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A missense mutation disrupting a dibasic prohormone processing site in pro-opiomelanocortin (POMC) increases susceptibility to early-onset obesity through a novel molecular mechanism

Benjamin G. Challis[1,†], Lynn E. Pritchard[2,†], John W.M. Creemers[3,†], Jerome Delplanque[4], Julia M. Keogh[1], Jian'an Luan[5], Nicholas J. Wareham[5], Giles S.H. Yeo[1], Sumit Bhattacharyya[1], Phillipe Froguel[4,6], Anne White[2], I. Sadaf Farooqi[1] and Stephen O'Rahilly[1,*]

[1]University Departments of Medicine and Clinical Biochemistry, Addenbrooke's Hospital, Cambridge CB2 2QQ, UK, [2]School of Biological Sciences and Faculty of Medicine, Stopford Building, University of Manchester, Manchester M13 9PT, UK and AstraZeneca, Mereside, Alderley Park, Cheshire SK10 4TG, UK, [3]Laboratory for Molecular Oncology, Department for Human Genetics, University of Leuven and Flanders Interuniversity Institute for Biotechnology, Herestraat 49, B-3000 Leuven, Belgium, [4]Institute of Biology–CNRS 8090, Pasteur Institute of Lille, CHRU of Lille, France, [5]Institute of Public Health, Cambridge, UK and [6]Barts and The London Genome Centre, Queen Mary's, University of London, UK

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The functional loss of both alleles of the human pro-opiomelanocortin (*POMC*) gene leads to a very rare syndrome of hypoadrenalism, red hair and early-onset obesity. In order to examine whether more subtle genetic variants in *POMC* might contribute to early-onset obesity, the coding region of the gene was sequenced in 262 Caucasian subjects with a history of severe obesity from childhood. Two children were found to be heterozygous for a missense mutation, R236G, which disrupts the dibasic cleavage site between β melanocyte-stimulating hormone (β-MSH) and β-endorphin. β-TC3 cells transfected with the mutant *POMC* cDNA produced a mutant β-MSH/β-endorphin fusion protein. This fusion protein bound to the human melanocortin-4 receptor (hMC4R) with an affinity similar to its natural ligands, but had a markedly reduced ability to activate the receptor. This variant co-segregated with early-onset obesity over three generations in one family and was absent in 412 normal weight UK Caucasian controls. Combining the results in UK Caucasians with a new case–control study in French subjects and three previously published reports, mutations disrupting this processing site were present in 0.88% of subjects with early-onset obesity and 0.22% of normal-weight controls. These results suggest that the R236G mutation may confer an inherited susceptibility to obesity through the production of an aberrant fusion protein that has the capacity to interfere with central melanocortin signalling.

INTRODUCTION

The adipocyte-derived hormone leptin mediates its anorexic and metabolic effects, at least in part, by inducing the expression of pro-opiomelanocortin (POMC)-derived melanocortin peptides in neurons of the hypothalamic arcuate nucleus (1–3). Activation of the melanocortin 4-receptor (MC4R) by melanocortin peptides or synthetic agonists suppresses food intake, while the endogenous antagonist AgRP (Agouti-related peptide), or synthetic antagonists, increase feeding (4). *MC4R*$^{-/-}$ mice are hyperphagic, obese and hyperinsulinemic, and heterozygous littermates are also obese (5). In humans, mutations in this gene represent the most common form of severe monogenic obesity thus far described, with most

*To whom correspondence should be addressed at: University Departments of Medicine and Clinical Biochemistry, Box 157, Addenbrooke's Hospital, Cambridge CB2 2QR, UK. Tel: +44 1223336855; Fax: +44 1223330598; Email: sorahill@hgmp.mrc.ac.uk
†The authors wish it to be known that, in their opinion, the first three authors should be regarded as joint First Authors.

affected individuals being heterozygous for nonsense or missense mutations (6,7). These observations suggest that signalling through MC4R is tightly regulated and a full complement of receptors is required for normal energy homeostasis. The importance of POMC in the regulation of body fat mass is illustrated by the observation that mice and humans homozygous for loss-of-function mutations in the *POMC* gene develop severe obesity in addition to the expected adrenocorticotrophic hormone (ACTH) deficiency and hypopigmentation (8,9). In both species, heterozygotes appear to be unaffected. Although complete POMC deficiency is rare, four linkage studies have identified a quantitative trait locus (QTL) determining obesity-related traits in a region of chromosome 2 encompassing the *POMC* gene, suggesting that genetic variation at this locus may be associated with more common forms of human obesity (10–13). However, no consistent associations of common polymorphisms in the region of POMC with adiposity have been described (14).

In a mutation screen of *POMC* in a large cohort of UK Caucasian subjects with early-onset obesity, a missense amino acid substitution (R236G) that disrupts the dibasic processing site between β melanocyte-stimulating hormone (β-MSH) and β-endorphin was found in two unrelated probands. Combining the results of two independent case–control studies with previously published results, this mutation appears to confer an increased the risk of early-onset obesity. Functional studies suggest that the R236G mutation may contribute to the development of obesity through the production of an aberrant fusion protein that can interfere with signalling through the MC4R.

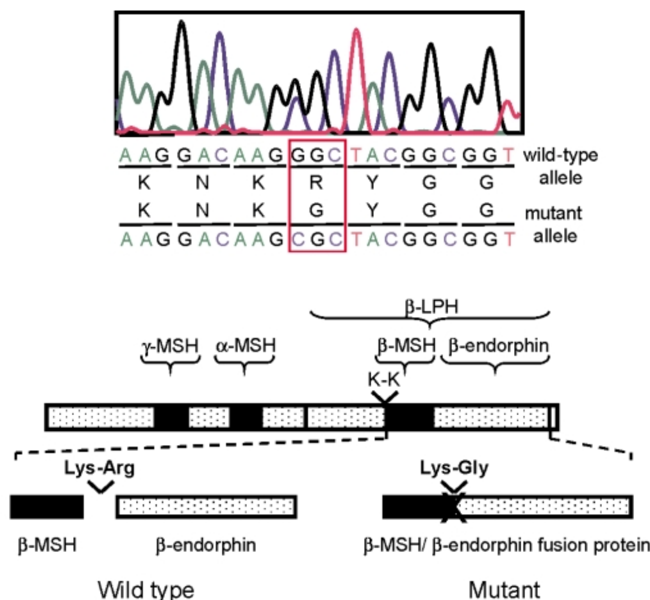

Figure 1. Identification of a cleavage site mutation in *POMC*. Sequence chromatogram derived from subject B showing a G→C substitution resulting in a heterozygous substitution of arginine for glycine at amino acid 236 of POMC. The mutation is predicted to disrupt the prohormone cleavage site (Lys235–Arg236) between β-MSH and β-endorphin (denoted by the 'X'), resulting in a β-MSH/β-endorphin fusion protein. The Lys215–Lys216 dibasic cleavage site is denoted by K–K. MSH, melanocyte-stimulating hormone; β-LPH, β-lipotropin.

RESULTS

Mutational screening of *POMC* in subjects with severe early-onset obesity

The coding regions of the *POMC* gene were examined by direct sequencing in 262 unrelated British Caucasian subjects with severe early-onset obesity. In all cases, obesity was manifest before the age of 10 years and the average body mass index (BMI) of the cohort was >4SD above the age- and gender-adjusted mean. A total of nine heterozygous sequence variants were identified, including three silent variants (C7659T, Ser94Ser; C7726T, Leu116Leu; C7965T, Ala195Ala), three missense mutations (A7654G, Ser93Gly; A8021G, Glu214Gly; C8086G, Arg236Gly), a 9 bp insertion (AGC AGC CGC, Ser-Ser-Gly) between codon 99 and 100, and two variants within the 3′-untranslated region (3′-UTR) (ΔT8211 and C8246T). Of the nine sequence variants, two have not been previously reported (Ser93Gly and ΔT8211). Ser93Gly did not co-segregate with obesity in family members studied (data not shown). The significance of the 3′-UTR sequence variant identified in one obese subject is, as yet, unclear. Two probands were heterozygous for the missense amino acid substitution R236G. Arginine 236 (R236) is highly conserved among all species for which the POMC sequence is known, including human, pig, mouse, rat and sea lamprey. R236G is predicted to disrupt a dibasic processing site between β-MSH and β-endorphin (Fig. 1).

Subject A is an obese 14-year-old girl (BMI standard deviation score (sds) = 3.2) with a history of hyperphagia and early-onset obesity (Fig. 2A). No family members were available for study. Subject B is a 13-year-old boy with early-onset obesity (BMI sds = 2.9). He had a history of hyperphagia but no other features suggestive of a recognized obesity syndrome. Both children were hyperinsulinaemic, but had normal lipid profiles, thyroid function and serum leptin levels appropriate for the degree of obesity. In the family of subject B, of the 16 relatives available for study, all 5 subjects heterozygous for the mutation were obese, while only 1 of the 11 wild-type subjects was obese, and this subject' is obesity was of late onset (Fig. 2B). None of the heterozygous subjects had red hair or any evidence of adrenal impairment. All the heterozygotes had a history of obesity since childhood and, of note, all were reported to have been tall as children—a finding previously noted in subjects with *MC4R* mutations (6).

The presence of this mutation removes a restriction site for *Hha*I, and this was used to design a PCR–RFLP assay. Using this assay, the mutation was not found in 412 unrelated non-obese British Caucasian control subjects (15). In an independent study of 182 French subjects with early-onset obesity (mean BMI = 31.88 ± 6.23; mean age = 11.56 ± 2.96 years) and 384 normal-weight controls (mean BMI = 27.8 ± 2.4; mean age = 56.8 ± 13.5 years), the mutation was present in 1.65% of cases and 1.04% of controls. Three previous studies (16–18) have examined the POMC gene in early-onset obesity in Italian, German and Danish subjects respectively. Echwald *et al.* (18) have reported a single obese subject with a different missense mutation (R236Q) disrupting the same cleavage site. This mutation was absent from control subjects. Table 1 shows

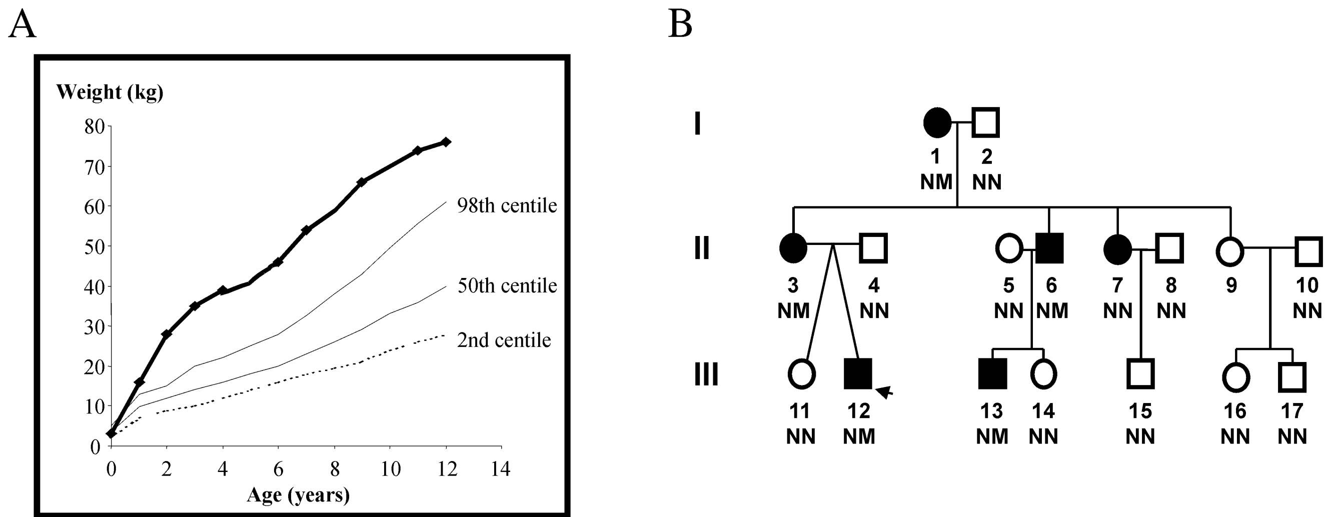

Figure 2. Relationship of R236G with inherited early-onset obesity. (**A**) Weight chart of subject A, showing early development of severe obesity. (**B**) Family tree of subject B (arrow), showing co-segregation of R236G with obesity. Filled symbols represent obese individuals (BMI >30 kg/m^2 for adults, BMI sds score >2.5 for children). One subject (II-7) developed obesity in later life, in contrast to the early-onset obesity seen in all other obese subjects in this family. N, normal allele; M, mutant allele.

Table 1. Frequency of mutations affecting arginine 236 in obese and non-obese subjects

Study	Obese children and adolescents			Non-obese controls		
	Ethnicity	*N*	Prevalence	Ethinicity	*N*	Prevalence
Present study	UK Caucasian	262	2/262 (0.76%)	UK Caucasian	412	0/412 (0%)
Present study	French Caucasian	182	3/182 (1.65%)	French Caucasian	384	4/384 (1.04%)
del Guidice *et al.* (16)	Italian	87	1/87 (1.15%)	—	—	—
Echwald *et al.* (18)	Danish	156	1/156 (0.6%)	Danish	585	0/585 (0%)
Hinney *et al.* (17)	German	96	0/96 (0%)	German	106	0/106 (0%)
Totals		783	7/783 (0.88%)[a]		1487	4/1487 (0.26%)[a]

Statistical significance was not reached for any of the studies analysed individually. Pooling the data from these studies revealed a prevalence of mutations of 0.88% in subjects with early-onset obesity and 0.22% in normal-weight controls.
[a]$P = 0.046$, odds ratio = 3.34 (90% confidence interval 1.02–12.26).

the frequency of mutations affecting arginine at position 236 in all studies reported to date. Pooling the data from of these studies revealed a prevalence of mutations of 0.88% in subjects with early-onset obesity and 0.22% in normal-weight controls (odds ratio = 3.34, 90% confidence interval 1.02–12.26, $P = 0.046$).

R236G prevents the correct processing of POMC and results in an aberzrant fusion protein

R236G comprises part of a conserved dibasic amino acid cleavage site, Lys235–Arg236, that is recognized by the subtilisin-like serine protease prohormone convertase 2 (PC2) and to a lesser extent PC1 (19,20). Normally, endoproteolytic cleavage at this site is an essential step in the release of the POMC-derived peptides β-MSH and β-endorphin. To examine the effect of R236G on POMC processing, β-TC3 cells were transfected with C-terminally epitope-tagged mutant or wild-type *POMC* cDNA, either alone or co-transfected with PC2 and its chaperone, 7B2 (Fig. 3). Immunoprecipitation of radiolabelled Myc-tagged POMC-related peptides from cells expressing wild-type POMC produced a 4 kDa protein, the size expected for β-endorphin (Fig. 3: lane 2). In addition, uncleaved POMC and partially cleaved intermediates were observed—most likely due to saturation of the endogenous processing enzymes. Co-transfection with PC2/7B2 resulted in considerably reduced amounts of these intermediates (Fig. 3: lane 4). When the mutant POMC construct was transfected, processing of POMC into mature β-endorphin was not observed (Fig. 3: lanes 3 and 5). However, a 6.5 kDa species was identified corresponding to the size of a β-MSH/β-endorphin fusion protein that would be the predicted product from aberrant processing at the mutant Lys235–Gly236 cleavage site (Fig. 3). The presence of β-lipotropin (Fig. 3: lanes 2, 3 and 5) probably reflects inefficient cleavage at the N terminus of β-MSH (Lys215–Lys216) in both wild-type and mutant POMC. However, cleavage at the Lys235–Arg236 site prevents the

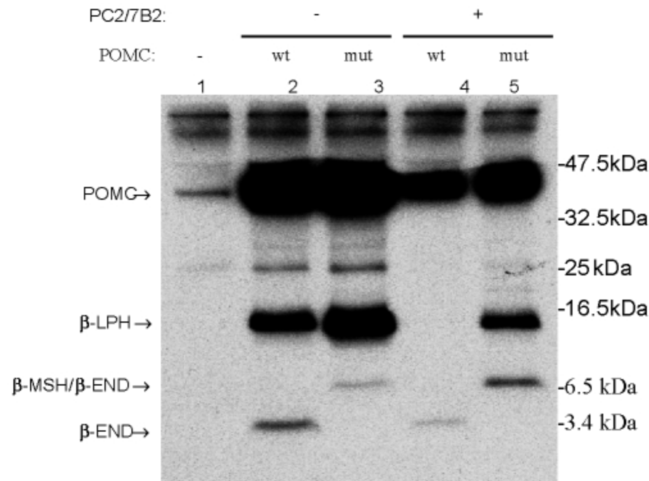

Figure 3. Arg236Gly prevents the correct processing of POMC and results in a mutant β-MSH/β-endorphin fusion protein. β-TC3 cells were transfected with an empty vector (lane 1), C-terminally, Myc epitope-tagged wild-type POMC or R236G POMC alone (lanes 2 and 3) or together with PC2/7B2 (lanes 4 and 5). [^{35}S]Methionine-labelled proteins were immunoprecipitated with anti-Myc antibody and electrophoresed on a 16.5% Tris/Tricine SDS–PAGE gel. Wild-type POMC is correctly processed to β-endorphin (3.4 kDa). The mutant POMC is unable to generate any normal β-endorphin but produces a 6.5 kDa species corresponding to the predicted size for a β-MSH/β-endorphin fusion protein. Higher-molecular-weight bands correspond to β-lipotropin (13 kDa) and unprocessed intermediates.

generation of this fragment in wild-type POMC in the presence of recombinant PC2/7B2 (Fig. 3: lane 4).

β-MSH/β-endorphin fusion protein is capable of binding hMC4R with high affinity

In order to examine the properties of this β-MSH/β-endorphin fusion protein, a synthetic peptide corresponding in sequence to the predicted mutant product was synthesized. For competition binding assays, membranes prepared from CHOK1 cells stably expressing hMC4R were incubated with [^{125}I]NDP–α-MSH in the presence of increasing concentrations of NDP–α-MSH (a synthetic, highly potent α-MSH analogue), α-MSH, β-MSH or the β-MSH/β-endorphin fusion protein. As expected NDP–α-MSH, had the highest binding affinity for hMC4R ($IC_{50} = 1.46$ nM), whereas the fusion protein ($IC_{50} = 87.4$ nM) exhibited a similar binding affinity for the receptor when compared with the natural ligands, α-MSH ($IC_{50} = 115$ nM) and β-MSH ($IC_{50} = 75.7$ nM) (Fig. 4A).

β-MSH/β-endorphin fusion protein is a weak agonist for hMC4R

To assess the ability of the ligands to generate cAMP through activation of hMC4R, CHOK1 cells stably expressing hMC4R and a pCRE/β-galactosidase construct were treated with increasing concentrations of ligand and assayed for β-galactosidase activity. NDP–α-MSH was highly potent at MC4R ($EC_{50} = 0.634$ nM). α-MSH ($EC_{50} = 10.3$ nM) and β-MSH ($EC_{50} = 8.66$ nM) produced superimposable dose–response curves. In contrast, the ability of the fusion peptide to activate MC4R was markedly reduced ($EC_{50} = 94.7$ nM) when compared with the natural ligands (Fig. 4B).

DISCUSSION

The mutation R236G in POMC is notable as a rare example of a naturally occurring mutation of a processing site in a human prohormone. Such dibasic processing sites are usually highly conserved, and Lys235–Arg236 is no exception, with the equivalent dibasic motif being conserved in all species studied to date, including the sea lamprey. When studied in transfected β-TC3 cells, the mutation results in the complete absence of processing between β-MSH and β-endorphin and the production of an aberrant, non-native fusion peptide containing the sequences of both β-MSH and β-endorphin. While statements about the presence or absence of processing products can be made with confidence, the stoichiometry of conversion is likely to be very different in transfected β-TC3 cells compared with native neurons. Thus, the low efficiency of production of the fusion peptide in our experimental system is unlikely to quantitatively reflect the situation in arcuate nucleus neurons *in vivo*. To the best of our knowledge, mutations affecting a processing site of proinsulin are the only other known examples of inherited defects in prohormone processing due to mutations in a dibasic processing site (21–24). Of note, however, inherited defects in the prohormone processing enzymic apparatus itself have been associated with obesity in both mice (25) and humans (26).

How convincing is the genetic evidence for a link between mutations at the Lys235–Arg236 dibasic site and early-onset obesity? We originally detected the R236G mutation in two of 262 morbidly obese UK Caucasian children and not in 412 non-obese UK Caucasians. Family members of one proband were available for study, and in this family the mutation appeared to co-segregate with early-onset obesity across three generations. However, we did find this mutation in normal-weight French subjects, albeit at a somewhat lower frequency than in French early-onset obese subjects. Notably, in two other studies of the *POMC* gene in independent populations, mutations disrupting this processing site have been found in early-onset obese, but not normal-weight, subjects (16,18). This includes an independent mutational event, R236Q, in an obese Danish subject. With low-frequency variants such as these, it is very difficult to obtain genetic epidemiological data that produce an unequivocal answer. Nonetheless, by pooling the data from five studies that have examined the *POMC* gene in early-onset obese subjects, we found a statistically significant increased risk of obesity in carriers with mutations that disrupt R236. These data, when taken together with the extreme conservation of arginine at position 236 and the clear evidence for likely functional consequences of its disruption, make a link between the genotype and susceptibility to obesity very likely. Indeed, it is possible that the genetic epidemiological studies thus far undertaken, in which control subjects tended to be older than cases, may have underestimated the effects of this mutation. In subjects with MC4R mutations, the maximal impact on body weight occurs in childhood, with the phenotype lessening in severity with age (6). If this is also the case with the Lys235–Arg236

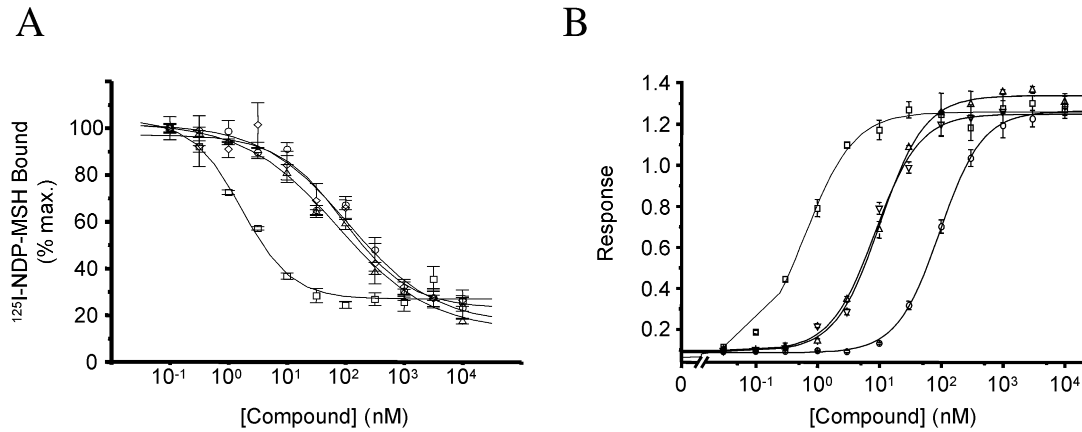

Figure 4. Properties of the β-MSH/β-endorphin mutant peptide at the human MC4R. (**A**) Receptor binding. Membranes isolated from CHOK1 stably expressing hMC4R were incubated with [^{125}I]NDP–α-MSH in the presence of increasing concentrations of ligand. The binding affinity of the mutant peptide [IC_{50} = 84.7 nM (38.3–197 nM)] was comparable to that of α-MSH [IC_{50} = 115 nM (54.9–241 nM)] and β-MSH [IC_{50} = 75.7 nM (48.6–118 nM)]. Datapoints represent means of triplicate measurements ± SD. Squares, NDP–α-MSH; upright triangles, α-MSH; inverted triangles, β-MSH; circles, β-MSH/β-endorphin fusion peptide. (**B**) Receptor activation. CHOK1 cells stably expressing hMC4R and a pCRE/β-galactosidase reporter construct were treated with increasing concentrations of ligand. The EC_{50} for the mutant peptide [94.7 nM (87.2–103 nM)] was ~10–11-fold higher then that seen with the natural ligands, α-MSH [EC_{50} = 10.3 nM (9.57–11.1 nM)] and β-MSH [EC_{50} = 8.66 nM (7.55–9.93 nM)]. Datapoints represent means of quadruplicate measurements ± SD. Squares, NDP–α-MSH; upright triangles, α-MSH; inverted triangles, β-MSH; circles, β-MSH/β-endorphin fusion peptide.

variant then the failure to age-match cases and controls could act to obscure a true effect. The low prevalence of this mutation means that it cannot in any way be postulated as a basis for the positive linkage data concerning the locus on 2q21 encompassing the *POMC* gene and familial obesity or related traits. Indeed, several studies of common polymorphisms in or near the *POMC* gene have failed to demonstrate any significant association with obesity or related intemediate traits (14,18). However, those data are not directly germane to the question of whether uncommon, but not vanishingly rare, variants at R236 can confer powerful, if not fully penetrant, susceptibility to early-onset obesity.

In mechanistic terms, how might this variant affect body fat mass? Hypothalamic signalling through MC4R is a highly regulated nodal point in the control of mammalian energy homeostasis. This is graphically illustrated by the observation that mice and humans with heterozygous null mutations at the MC4R locus are markedly obese (5,6). Given the importance of MC4R in energy homeostasis, it is not surprising that regulation of signalling at this receptor is complex and subtle. It involves the interplay of at least two high-affinity agonists, α- and β-MSH, and a lower-affinity agonist, γ-MSH, as well as the secreted receptor antagonist, AgRP (27). While the central melanocortin system is intolerant to the loss of a single MC4R allele, it appears to tolerate loss of one copy of the *POMC* gene (9). It is therefore surprising that a heterozygous missense mutation in *POMC* appears to predispose to obesity. Our data suggest that in subjects with this particular mutation, not only do they lose one functional copy of β-MSH, but it is replaced by a fusion protein that has the ability to compete for binding to the MC4R with an affinity similar to that of the native ligand. However, as the ability of the fusion protein to activate MC4R signalling is reduced, its presence is likely to shift the balance towards supressed MC4R signalling and thus predispose to obesity (Fig. 5).

While we have concentrated on the examination of the effects of this mutation on MC4R signalling, it is conceivable that R236G might affect other hypothalamic signalling events relevant to energy balance. Thus, interference with MC3R signalling may also have some negative impact on weight homeostasis (28,29). Additionally, mice in which the β-endorphin peptide has been selectively deleted have a modest increase in fat mass, which develops later in life (30). It is possible that the R236G mutation may also interfere with normal β-endorphin signalling in the brain. In this regard, it is notable that there was no clinical evidence for altered pain sensation in the affected subjects.

In summary, heterozygous mutations disrupting the dibasic processing site, Lys235–Arg236, in POMC have now been reported in obese probands from four different ethnic groups. Mutations that disrupt this dibasic site appear to confer an increased risk of early-onset obesity. These mutations are likely to interfere with hypothalamic MC4R signalling through the production of a secreted mutant protein that can compete with natural peptide ligands at their target receptor. While mutations affecting processing sites in proinsulin have been described previously, there has been no suggestion that the mutant product of proinsulin processing might actively interfere with signalling of normal insulin through the insulin receptor (31). Thus, the suggested molecular mechanism whereby the R236G mutation in POMC increases susceptibilty to obesity appears to be novel among inherited endocrine diseases.

MATERIALS AND METHODS

PCR amplification and sequencing

Exons 2 and 3 of *POMC* were PCR-amplified in two separate reactions from genomic DNA isolated from whole blood

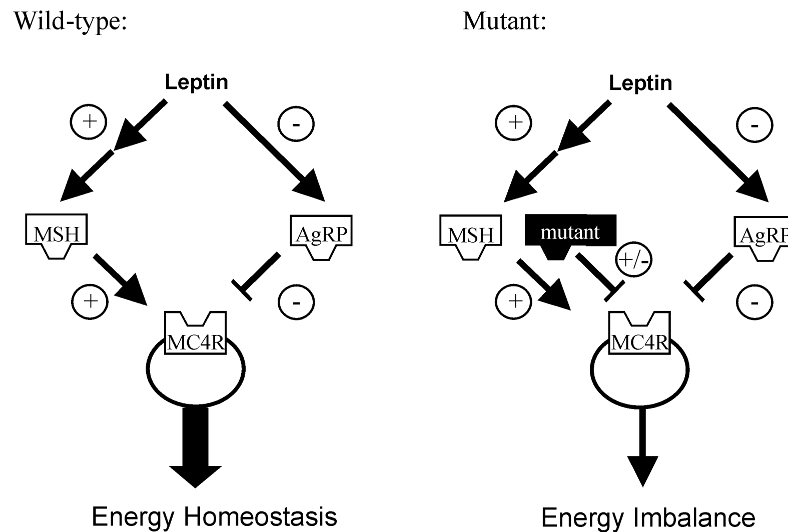

Figure 5. The β-MSH/β-endorphin fusion peptide reduces the effectiveness of the natural agonists by competing for binding at MC4R. Under normal conditions, in response to positive energy balance, plasma leptin levels are increased and act on two distinct hypothalamic neuronal cell populations, inducing the expression of POMC-derived melanocortins and suppressing the expression of AgRP. Melanocortin peptides activate the melanocortin 4 receptor, whereas AgRP antagonizes the receptor. In the presence of the R236G variant in POMC, a mutant β-MSH/β-endorphin fusion peptide is produced. The fusion peptide acts as an agonist with low potency that reduces the effectiveness of the natural agonists by competing for binding at MC4R. This results in reduced signalling through the receptor, thus predisposing to obesity.

with the following primer pairs: POMC X2 Fwd: 5′-TCCTGGTGAGTGGCCAACATT-3′, POMC X2 Rev: 5′-CCAGCTCCAGTCCCATCTAAT-3′, POMC X3 Fwd: 5′-GCTTGACACGCCCGACACTGT-3′ and POMC X3 Rev: 5′-GCATGGAAACCACTGTGCTCC-3′. PCR was performed using BioTaq (Bioline, London) and carried out under standard conditions, with 35 cycles of 95°C for 30 s, 57°C for 30 s and 72°C for 50 s. For exon 2, the primers used for PCR amplification were also used for bidirectional sequencing. Three primers (POMC exon 3A: 5′-CGGCCAGGGCCTAGG-CGCAG-3′, POMC exon 3B: 5′-GCGAAGACTGCGGCC-CGCTG-3′ and POMC exon 3C: 5′-CCTACAGGATGGAG-CACTTC-3′) were used to sequence the PCR product generated for POMC exon 3. All cycle sequencing reactions were performed using an annealing temperature of 50°C. Sequencing was carried out using BigDye terminator chemistry (Perkin-Elmer, Foster City, CA) and electrophoresed on an ABI 377 automated DNA sequencer. Sequences were assembled and examined using Sequencher software (Gene Codes, Ann Arbor, MI). Nucleotides and amino acids were numbered according to GenBank accession nos V01510 and NP_000930 respectively.

RFLP analysis

Genotyping for R236G was done by amplifying a segment of the POMC gene from genomic DNA with primers Fwd: 5′-GCACAGCCTGCTGGTGGCGGCC-3′ and Rev: 5′-CTTGTAGGCGTTCTTGATGATGG-3′ flanking the site of the mutation. Reaction conditions were as follows: BioTaq (2 U/100 µl), NH_4^+ buffer, dNTP (200 µM each) and Mg^{+2} (1.5 mM). Reactions were cycled 35 times at 95°C for 30 s, 63°C for 30 s and 72°C for 45 s. R236G removes a *Hha*I recognition site, and 5 U of *Hha*I was added per 20 µl of PCR product and incubated at 37°C for 5 h in accordance with supplier's instructions. Digests were electorphoresed in 0.5× TBE on a 2.0% agarose gel (Life Technologies). The odds ratio was calculated using a one-sided Fisher's exact test (Epi Info 6).

Construction of expression vector

A clone containing human *POMC* cDNA was obtained from the IMAGE. Consortium Library (IMAGE ID 2075537). *POMC* cDNA was amplified with primers containing an *Eco*RI restriction site at the 5′ ends (POMC cDNA Fwd: 5′-CCGGAATTCGAGAGCAGCCTCCCGAGACAGAGCC-3′ and POMC cDNA Rev: 5′-CCGGAATTCCCTCACTCG-CCCTTCTTGTAGGCG-3′) under the following conditions: primers (0.2 µM), BioTaq (2 U/100 µl), NH_4^+ buffer, dNTP (200 µM each) and Mg^{+2} (1.5 mM). Reactions were cycled 30 times at 95°C for 30 s, 63°C for 30 s and 72°C for 1 min. A 0.9 kb *Eco*RI–*Eco*RI fragment of human POMC was subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen), and the point mutation was introduced according to the instructions of the QuikChange Mutagenesis Kit (Stratagene). The same mutagenesis protocol was used to add a c-Myc epitope tag to wild-type and mutant constructs, between codons 262 and 263, thus avoiding its removal as the result of cleavage at the Lys263–Lys264 processing site. Incorporation of the point mutation and epitope tag was confirmed by nucleotide sequencing.

Metabolic labelling and immunoprecipitation

5–6 × 10^6 β-TC3 cells in 60 cm^2 dishes were transfected using 12 µg DNA and 36 µl lipofectAMINE reagent (Invitrogen Life

Technologies). The next day, cells were metabolically labelled for 1 h with 1 mCi [^{35}S]methionine (ICN; specific activity 1175 Ci/mmol) in 5 ml methionine-deficient RPMI-1640 medium (Sigma), and chased for 1 h by the addition of excess unlabelled methionine. Cells were lysed in 5 ml lysis buffer [50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100 andComplete protease inhibitors (Roche Molecular Biochemicals)]. All samples were precleared with preformed complexes of protein G sepharose (Pharmacia Biotech) and mouse anti-POMC antibody directed against the N terminus of POMC. Specific immunoprecipitation was performed with monoclonal antibody 9E10, directed against the c-Myc epitope. Immunoreactive proteins were subject to 16.5% Tris/Tricine SDS–PAGE.

Radioligand binding assays

The β-MSH/β-endorphin mutant peptide was synthesized (Alpha Diagnostic International, USA) and found to be >99% pure by HPLC. Mass spectrometry confirmed the composition of the peptide. Appropriate dilutions of α-MSH (Sigma), β-MSH (Sigma) and mutant peptide were prepared in 1× assay buffer [25 mM HEPES–KOH pH 7.0, 1.5 mM $CaCl_2$, 1 mM $MgSO_4$, 100 mM NaCl, 0.2% BSA, 1% DMSO and one Complete protease inhibitor tablet (Roche)]. Peptides were aliquoted into 96-well polypropylene well plates (Fisher), along with 15 µg of MC4R CHOK1 cell membranes and 0.01 µCi [^{125}I]NDP–α-MSH in 1× assay buffer. Plates were incubated at 37°C for 2 h. Contents of the assay plates were then transferrred to 96-well filter plates (Fisher), using a 96-well plate harvester (Brandel) following manufacturer's instructions. Filter plates were dried at 37°C for 30 min, and 50 µl of Microscint 40 (Packard) was added to each well. Total counts per minute per well were established using a microplate scintillation counter (Packard).

Reporter assays

CHOK1 cells were stably transfected with full-length human MC4R and a cAMP reporter construct consisting of a cAMP response element and three vasoactive intestinal peptide (VIP) enhancer elements upstream of a *lacZ* reporter gene (kindly provided by Drs M. Needham and D. Scanlan, AstraZeneca, Cheshire, UK). Cells were grown to complete confluence in a T_{175} flask for 5 days in Dulbecco's modified Eagle's medium (Sigma), 10% fetal calf serum, 1% HT supplement (Gibco), 1% non-essential amino acids (Gibco), 200 g/ml G418 (Gibco) and 500 g/ml hygromycin B (Roche). Appropriate dilutions of α-MSH (Sigma), β-MSH (Sigma) and mutant peptide (Phoenix) were prepared in indicator-free DMEM and aliquoted into each well, and the plate was incubated for 5 h at 37°C/5% CO_2. cAMP was detected by addition of 1 mM chlorophenol red-β-D-galactopyranoside (CPRG) (Roche) in buffer containing a final concentration of 40 mM Na_2HPO_4, 40 mM NaH_2PO_4, 7 mM KCl and 0.7 mM $MgSO_4 \cdot 7H_2O$. β-Galactosidase converts CPRG to give a red colour. Results were quantified by reading absorbance at 590 nm on a Spectrafluor (Tecan) plate reader.

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