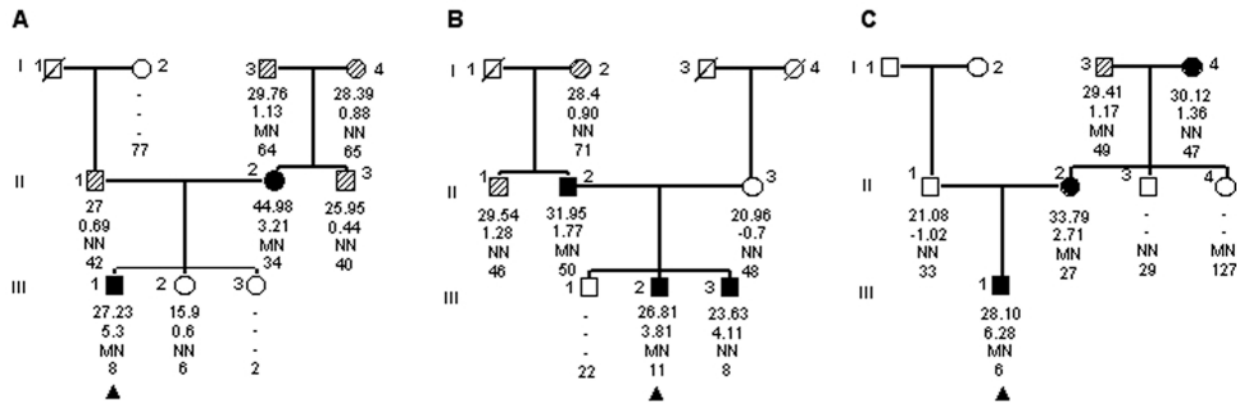


Figure 2. Pedigrees, mutation screening and phenotypes in the available family members of MC4R mutation carriers. (A) Ser127Leu family; (B) Ala244Glu family; (C) Pro299His family. Arrows indicate the probands. Hatched symbols represent overweight status. Filled symbols denote obesity. The first line of the symbols corresponds to the BMI (kg/m²). The second line shows the BMI Z-score. The third line shows the genotype: N, normal allele; M, mutant allele. The fourth line shows the age of the subject.

Table 1. Sequence variants reported in early onset obesity. MC4R variants, study and BMI (kg/m²) are indicated. For comparison purpose and when data were available, BMI standard deviation score (Z-score) was calculated using French population data (34). BMI Z-score is derived from age- and gender-specific means. Age of onset is the age at which the BMI curve of the patient first reached the 95th percentile on the French reference BMI curve (34). Functional characteristics of MC4R variants are reported. Mean \pm SEM of the EC50 derived from α -MSH dose-response curve are indicated for each variant studied as well as the ratio PE/GFP, expressed as a percentage of the value obtained in the same experiment for the wtMC4R and representing membrane expression (see Materials and Methods and the legend for Figures 4 and 5). Mean \pm SEM of the ratio PE/GFP are calculated from at least two independent experiments

Mutations	Study	BMI (kg/m ²)	Z-score (SD)	Age of onset (years)	α -MSH response, EC50 (nM)	Membrane expression, PE/GFP (%wt)
wt	Present				1.04 \pm 0.16	100
Ser127Leu	Present	27.2	5.3	3.5	28.7 \pm 11.29	101 \pm 8
Ala244Glu	Present	26.6	3.72	2	6.55 \pm 0.91	90 \pm 7
Pro299His	Present	26.5	6.28	1	No response	18 \pm 7**
Val50Met	(13)	24.1	5.4	1	1.64 \pm 0.64	69 \pm 10*
Ser58Cys	(13)	33	4.2	4	15.88 \pm 7.16	50 \pm 6**
Ile102Ser	(13)	34.8	4.1	1	No response	57 \pm 5**
Ile170Val	(13)	33.2	4.4	4	ND	84 \pm 3*
Δ CTCT at codon 211	(9)	28	NP	NP	ND	ND
Ser30Phe/Gly252Ser	(10)	34.1	4.13	NP	4.37 \pm 1.65	60 \pm 5**
Tyr35Stop/Asp37Val	(10)	31.29	5.16	NP	No response	NP
Pro78Leu	(10)	26.5	4.3	NP	No response	31 \pm 5**
Δ CTCT at codon 211	(10)	42.06	4.26	NP	No response	NP
Ile317Thr	(10)	36.5	4.07	NP	2.42 \pm 0.77	66 \pm 8**
Arg165Trp	(10)	26.8	3.45	NP	ND	26 \pm 6**
GT ins at codon 279	(24)		3.91	NP	No response	NP
Asn62Ser (homozygous)	(24)		4.85	NP	ND	35 \pm 0**
Arg165Gln	(24)		NP	NP	ND	ND
Val253Ile	(24)		NP	NP	3.65 \pm 1.19	90 \pm 7
Cys271Tyr	(24)		3.04	NP	ND	38 \pm 9**

wt, wild-type. ND, not determined in this study. NP, not published.

of the previously described cohorts of severely obese children (9,10,24), indicating the poor predictive value of these clinical criteria for detecting MC4R mutation carriers. Only one recent report describes a significantly lower frequency of MC4R mutations in a cohort of obese children from Italy (27). However, in this report the studied population had a lower mean BMI (as evaluated by the Z-score using the same standard curves) and the authors used a screening method that has a lower sensitivity which allows for the possibility of false negative results.

More rigorous proof that the identified mutations are responsible for the disease being studied requires functional studies of the expressed mutated gene product. This had previously not been completed for most of the childhood obesity-associated MC4R mutations. Our demonstration that all childhood obesity-associated MC4R mutations display an altered response to the physiological agonist α -MSH provides an additional strong argument for their pathogenic significance. Decreased α -MSH activation of the mutated MC4R in our

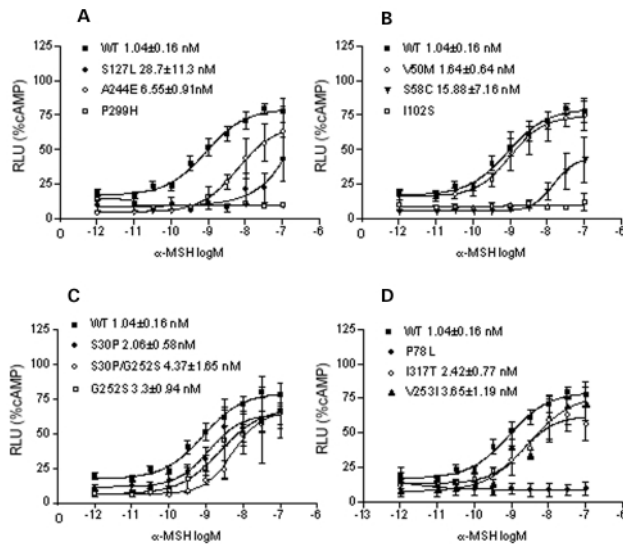


Figure 3. α -MSH activation of obesity-associated MC4R mutants. Activity of the receptors is assayed by analyzing their ability to activate the expression of a cAMP-induced luciferase reporter gene. WT and mutant MC4R are transiently transfected in a stable HEK 293 cell line expressing pCRE-luciferase. Thirty-six hours after transfection, cells are stimulated for 6 h at 37°C with medium alone, increasing amounts of α -MSH or 8Br-cAMP (1 mM), after which luciferase activity is measured. Data obtained are normalized to 8Br-cAMP (1 mM) maximal response. Data points represent mean \pm SEM of at least three independent experiments performed in triplicates. Mean \pm SEM of the EC50 (nM) is indicated for each variant. The WT receptor activation curve is shown on all graphs for comparison with (A) mutations S127L, A244E, P299H; (B) mutations V50M, S58C and I102S; (C) double mutation S30P/G252S, and mutations S30P and G252S; (D) mutations P78L, I317T, V253I.

assay can be due to a decreased cell surface expression of MC4R, to a decreased affinity of the receptor for α -MSH, to a decrease in signal transduction, or to a combination of these defects. Systematically determining the nature of the alterations caused by obesity-associated MC4R mutations will allow the development of a functional classification of this condition. This, in turn, is a prerequisite for clarifying the genotype-phenotype relationship of this condition and for eventually adapting the preventive and pharmacologic interventions to the specific molecular defects.

To determine if obesity-associated MC4R mutations lead to intracellular retention of the receptor, we developed a simple, efficient and sensitive method that allows for a rapid comparison of the cell surface expression of mutated MC4Rs to that of the wild-type receptor. By using this detection method, we demonstrate here that, together with the described frameshift mutations, 81.3% of MC4R missense mutations associated with childhood obesity cause partial or complete intracellular retention of the receptor. Our results also suggest that intracellular retention of the mutated protein correlates with an early onset of obesity in the probands. Interestingly, using this assay, we also find that Leu250Gln, an obesity-associated mutation previously demonstrated to be constitutively active (12), is partially intracellularly retained. This further suggests that intra cellular retention of MC4R mutants is implicated in the pathophysiology of obesity caused by MC4R mutations.

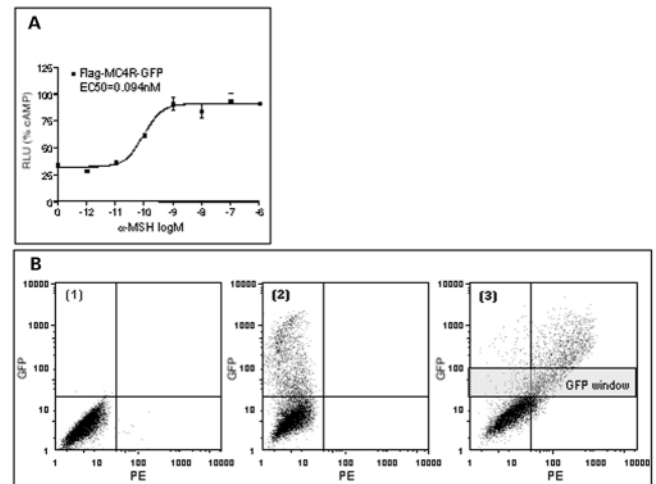


Figure 4. Flow cytometry analysis of MC4R cell surface expression. To evaluate MC4R cell surface expression relative to total expression of the receptor, we developed a method based on immunostaining and fluorescence detection by flow cytometry. We constructed a chimeric receptor by adding an extracellular N-terminal flag epitope and an intracellular C-terminal GFP. (A) To assay the effect of α -MSH on this Flag-MC4R-GFP, this chimeric MC4R was transiently transfected in a stable HEK 293 cell line expressing pCRE-luciferase. Thirty-six hours after transfection, cells are stimulated with different concentrations of α -MSH for 6 h at 37°C. Results are normalized to cAMP maximal response. Numbers are mean \pm SEM of triplicates. (B) HEK 293 cells were transfected with the chimeric MC4R WT and immunostained at 4°C with a mouse antiflag antibody (M2) and a PE-conjugated antimouse in the absence of detergent. GFP and PE emissions were analyzed in each individual cell by flow cytometry with a FACS[®] Calibur Beckton-Dickinson flow cytometer (Beckton-Dickinson Immunocytometry Systems, San Jose, CA, USA), and results analysed using the software CellQuest (Beckton-Dickinson Immunocytometry Systems, San Jose, CA, USA). Results are shown as a dot plot: (1) untransfected HEK 293 cells; (2) HEK 293 cells transfected with WT chimeric MC4R and immunostained without antiflag antibody; (3) HEK 293 cells transfected with WT chimeric MC4R and immunostained with both antibodies. GFP emission represents MC4R total expression and PE emission represents cell surface MC4R expression. To limit artifacts caused by receptor overexpression, we limited our analysis to cells expressing low levels of the receptor as defined by the GFP window shown.

In a previous study, Ho and MacKenzie had shown that two frameshift MC4R mutations implicated in early onset obesity were intracellularly retained (25). For these studies the authors used immuno-cytofluorescence using N-Terminal epitope-tagged receptors in the presence or absence of a permeabilizing agent. While broadly used and well suited for qualitative analysis, this technique is difficult to adapt to quantitative analysis and does not allow for the measurement of cell surface expression versus total expression in the same cell. In contrast, the FACS-based approach presented here allows the rapid quantitation of MC4R membrane expression on a cell-by-cell basis. This method could be easily applied to the study of cell surface expression of any transmembrane protein.

Defective intracellular protein transport is an increasingly recognized anomaly of hereditary disease-causing mutations. Examples include the failure of proper transport of low density lipoprotein (LDL) receptor in some types of familial hypercholesterolemia (28), and the cystic fibrosis transmembrane conductance regulator (CFTR) in cystic fibrosis (29). Of particular relevance is the implication of rhodopsin mutations in retinitis pigmentosa (RP) (26,30–32). Rhodopsin is a

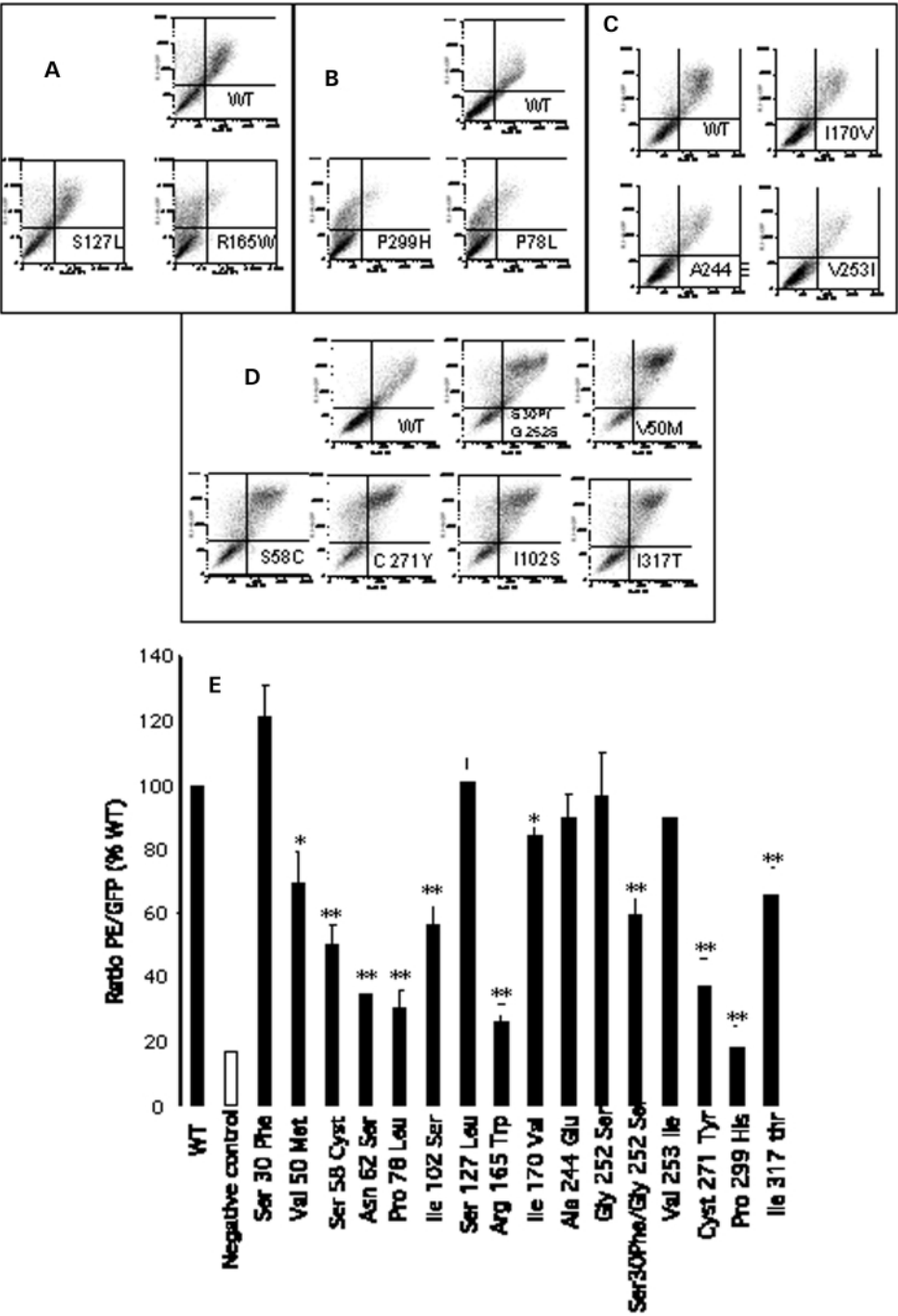


Figure 5. Effect of childhood obesity-associated MC4R mutations on membrane expression of the receptor. Twenty-four hours after transfection with chimerical Flag/GFP WT or mutant MC4R, HEK 293 cells were incubated at 4°C with monoclonal anti-Flag antibody (M2) and PE conjugated anti-mouse IgG. Cell emission of GFP and PE was measured by flow cytometry as described in Figure 4. Representative dot plots are shown for WT MC4R and (A) S127L and R165W; (B) P299H and P78L; (C) I170V, A244E, V253I; (D) S30P/G252S, V50M, S58C, C271Y, I102S, I317T. The ratio PE emission/GFP emission, representing MC4R membrane expression relative to MC4R total expression, was calculated in each individual cell belonging to the pre-determined GFP window using FlowJo software (Tree Star Inc., San Carlos, CA, USA). Results are expressed as a percentage of the value obtained in the same experiment for the WT MC4R and are represented on (E). The numbers represent mean \pm SEM of three independent experiments. The negative control is the ratio PE/GFP calculated in the absence of primary anti-Flag antibody. * $P < 0.05$; ** $P < 0.01$.

G-protein coupled receptor expressed in specialized neuronal photoreceptor cells. As for MC4R, mutations in rhodopsin can cause either a dominant or recessive form of RP. The vast majority of the 150 dominant RP-causing rhodopsin mutations

described to date are so called class II mutations that are intracellularly retained. Transgenic expression in rodents of such mutants has subsequently demonstrated their intracellular accumulation and role in photoreceptor degeneration (31,33).

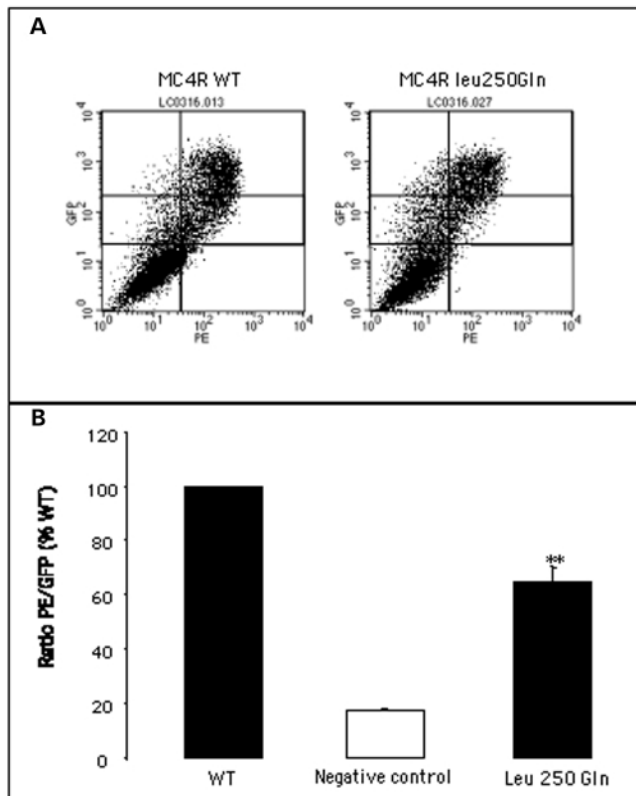


Figure 6. Effect of Leu 250 Gln mutation on MC4R membrane expression. Twenty-four hours after transfection with chimerical Flag/GFP WT, or MC4R L250Q, HEK 293 cells were treated as described in Figure 5. (A) Representative dot plots are shown for WT MC4R and L250Q. (B) The ratio PE emission/GFP emission, representing MC4R membrane expression relative to MC4R total expression, is expressed as a percentage of the value obtained in the same experiment for the WT MC4R. The numbers represent mean \pm SEM of three independent experiments. The negative control is the ratio PE/GFP calculated in the absence of primary anti-Flag antibody.

Our finding that childhood obesity-associated MC4R mutations have a decreased membrane expression in a heterologous expression system leads us to speculate that MC4R mutations could act through a dominant negative neurodegenerative disease mechanism. Such a mechanism would have major implications with respect to the early detection of MC4R mutation carriers and the approaches to therapeutic intervention for this condition. Further studies will be necessary to demonstrate a defective processing of MC4R mutations as well as neuronal degeneration upon transgenic expression of MC4R mutants.

MATERIALS AND METHODS

Subjects and phenotypes

Patients were recruited in France through an advertisement campaign and a toll-free number. Inclusion criteria were a body mass index (BMI) above the 99th percentile for ages below age 8 years according to the French standard BMI curve (34) and a family history of obesity (at least one sib with a BMI above the 95th percentile). The clinical characteristics of the

cohort are as follows: $n = 172$ (sex ratio = 85 male/87 female), age = 12.6 ± 3.2 years (4.9–22.4), BMI = 32 ± 6.3 kg/m² (21.6–69.4), Z-score of BMI = 4.3 ± 1 SD (2.1–7.6), obesity onset = 3.3 ± 2.2 years (0.2–16), age at BMI rebound = 2.1 ± 1.5 years (0.2–8.0). Informed consent was obtained for all subjects and the protocol was approved by the Local Ethics Committee (CCPPRB Lille). Genomic DNA was extracted from peripheral leukocytes for all subjects.

Direct nucleotide sequencing of the MC4R gene

Two primers, MC4R-AF (5'-ATCAATTCAGGGGGACACTG-3') and MC4R-ER (5'-TGCATGTTCTATATTGCGTG-3') were used in a PCR reaction to amplify the entire coding region of the MC4R gene as described (12). The sequencing reaction was performed with the BigDye Terminator kit (Applied Biosystems, Foster City, CA, USA) under the standard manufacturer's conditions. Each PCR product was sequenced using MC4R-AF, MC4R-ER and two internal primers, MC4R-CF (TGAGCTCCTTGCTTGATC) and MC4R-CR (GGCCATCAGGAACATGTGGA). Sequencing was performed on an ABI PRISM[®] 3700 automated DNA sequencer (Applied Biosystems, Foster City, California, USA).

Genomic amplification and construction of wild-type and mutant MC4R expression vectors

Since MC4R is a single exon gene, we amplified the variant and wild-type MC4R genes from the available genomic DNA of the patients carrying the mutations Ser127Leu, Ala244Glu and Pro299His (this paper) and Val50Met, Ser58Cys, Ile102Ser (13) using the primers MC4R-AF and MC4R-ER. PCR products were cloned into the pcDNA3/MC4R expression vector (Invitrogen, Carlsbad, CA, USA). Mutations Ser30Phe, Gly252Ser, Pro78Leu and Ile317Thr were obtained by site-directed mutagenesis of pcDNA3 MC4R WT (QuickChange Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA, USA). Construction of the MC4R mutants Arg165Trp and Ile170Val in pcDNA3 have been described previously (12).

To construct the Flag-MC4R-GFP fusion protein used in the membrane expression assay, wild-type and mutant MC4Rs were amplified by PCR from the pcDNA3 expression vector using a primer containing the Flag epitope sequence (MDYKDDDDK) added in 5' of MC4R-AF and the primer MC4R-ER. PCR products were subcloned in pEGFP-N1 (Clontech, Palo Alto, California, USA). All expression vectors were sequenced to establish the presence of the mutation and the absence of PCR-induced mutations.

α -MSH response of childhood obesity-associated MC4R mutants

HEK 293 cells were maintained in α -MEM supplemented with 10% calf serum (HYCLONE), L-glutamine, penicillin/streptomycin. A stable cell line (HEK 293) expressing a cAMP-inducible luciferase reporter gene (35) was established by co-transfection (ratio 10/1) of pCRE/luciferase plasmid (pCRE-luc) and a plasmid containing Blastidicine resistance. Single isolated clones were grown in 5 μ g/ml of Blastidicine

S (Invitrogen, Carlsbad, California, USA), tested and selected for a low basal luciferase activity together with a significant 8Br-cAMP inducible luciferase activity. The day before transfection, 190 000 cells/well were seeded in coated poly L-lysine (Sigma, St Louis, MO, USA) and collagen type I rat tail (BD Bioscience, Bedford, MA, USA) six-well plates. The stable cell line expressing pCRE/luc was transfected with 8 µg of wild-type or variant MC4R expression vector using 8 µg (0.2 nmol) of polyethylenimine 25 kDa (PEI) per well. Twenty-four hours after transfection, cells were split into 96-well plates and incubated overnight in MEM α medium. At 36 h post-transfection, cells were washed and incubated in stimulation medium (MEM α medium containing 0.1 mg/ml BSA and 0.25 mM isobutylmethylxanthine) and stimulated with different concentrations of α -MSH (Sigma, St Louis, MO, USA) for 6 h at 37°C in a 5% CO₂ incubator. Luciferase activity, representing cAMP produced in response to α -MSH, was assessed using the Steady-Glo Luciferase Assay System (Promega, Madison, WI, USA) and a microplate luminescence counter (Packard Instrument, Downers Grove, IL, USA). Results were normalized to the maximal stimulation by 8Br-cAMP. Data points represent the mean of at least two independent experiments performed in triplicate.

Cell surface expression of wild-type and mutated MC4Rs

HEK 293 or Neuro 2A cells were transfected in six-well plates with 8 µg of Flag-GFP chimerical wild-type or mutant MC4R using 8 µg (0.2 nmol) PEI per well. Twenty-four hours after transient transfection, cells were harvested and rinsed in ice-cold MEM medium containing 0.1% BSA. Cells were incubated with monoclonal anti-Flag antibody (M2, Sigma, St Louis, MO, USA) in MEM medium with 0.1% BSA at 4°C for 1 h. Under these conditions the primary antibody binds only to receptors located at the cell surface. After this incubation, cells were washed with ice-cold MEM medium 0.1% BSA and incubated with phycoerythrin-conjugated anti-mouse (Caltag, Burlingame, CA, USA) in MEM medium with 0.1% BSA at 4°C for 45 min. Cells were then washed with ice-cold MEM medium with 0.1% BSA and re-suspended in ice-cold FACS medium (MEM medium 0.3% fetal calf serum, 5% dissociation buffer, 1 µg/ml propidium iodide). Cells were analyzed through a FACS[®] Calibur Beckton-Dickinson flow cytometer (Beckton-Dickinson Immunocytometry Systems, San Jose, CA, USA), set to detect GFP, PE and PI fluorescence. Using the Cell Quest software, 10 000–15 000 cells were analyzed for each sample. The ratio PE emission/GFP emission was determined for each individual cell for a pre-determined GFP emission level using the FlowJo software (Tree Star Inc., San Carlos, CA, USA). ANOVA analysis of the data was performed using JMP3 software (SAS Institute Inc., Cary, NC, USA).

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