A Single-Base Mutation in the Peroxisome Proliferator-Activated Receptor γ4 Promoter Associated with Altered in Vitro Expression and Partial Lipodystrophy

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Familial partial lipodystrophy (FPLD) results from coding sequence mutations either in LMNA, encoding nuclear lamin A/C, or in PPARG, encoding peroxisome proliferator-activated receptor γ (PPARγ). The LMNA form is called FPLD2 (MIM 151660), and the PPARG form is called FPLD3 (MIM 604367). We now report a 21-yr-old female with FPLD and no coding sequence mutations in either LMNA or PPARG. She was heterozygous for a novel A>G mutation at position −14 of intron B upstream of PPARG exon 1 within the promoter of the PPARγ4 isoform. Her less severely affected father, who had features of the metabolic syndrome and a paucity of limb and gluteal fat, was also heterozygous for −14A>G. This mutation was absent among 600 alleles from normal Caucasians. A minimal bearing the mutation had significantly reduced promoter activity when used to drive reporter expression in in vitro expression in two cell lines, compared with the wild-type sequence. This is the first report of a human mutation in the promoter of a PPARγ isoform. Because the mutation affects PPARγ4 expression and is associated with FPLD, this implies that PPARγ4 might be important for fat depot distribution and metabolism in vivo. (J Clin Endocrinol Metab 89: 5655–5660, 2004)
**Proband’s father.** Her father was 56 yr old and apparently healthy, although he reported a sister and paternal female first cousin with similar physical appearance to the proband. On examination, he was overweight: weight, 94 kg; height, 186 cm; and BMI, 27.2 kg/m². The circumferences of his waist and hip were 110 and 103 cm, respectively, with a ratio of waist to hip circumference of 1.07. His resting blood pressure was 132/78 mm Hg. Like his daughter, he had relative depletion of sc fat from the arms, legs, and gluteal area, with limited truncal fat, although the phenotype was much less severe than that of his daughter. Fasting plasma determinations included: glucose, 4.8 mmol/liter (86 mg/dl); insulin, 8 mU/liter; C-peptide, 0.74 nmol/liter; total cholesterol, 5.8 mmol/liter (224 mg/dl); triglycerides, 1.7 mmol/liter (153 mg/dl); high-density lipoprotein cholesterol, 1.0 mmol/liter (38 mg/dl); and low-density lipoprotein cholesterol, 4.07 mmol/liter (158 mg/dl). Based on the 2001 NCEP criteria (14), he had the metabolic syndrome. DNA sequence of the exons, intron-exon boundaries, and flanking regions of her LMNA gene, using a described procedure (15), was normal.

**Other subjects.** The proband’s 47-yr-old mother and 18-yr-old brother were each clinically and biochemically normal, with normal fat distribution. Neither had clinical or biochemical criteria for diagnosis of the metabolic syndrome. Three hundred additional control DNA samples of
normal subjects of European descent without diabetes or the metabolic syndrome were studied to determine mutation frequency.

**DNA analysis**

Participants from The Netherlands provided informed consent, and all genetic analyses were performed with approval from the University of Western Ontario Ethics Review Board. PPARG was amplified from the proband’s genomic DNA. Oligonucleotides were designed using genomic DNA sequences of human PPARG obtained from GenBank accession numbers AB005520–AB005526, AF548332, and AY043357 and from published sequences (8, 9, 16). PPARG primers spanned all translated exons, namely exon B and exons 1–6, with more than 50 nucleotides at each intron-exon boundary, 400 bp of the PPARG2 promoter, 660 bp that spanned the PPARG3 promoter and exon A2, and the 433-bp PPARG4 promoter. Amplified fragments were purified on a 2% agarose gel (QiAquick Gel Extraction Kit; Qiagen, Mississauga, Ontario, Canada). Direct DNA sequencing was carried out using the dideoxynucleotide chain termination method with the designed primers, with electrophoresis on a Prism 377 Automated DNA Sequencer and analysis using Sequence Navigator software (both from Applied Biosystems, Mississauga, Ontario, Canada). To detect rapidly the PPARG4 promoter mutation in three first-degree relatives of the proband and in 300 control DNA samples, an allele-specific method called SNaPshot (Applied Biosystems) was used (17). The sequence of the common amplification primer was 5'-CTG GGA TAA CAC GTG TGA GCC A. The sequence of the bracketing primer for the 3'-translated promoter was 5'-TTT CTG AAA GGA AAA ACA GAC TAG CTG TG. The sequence of the bracketing primer for the mutant promoter was 5'-TTT CTG AAA GGA AAA ACA GAC TAG CTG TG (mutated nucleotide shown in bold and underlined). The SNaPshot primer sequence was 5'-GGA CTT AAC TTC ACA GCT AGT CT. The procedure was performed as described, with fragment analysis using GeneScan Software (Applied Biosystems).

**In vitro expression studies**

**Expression vector constructs.** The PPARG4 promoter was amplified from subjects with and without the −14A>G mutation using primers. All sequences for promoter studies were derived from GenBank number AY043357. Purified 436-bp products representing wild-type and mutated PPARG4 promoter were ligated into the pCR2.1 vector (Invitrogen, Carlsbad, CA). Plasmid DNA was prepared using the Qiagen kit (Qiagen), and sequencing identified a 433-bp PCR fragment representing the wild-type promoter (9). All other primer sequences for promoter studies were derived from GenBank number accession numbers AB005520–AB005526, AF548332, and AY043357.

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**Statistical analysis**

SAS version 6.12 (SAS Institute, Cary, NC) was used for statistical analysis. A two-way ANOVA was performed using the general linear model computing procedure of SAS, with independent variables being single-nucleotide polymorphism type (wild-type or mutation) and experiment number (first, second, or third experiment). The dependent variable was RLU/B-Gal. Because transfection was done in triplicates in each experiment, each ANOVA cell had three data points.

**Results**

**Identification of mutation in promoter of PPARG4 isoform**

In the genome of the proband, we found a heterozygous nucleotide substitution in PPARG intron B, −14A>G upstream of exon 1 within the PPARG4 promoter (9). All other regions were free of DNA sequence changes. By genotyping, we found that this mutation was also present in the heterozygous father but was absent from the genomes of her unaffected mother and brother and also from 300 normal Caucasian controls. Furthermore, our DNA sequence analysis revealed that the PPARG4 promoter consists of 433 bp rather than 432 bp, as previously published (9), with the difference in size attributable to an extra A nucleotide at position −383 of intron B. This 433-bp sequence length was confirmed in an additional 10 control DNA samples.

**PPARG4 promoter activity**

Adjusted (least squares) means of normalized luciferase activities from triplicate experiments performed on three different days are shown in Fig. 3. Compared with wild-type promoter, the mutant promoter had 60% decreased normalized luciferase activity in 3T3 cells (P < 0.05) and 70% decreased activity in 3T3-L1 cells (P < 0.0001).

**Discussion**

In the PPARG gene of a proband with the FPLD phenotype and type 2 diabetes and marked fat repartitioning, we found the −14A>G heterozygous mutation in the promoter of PPARG4. The clinical phenotype of the subject was notable for a relative excess of sc fat centrally in the upper body and a relative paucity of fat in the gluteal region and extremities, especially dorsally (Fig. 2). This mutation was also found in the genome of her less severely affected father but neither in her clinically normal mother nor brother nor among the genomes of 300 normal Caucasian control subjects. In vitro expression studies showed that this mutation resulted in a significant reduction in promoter activity in both 3T3 and 3T3-L1 cell lines, the latter of which is an adipogenic cell line derived from mouse Swiss 3T3 fibroblasts (19, 20). The findings suggest that noncoding mutations in PPARG are associated with partial lipodystrophy and implicate the PPARG4 isoform as being potentially important in adipocyte biology. Because the mutation is in PPARG, this patient and father have FPLD3.

Differential splicing of PPARG gives rise to four distinct
mRNA isoforms, called PPARγ1, γ2, γ3, and γ4, respectively (Fig. 1). PPARγ1, γ3, and γ4 mRNA species give rise to the identical 477-amino-acid protein, whereas PPARγ2 mRNA gives rise to a 505-amino-acid protein with 28 extra N-terminal amino acids (8–10). Although the expressed protein is the same for each mRNA isoform, the differences in the promoters and noncoding exons result in differential tissue expression. For instance, the well-studied PPARγ2 isoform is primarily expressed in fat and is considered to be the key isoform related to adipose metabolism. In contrast, the recently characterized PPARγ4 isoform, though also expressed in fat, has largely unknown metabolic attributes. Expression studies to identify tissue distribution and relative quantity of the PPARγ4 mRNA have not been possible because the PPARγ4 nucleotide sequence is common to all four PPARγ mRNA isoforms. At minimum, it is known that PPARγ4 mRNA is present in human adipose tissue, because it was detected there by primer extension analysis of total RNA (9). The current findings indicate that deficiency of the PPARγ4 isoform resulting from diminished expression in carriers of the −14A>G mutation is associated with partial lipodystrophy.

The PPARγ4 promoter contains a TATA-like sequence at nt −170 to −166, an AP-1 site at position nt −187 to −184, and a retinoic acid receptor-related orphan receptor response element (RORE) at position nt −303 to −298, all relative to the start site of transcription (9). The latter element has been proven to be regulatory, because overexpression of RORA1, which specifically binds to RORE, resulted in a 40-fold increase in promoter activity in transient transfection assays in several cell lines (9). However, the −14A>G mutation is not located within RORE or in any potential regulatory element. Instead, the mutation is very close to the transcription initiation site and, as such, may affect the transcription initiation complex machinery. Alternatively, an unknown transcription factor might fail to bind and transactivate transcription in the presence of the −14A>G mutation. Detection of such a DNA binding protein would require EMSA, which is beyond the scope of this report. However, our findings highlight the importance of more careful characterization of the PPARγ4 promoter.

The proband’s phenotype was consistent with that seen in other female heterozygotes for coding sequence mutations in PPARγ. To date, four missense mutations in PPARγ resulting in PPARγ deficiency have been shown to cause partial lipodystrophy, namely R425C (2), F388L (3), and V290M and P467L (4, 5). Clinical features of these patients and the two subjects with the −14A>G mutation are shown in Table 1. Careful examination of subphenotypes (also called phenomic examination) has shown that patients with PPARγ mutations (FPLD3) have a distinctive phenotype including a specific distribution of adipose tissue loss, relatively severe biochemical and clinical signs of insulin resistance, and relatively early onset of type 2 diabetes. There have been suggestions that the biochemical phenotype in PPARγ deficiency is out of proportion to the extent of adipose loss, compared with LMNA-associated partial lipodystrophy (1, 3). This would suggest that, in addition to adipose tissue redistribution, PPARγ mutations may have additional independent effects on metabolism.

Furthermore, there appeared to be a difference in phenotypic severity in the PPARγ −14A>G proband and her father. Specifically, the proband’s father had a relatively mild clinical and biochemical phenotype, which contrasted with the proband’s more striking phenotype. This appears to be...
TABLE 1. Clinical and biochemical features in FPLD3 subjects with PPARG mutations

<table>
<thead>
<tr>
<th>PPARG F388L</th>
<th>PPARG R425C</th>
<th>PPARG V290M</th>
<th>PPARG P467L</th>
<th>PPARG Y4 promoter - 14A &gt; G</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>44.3 ± 6.1</td>
<td>1 subject</td>
<td>1 subject</td>
<td>21</td>
</tr>
<tr>
<td>% Female</td>
<td>2 of 4 subjects</td>
<td>1 subject</td>
<td>1 subject</td>
<td>2 and 56</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>N. European</td>
<td>Hispanic</td>
<td>N. European</td>
<td>N. European</td>
</tr>
<tr>
<td>Limb fat</td>
<td>Absent, mainly distal</td>
<td>Absent, mainly distal</td>
<td>Decreased, mainly distal</td>
<td>Decreased</td>
</tr>
<tr>
<td>Facial fat</td>
<td>Present</td>
<td>Present</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>Glutel fat</td>
<td>Present</td>
<td>Present</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>Truncal fat</td>
<td>Increased</td>
<td>Increased</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>Lipodystrophy onset</td>
<td>Females: −15; males: unknown</td>
<td>Early adult</td>
<td>Mid-adult</td>
<td>Early adult</td>
</tr>
<tr>
<td>Diabetes onset (yr)</td>
<td>45.5 ± 6.7</td>
<td>32</td>
<td>17</td>
<td>30.0 ± 4.0</td>
</tr>
<tr>
<td>Hypertension</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Acanthosis nigricans</td>
<td>2/4</td>
<td>Absent</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Hirsutism</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Hepatic steatosis</td>
<td>Absent</td>
<td>Present</td>
<td>Dysmenorrhea</td>
<td>Dysmenorrhea</td>
</tr>
<tr>
<td>Polycystic ovaries</td>
<td>1 of 2 women</td>
<td>Absent</td>
<td>Dysmenorrhea</td>
<td>Dinessenorrhea</td>
</tr>
<tr>
<td>Body mass index</td>
<td>1.0-fold</td>
<td>0.9-fold</td>
<td>1.1-fold</td>
<td>1.0-fold</td>
</tr>
<tr>
<td>Insulin</td>
<td>2.7-fold</td>
<td>1.6-fold</td>
<td>4.6-fold</td>
<td>4.8-fold</td>
</tr>
<tr>
<td>MetS criteria met</td>
<td>Yes, all four</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes, both</td>
</tr>
</tbody>
</table>

MetS, Patient meets criteria for metabolic syndrome as proposed by the National Cholesterol Education Program (14); values for continuous traits are mean ± SEM. Explanation of fold changes for quantitative variables: 1) for PPARG F388L mutation, group means for mutation carriers were compared with group means for family controls from Ref. 3. For other PPARG mutations, individual values or mean for two observations were compared with the upper limit value for normal reference ranges.

Consistent with observations in the small numbers of PPARY-deficient subjects studied so far (2–5). For instance, female carriers of the PPARG F388L mutation appeared to be more severely affected, with respect to anthropometry, hypertension, and dyslipidemia, than male carriers (3). Similar, between-sex differences in phenotype severity have been noted in LMNA-related partial lipodystrophy (1, 21). Although interesting, the anecdotal nature of the between-sex differences in phenotypic severity in PPARY deficiency and in partial lipodystrophy precludes speculation regarding potential mechanisms. Ascertainment of additional subjects and families and specific physiological studies are required.

Finally, we cannot exclude the possibility that there is a defect at another genetic locus that interacts with the PPARY4–14A>G mutation. For instance, Savage and colleagues (22) described heterozygosity for a loss-of-function premature stop mutation in PPARG in a large Caucasian European kindred. However, in that family, the PPARG defect alone was not associated with an abnormal metabolic phenotype. Instead, subjects who were doubly heterozygous for the PPARG defect together with a defect in a separate unrelated gene had severe insulin resistance (22). In an analogous manner, therefore, it is possible that the haploinsufficiency resulting from the PPARY4–14A>G mutation requires a second genetic defect for the affected subjects to express their disease. In our sample, the search for such a second defect is limited by the small family size.

In summary, we have found a rare loss-of-function mutation in the promoter of PPARY4 in a proband with partial lipodystrophy. This extends the range of PPARG mutations (23) and indicates that noncoding mutations in PPARG are associated with the FPLD3 subtype of partial lipodystrophy. Furthermore, the findings provide additional confirmation of the concept that PPARY deficiency is a cause of partial lipodystrophy with associated metabolic disturbances. Finally, the findings implicate the PPARY4 isoform as being potentially important in adipocyte biology.

Acknowledgments

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References

20. Green H, Kehinde O 1976 Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. Cell 7:105–113