Obesity and impaired prohormone processing associated with mutations in the human prohormone convertase 1 gene

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Human obesity has an inherited component, but in contrast to rodent obesity, precise genetic defects have yet to be defined[1]. A mutation of carboxypeptidase E (CPE), an enzyme active in the processing and sorting of prohormones, causes obesity in the *fat/fat* mouse[2,3]. We have previously described a woman with extreme childhood obesity (Fig. 1), abnormal glucose homeostasis, hypogonadotrophic hypogonadism, hypocortisolism and elevated plasma proinsulin and pro-opiomelanocortin (POMC) concentrations but a very low insulin level, suggestive of a defective prohormone processing by the endopeptidase, prohormone convertase 1 (PC1; ref. 4). We now report this proband to be a compound heterozygote for mutations in *PC1*. Gly→Arg^{483} prevents processing of proPC1 and leads to its retention in the endoplasmic reticulum (ER). A→C^{+4} of the intron-5 donor splice site causes skipping of exon 5 leading to loss of 26 residues, a frameshift and creation of a premature stop codon within the catalytic domain. PC1 acts proximally to CPE in the pathway of post-translational processing of prohormones and neuropeptides. In view of the similarity between the proband and the *fat/fat* mouse phenotype, we infer that molecular defects in prohormone conversion may represent a generic mechanism for obesity, common to humans and rodents.

PC1 is difficult to access for direct study because its expression is restricted to neuroendocrine tissues. Therefore, the PC1 gene of the proband was analysed. Informed consent and approval by the Cambridge Local Research Ethics Committee was obtained for all studies.

The 14 exons of the proband's *PC1* were characterized by PCR and single-strand conformation polymorphism (SSCP) analysis[5,6]. A variant was detected in exon 13 (Fig. 2*a*) and direct sequencing revealed it to be a heterozygous missense mutation, Gly→Arg^{483} (GGG→AGG^{483}) (Fig. 2*b*). This mutation, which removes a restriction site for *Nla*IV, was absent in 85 unrelated British Caucasian subjects (Fig. 2*c*).

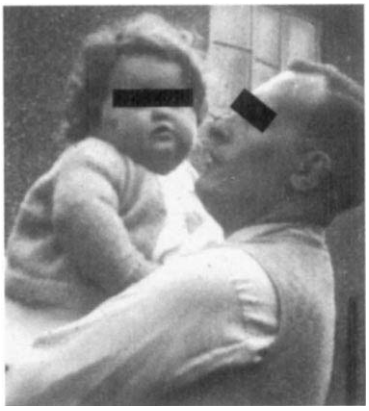

Fig. 1 Severe early-onset obesity. The proband aged 3 years, weighing 36 kg, with her father (now deceased). This photograph is reproduced with the written informed consent of the proband.

The presence of this substitution in three of the proband's four children, all of whom were clinically unaffected, suggested the possibility of an undetected mutation in the other allele. Hence, all 14 exons and intron/exon boundaries of *PC1* were directly sequenced and a heterozygous A→C transversion was found at position +4 of the donor splice site[7] of intron 5 (Fig. 3*a*). This mutation was inherited by the one child who did not inherit the Gly→Arg^{483} mutation, confirming their heteroallelism. The effect on RNA splicing of analogous mutations in other genes is varied, and demonstration of the consequences requires examination of mRNA[8]. Proband mRNA was obtained from a biopsy of duodenal mucosa, which contains endocrine L cells, taken during endoscopic investigation of an unrelated problem. Using PCR

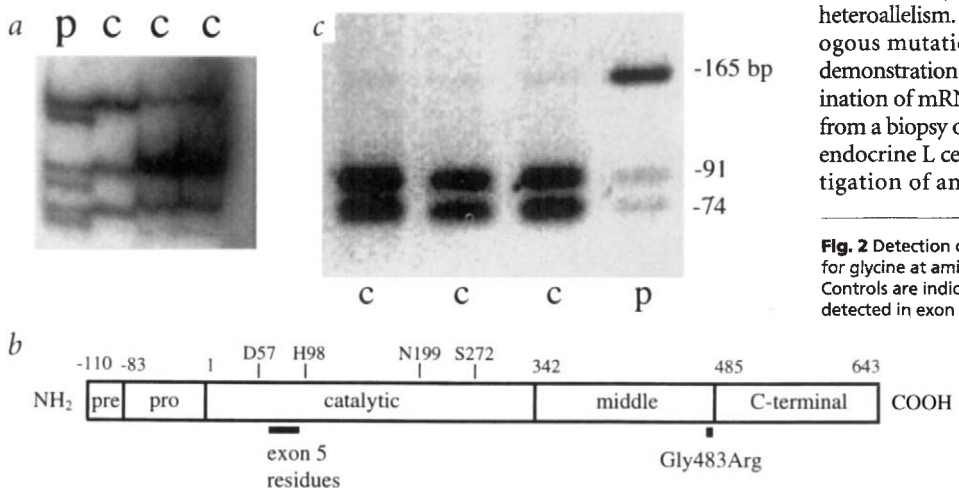

Fig. 2 Detection of a heterozygous substitution of arginine for glycine at amino acid 483 of PC1 in the proband (p). Controls are indicated by '*c*'. ***a***, A variant SSCP was detected in exon 13 of *PC1*. ***b***, The site of the Gly→Arg^{483} mutant in relation to domains and key residues of pro-PC1. The region encoded by exon 5 is also shown. ***c***, This mutation removes an *Nla*IV restriction site in one of the proband's *PC1* alleles. Electrophoresis of *Nla*IV-digested PCR products of genomic DNA, exon 13.

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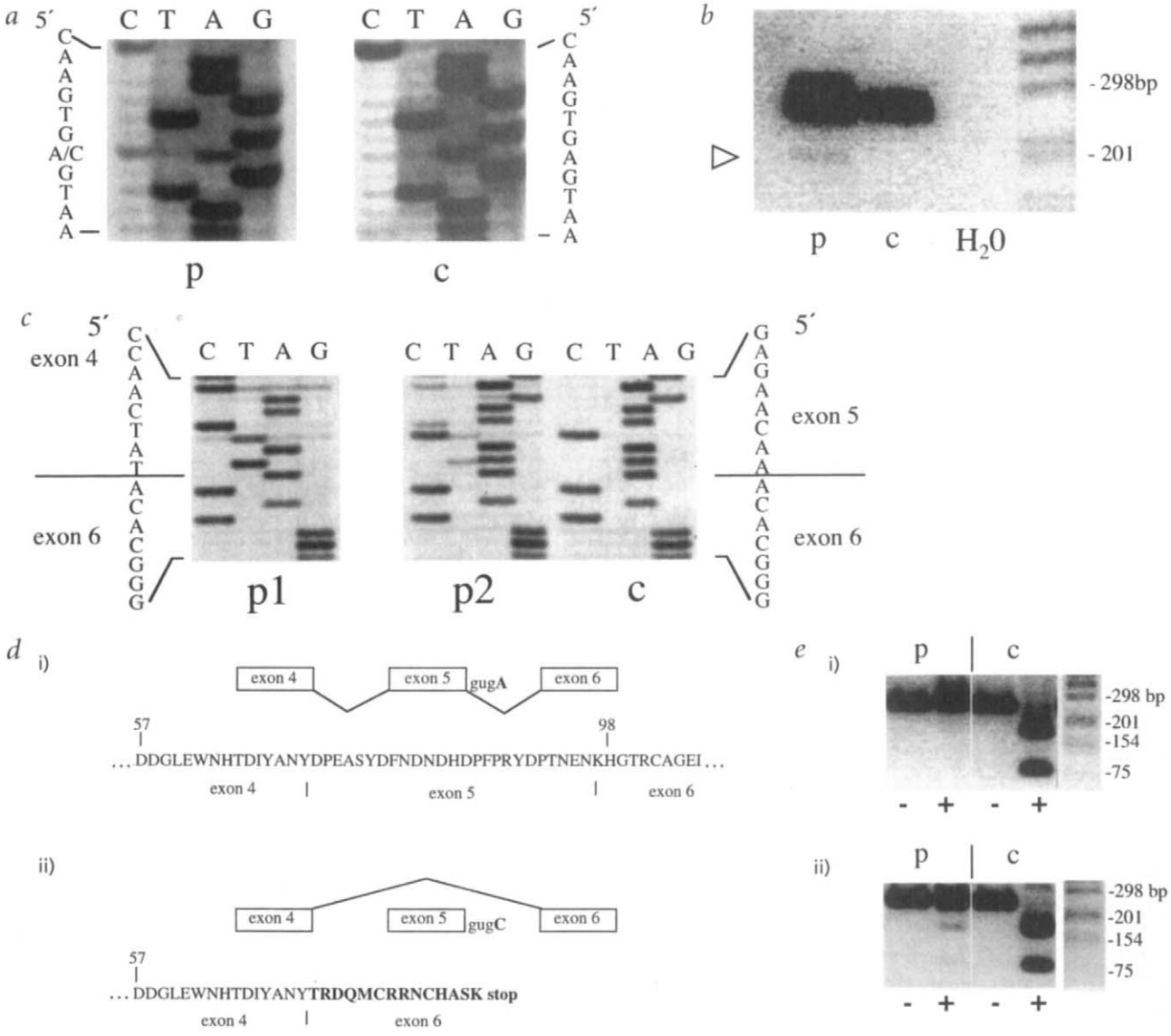

Fig. 3 Detection of a heterozygous splice-site mutation in the other PC1 allele and demonstration of exon skipping in mRNA of the proband (p). Controls are indicated by 'c'. ***a***, Heterozygous A to C transversion at position +4 of the intron 5 donor splice site. ***b***, Electrophoresis of proband cDNA, PCR-amplified using primers to exons 4 and 6, reveals a short product, represented by a faint band (indicated by an arrow), in addition to the expected one. ***c***, Sequencing of this short cDNA species (P1) shows complete absence of exon 5. The wild-type sequence of the larger, more abundant cDNA (P2) indicates normal splicing of exon 5 in the other allele. ***d***, Wild type (i) and mutant (ii) splicing. The skipping of exon 5 leads to deletion of 26 amino acids, a reading frameshift and the introduction of a premature stop codon. ***e***, Negligible full-length mRNA was generated by the mutant splice site allele. cDNA was amplified by PCR with primers designed to amplify sequence from exon 12 and 13. (i) or 4 and 13 (ii). The alleles amplified were identified by nested PCR, using primers in exons 12 and 13, followed by *Nla*IV digestion. (i) The Gly→Arg^{483} allele, identifiable by its loss of restriction digestion, is amplified because it contains exon 5. Although the mutant splice site allele retains this restriction site, digestion products are not seen because the cDNA of this allele fails to amplify; none of it contains exon 5. (ii) cDNA, even if without exon 5, possesses exon 4. PCR with an exon 4 primer therefore amplifies both alleles, confirming that the absence of restriction digestion in (i) is not a technical artefact. +, *Nla*IV digest; –, negative control.

primers designed to amplify sequence between exons 4 and 6, a product of the expected size and another, approximately 80 bp shorter one (Fig. 3*b*), were generated. Sequencing showed this shorter species to contain no exon-5 (Fig. 3*c*). This would cause deletion of 26 residues from the translated protein and a frameshift resulting in the introduction of fourteen aberrant residues and a premature stop codon in the catalytic domain (Fig. 3*d*). Normal splicing and translation may not be completely prevented by donor splice site mutations[9]. In the proband, a residual amount of normal splicing would produce some mRNA that includes exon 5 from the allele containing the splice site mutation. None was found (Fig. 3*e*). The relatively low abundance of aberrantly spliced mRNA is consistent with the presence of a premature stop codon[9] (Fig. 3*b*,*e*).

Gly^{483} is highly conserved; it is present at the topologically equivalent position in all known prohormone convertases (R. Siezen, pers. comm.) (Fig. 2*b*). To examine the effect of the Gly→Arg^{483} mutation on PC1 function, CHO cells were transiently transfected with vectors expressing mutant and wild-type *PC1*.

Normally, the propeptide of PC1 is autocatalytically cleaved in the ER to produce mature PC1, which is subsequently truncated at its C-terminus[10] while in the secretory pathway. Consistent with previous reports[10], immunoprecipitation of PC1 from pulse-labelled cells expressing wild-type PC1 showed two species of 95 kD and 87 kD, representing pro-PC1 and mature PC1, respectively (Fig. 4*a*). In contrast, cells expressing Gly→Arg^{483} PC1 contained only the 95-kD isoform and aberrant material of low molecular weight, which

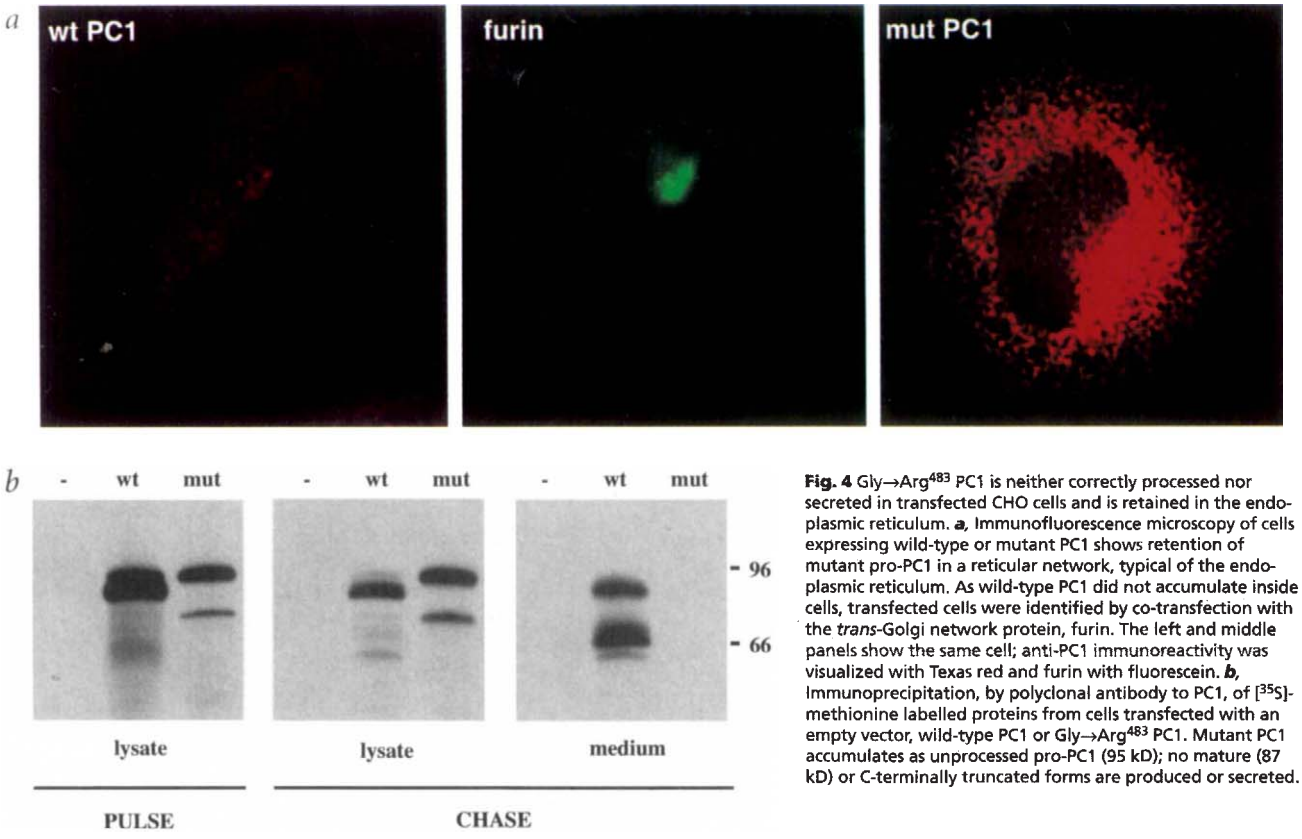

Fig. 4 Gly→Arg^{483} PC1 is neither correctly processed nor secreted in transfected CHO cells and is retained in the endoplasmic reticulum. ***a***, Immunofluorescence microscopy of cells expressing wild-type or mutant PC1 shows retention of mutant pro-PC1 in a reticular network, typical of the endoplasmic reticulum. As wild-type PC1 did not accumulate inside cells, transfected cells were identified by co-transfection with the *trans*-Golgi network protein, furin. The left and middle panels show the same cell; anti-PC1 immunoreactivity was visualized with Texas red and furin with fluorescein. ***b***, Immunoprecipitation, by polyclonal antibody to PC1, of [^{35}S]-methionine labelled proteins from cells transfected with an empty vector, wild-type PC1 or Gly→Arg^{483} PC1. Mutant PC1 accumulates as unprocessed pro-PC1 (95 kD); no mature (87 kD) or C-terminally truncated forms are produced or secreted.

may represent degradation products (Fig. 4*a*). After a 2.5-h chase period, only trace amounts of wild-type pro-PC1 were detectable and most of the intracellular immunoreactive material was mature PC1 (Fig. 4*a*). However, despite plentiful pro-PC1, mature PC1 was undetectable in cells with mutant PC1. The medium of cells expressing wild-type *PC1* contained immunoreactive PC1, corresponding to mature PC1 and C-terminally cleaved forms, but cells expressing Gly→Arg^{483} *PC1* failed to secrete any PC1-related products (Fig. 4*a*).

The location of retained mutant PC1 was determined by indirect immunofluorescence. Consistent with the rapid secretion of wild-type PC1, no compartment-specific staining was seen in cells expressing wild-type PC1—which contrasted with the reticular cytoplasmic staining of cells expressing Gly→Arg^{483} PC1 and suggesting retention in the ER (Fig. 4*b*). Failure of propeptide processing of mutant forms of the related convertases furin and PC2 similarly causes accumulation within the ER[11,12]. The failure of mutant PC1 to leave the ER would prevent access to the high calcium concentration and low pH within the granules of the regulated secretory pathway, conditions that are essential for PC1 enzymatic activity[13].

Given the almost-universal requirement for post-translational processing of prohormones and neurotransmitters[14], it is remarkable that the phenotype was not more severe. Other convertases may compensate for reduced PC1 activity (for example, PC2, PC5, PACE 4A and possibly furin[14]), as in the *fat/fat* mouse, where other carboxypeptidases partially compensate for deficient CPE activity[15]. There may be residual PC1 activity due to the proper processing and targeting of some Gly→Arg^{483} PC1 to the secretory granule, mutant PC1 retained in the ER, and the presence of some normal splicing of exon 5 despite the splice site mutation. The last seems unlikely because no full-length mRNA of this allele was detected.

Thus, the defects of prohormone processing seen in the proband are likely to result from her compound heterozygosity for deleterious mutations in *PC1*. The secretion of proinsulin instead of insulin can account for the impaired glucose tolerance and postprandial hypoglycaemia, given its partial insulin-like action, its longer biological half-life and high plasma concentrations after meals. Impaired processing of POMC probably underlies the proband's impaired adrenal function. Her hypogonadotropic hypogonadism may arise from impaired processing of hypothalamic hormones and neuropeptides related to gonadotropin-releasing hormone secretion.

Defects in leptin cause obesity in rodents[1], but the proband's fasting serum leptin concentration was 45 ng/ml, which is appropriate for her body mass index. The similarity of the proband and *fat/fat* mouse phenotypes is intriguing because PC1 and CPE cooperate in prohormone processing. Products of PC1 and CPE action have been implicated in the neuroendocrine control of energy balance and include αMSH[16,17] and GLP-1[18] derived from POMC and proglucagon, respectively. Further investigation may identify a common molecular mechanism underlying obesity associated with genetic defects in *CPE* and *PC1*.

Methods

PCR SSCP. Genomic DNA was prepared from peripheral white blood cells by a standard procedure using phenol, chloroform, alcohol before PCR[6] and SSCP with MDE gel (AT Biochem) at room temperature and 6% polyacrylamide gel at 4 °C[19].

Sequencing of genomic DNA. DNA was amplified by PCR with a biotinylated primer. Single-stranded DNA was captured and sequenced with Dynal beads, Sequenase v. 2.0 (USB) and ^{35}S α-dATP. The primers for PCR and nested sequencing follow (5'→3') and are in the order corresponding with their '5 biotinylated', 'sequencing' and 'amplification' purposes, their respective exons are indicated. (S) indicates sense and (AS) indicates antisense product. 1 (S) GTTTCTTGAAAGTGGAAACT, CCAGGAGTGGTCTAGAG, CAATATCGGAGTATAACTAC. 2 (S) TAAGCTAGAGTATTGGTTTG, TGAGTTTTAAACTAGTC, GTTGCCTATCTCTAAGTTAG. 3 (S) CATAGTCCTTCTGTAAGGTAC, AAGGGTACTGGAGATAG, CAATCCCTTCTTCTCACTGA. 4 (S) TGAGCACTGGAATGTGGATG, GGTTGGAACTGAAGTGCCCA, AAAGAGTAGAGTGACCCAAG. 5 (AS) TTTGAGCTATCAGCCAGGAT ACTG-

GAATGTGGATGAA, GTGGTATGGATGTTGTGCAT. 6 (S) TCACATTAAAATGGCAAGCT, TCATTCATATGCAAAAC, ACCTATGCCCCATTAATTCA. 7 (AS) TGTCCATGTACATACTGACA, ATTCCATGTAACCTAAG, ACATCAAGCTTAAGCGAATC. 8 (AS) TGTCCATGTACATACTGACA, GTCGTACCAAAGGTCAG, ACATCAAGCTTAAGCGAATC. 9 (S) TATCAAGCTTTCTGGGCCT, GCTGAGTTTCCTGGTCATAGCA, GCTGAGTTTCCTGGTCATAGCA. 10 (AS) ACTTTGGTCGAGCTTCCCCT, CAGAATGGCAAACATAG, TTGCTTCAAATTGTACATGC. 11 (AS) CGAAGGAAGTTTGGATATACT, GAAATCAACCTTAAAAG, CCCTAATTAATGATGAAATCAACC. 12 (AS) CGAAGGAAGTTTGGATATACT, AATCAGTTATTTGAATC, CCCTAATTAATGATGAAATCAACC. 13 (AS) ACACATACTAAATGTAGGTA, GGGTACAGCTTCACTGACTA, CCCTATCCATGTTTGACTTA. 14 (AS) ACACATACTAAATGTAGGTA, ACAACCACTTCAGACACAGG, GTGCAGACAGGAAAGATGTG.

Restriction enzyme analysis. Genomic DNA was amplified by PCR with primers designed to amplify between intron 12 (5'–ACACATACTAAATGTAGGTA–3') and exon 13 (5'–CTTCTGCATTCTGAACAGT–3') (0.5 μM). Reactions conditions were as follows: BioTaq (2 U/100 μl), NH_4 buffer, dNTP (100 μM each), Mg^+ (4.0 mM). Reactions were cycled 35 times, at 94 °C for 30s, 54 °C for 45 s and 72 °C for 30s, *Nla*IV (8 U) (New England Biolabs) per 100 μl of PCR product was then added and incubated at 37 °C for 3 h in accordance with the suppliers instructions. Digests were electrophoresed in 1× TBE on 2.5% agarose (Life Technologies) and stained with ethidium bromide.

Detection of exon skipping. Total RNA was extracted with TRIzol (Life Technologies) from duodenal mucosa ground under liquid nitrogen, according to the manufacturer's instructions. This was reverse-transcribed using an oligo-dT primer and MMLV reverse transcriptase (Stratagene) and then PCR-amplified with primers designed to amplify sequence between exons 4 (5'–AGCTGGACCTTCATGTGATA–3') and 6 (5'–CTTTGGAATTGTATGCAACT–3'). Conditions: primers 0.5 μM, BioTaq 2 U/100 μl, NH_4buffer, dNTP 200 μM each, Mg 2.0 mM. 94 °C 30 s, 56 °C for 30 s and 72 °C for 30s; forty cycles. Products were electrophoresed through a gel (2.5% agarose/1× TBE) and stained with ethidium bromide.

Sequencing of *PC1* cDNA. cDNA was amplified using PCR primers in exons 4 (5' biotin-AGCTGGACCTTCATGTGATA–3') and 7 (5'–TCCACAGTTTTCCCATCATC–3'). Conditions were as follows: primers (0.5 μM), Pfu (Stratagene; 2.5 U/100 μl), buffer as supplied, dNTP (200 μM each). Reactions were cycled forty times, at 94 °C for 30 s, 58 °C for 30 s and 75 °C for 50 s. PCR products were agarose-gel–purified before used as template in a second round of PCR (same primers), followed by further gel purification and extraction with QIAquick (Qiagen). These products were sequenced, using an antisense primer to exon 6 (5'–CATTGGAATTGTATGCAACT–3').

Nested PCR. cDNA was amplified by nested PCR in which the first round used a primer pair to exons 4 (5'–AGCTGGACCTTCATGTGATA–3') and 13 (5'–CTTCTGTCATTCTGAACAGT–3'), or a pair to exons 5 (5'–GGCTAGCTATGATTTTAATG–3') and 13 (5'–CTTCTGTCATTCTGAACAGT–3'). Conditions were as follows: BioTaq (Bioline; 2U/100 μl), NH_4 buffer, Mg^{2+} (1.5 mM), dNTP (200 μM). Reactions were cycled 40 times at 94 °C for 35 s, 57 °C for 30 s and 72 °C for 80 s. Alleles amplified using these primer combinations were verified by a second round of PCR using diluted products of the first reaction and primers to exons 12 (5'–GAGAACGGGATACATCTCCT–3') and 13 (5'–CTTCTGTCATTCTGAACAGT–3'), followed by *Nla*IV digestion.

Construction of expression vector. A 2.9-kb *Xba*I-*Xba*I fragment of human *PC1* (ref. 20) was subcloned in pALTER-1 (Promega). The Altered Sites II (Promega) *in vitro* mutagenesis system was used according to the manufacturer. Incorporation of the mutation was confirmed by nucleotide sequencing. Both wild-type *PC1* and mutant *PC1* were cloned in the eukaryotic expression vector pcDNA3 (Invitrogen). Chinese hamster ovary (CHO) cells were transfected using lipofectAMINE (Life Technologies).

Metabolic labelling and immunoprecipitation. Metabolic labelling and immunoprecipitation of PC1 were performed as described previously[21].

Indirect immunofluorescence. Transfected CHO cells were treated for 1 h with cycloheximide (100 mg/L) before fixation in 4% (w/v) paraformaldehyde. Immunofluorescence was performed with polyclonal anti-PC1, as described[22]. Slides were analysed with a Zeiss Axiophot microscope equipped with UV-optics. Images were recorded with a CE200A CCD-camera (Photometrics).

Serum leptin assay. Measurement of leptin concentration was performed in duplicate using a commercial radio-immunoassay (Linco Research).

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