A Single-Base Mutation in the Peroxisome Proliferator-Activated Receptor γ4 Promoter Associated with Altered in Vivo 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 either in 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 PPARγ 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 vivo 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 PPARγ expression and is associated with FPLD, this implies that PPARγ might be important for fat depot distribution and metabolism in vivo. (J Clin Endocrinol Metab 89: 5655–5660, 2004)

DUNNIGAN-TYPE FAMILIAL PARTIAL lipodystrophy (FPLD; MIM 151660) is considered to be a monogenic model of the common syndrome of insulin resistance, or metabolic syndrome (1). FPLD is a genetically heterogeneous autosomal dominant phenotype characterized by repartitioning of adipose tissue and is associated with multiple metabolic disturbances. FPLD results from coding sequence mutations either in LMNA, encoding nuclear lamin A/C, or in PPARG, encoding peroxisome proliferator-activated receptor γ (PPARγ) (1). The LMNA form is called FPLD2 (MIM 151660), and the PPARG form is called FPLD3 (MIM 604367). The presence of lipodystrophy in subjects with dysfunctional PPARG missense mutations, such as R425C, F388L, V290M, and P467L (2–5), and in PPARγ-deficient murine models (6, 7) has confirmed the central role of PPARγ in adipogenesis.

The tissue expression of PPARγ mRNA is complex: four PPARγ mRNA isoforms, called PPARγ1, γ2, γ3, and γ4 (8–10), result from different promoter usage and alternative splicing. A map of the mRNA isoforms and promoters of the PPARγ isoforms is shown in Fig. 1. PPARγ1 and PPARγ3 mRNAs are relatively widely expressed (8, 11, 12), whereas PPARγ2 mRNA is expressed exclusively in adipose tissue (8, 13), suggesting that it is functionally important for that tissue. Primer extension studies have confirmed that PPARγ4 mRNA is also present in adipose tissue (9). However, little is known of PPARγ4's potential role in adipocyte biology or metabolism. We now present human genetic evidence suggesting that the PPARγ4 isoform has a role in adipocyte biology. Specifically, in a subject with FPLD3, we found a rare PPARγ mutation within the PPARγ4 promoter that was associated with decreased promoter activity in two cell lines.

Subjects and Methods

Study subjects

FPLD proband. The proband was a 21-yr-old Dutch female. Menarche occurred at age 11, and she has had regular menstrual cycles since then. At about age 13, she was noted to have grossly abnormal fat distribution consistent with FPLD. At age 20, she was diagnosed with type 2 diabetes, and was treated with metformin (1500 mg daily). On examination, she was obese: weight, 109 kg; height, 178 cm; and body mass index (BMI), 34.4 kg/m². Waist and hip circumferences were 130 and 114.5 cm, respectively, with a ratio of waist to hip circumference of 1.14. Her resting blood pressure was 140/65 mm Hg. Clinically, she had excess sc fat on the face, neck, trunk, and abdomen with relative lack of sc fat on the gluteal region, arms, and legs. This was confirmed with magnetic resonance imaging (Fig. 2), which showed excessive and relatively symmetrical deposition of sc fat on the face, neck, and upper trunk, with disproportionate depletion of sc fat in the lower body, especially dorsally in the gluteal region and thigh. She had no acanthosis nigricans or hirsutism. Measurements in fasting plasma included: glucose, 8.5 mmol/liter (153 mg/dl); insulin, 19 mU/liter (normal, <11 mU/liter); C-peptide, 1.22 nmol/liter (normal, <0.9 nmol/liter); total cholesterol, 4.1 mmol/liter (159 mg/dl); triglycerides, 0.94 mmol/liter (85 mg/dl); high-density lipoprotein cholesterol, 0.88 mmol/liter (34 mg/dl); and low-density lipoprotein cholesterol, 2.83 mmol/liter (110 mg/dl). Based on the 2001 National Cholesterol Education Panel (NCEP) criteria (14), she 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.

**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 his 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

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**Fig. 1.** Organizational structure of the PPARG gene on chromosome 3p25 and the four PPARγ mRNA isoforms. *Arrows,* Start site of transcription for the specific mRNA isoform. Exons are indicated as boxes on the genomic map. The schematic structure of the four mRNA isoforms is shown under the genomic map. Exons A1 and A2 are untranslated, and exon B is translated. PPARγ1, γ3, and γ4 mRNAs translate into a 477-amino-acid protein. PPARγ2 mRNA translates into a 505-amino-acid protein with 28 extra amino acids at the N-terminal end.

**Fig. 2.** Magnetic resonance imaging (MRI) tissue scans of the proband at various anatomical sites. A, Sagittal scan of the neck, with the arrow showing a prominent dorsal suprascapular sc fat pad; B, cross-section at the level of the upper thorax, with the arrow showing a layer of dorsal sc fat measuring 4.84 cm at the thickest point; C, cross-section at the abdomen, showing a symmetrical circumferential layer of sc fat measuring 7.59 cm at the thickest point; D, cross-section at the right gluteal region, with the large arrow showing concavity of dorsolateral sc fat pad and the small arrow showing paucity of dorsal sc fat, measuring 0.5 cm at its thickest point; E, cross-section at the right midthigh, with the arrow showing paucity of dorsal sc fat, measuring 0.3 cm at its thickest point; F, cross-section of the right leg, showing a thin layer of sc fat. The scans are consistent with excess sc fat deposition centrally in the upper body, with relative lack of sc fat on extremities and gluteal region and preferential loss of fat dorsally in the lower body.
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. PPARγ was amplified from the proband’s genomic DNA. Oligonucleotides were designed using genomic DNA sequences of human PPARγ obtained from GenBank accession numbers AB005520–AB005526, AF548352, and AY043357 and from published sequences (8, 9, 16). PPARγ 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 PPARγ2 promoter, 660 bp that spanned the PPARγ3 promoter and exon A2, and the 433-bp PPARγ4 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 PPARγ4 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 CAG GTG TGA GCC A. The sequence of the bracketing primer for the mutant promoter was 5′-TTT CTG AAA GGA AAA ATA 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′-AGA CTG AAT TAC TTC ACA GGT AGT CT. The procedure was described as performed, with fragment analysis using GeneScan Software (Applied Biosystems).

In vitro expression studies
Expression vector constructs. The PPARγ4 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 PPARγ4 promoter were ligated into the pCR2.1 vector using the TA Cloning Kit protocol (Invitrogen, Carlsbad, CA). Plasmid DNA was prepared using the QiaGen kit (Qiagen), and sequencing identified a promoter sequence. After digestion with endonucleases ScaI and XhoI, the inserts were directionally subcloned in the luciferase reporter PGL3 basic vector (Promega, Madison, WI). After transfection and purification, sequencing was used to confirm orientation and fidelity of purified plasmid DNA samples for three types of constructs: 5′→3′ wild-type, 3′→5′ wild-type, and 3′→5′ mutation.

Cell lines and culture. Murine cell lines NIH/3T3 (CRL-1658) and 3T3-L1 (CL-173) were obtained from American Type Culture Collection (Manassas, VA). The overall strategy for in vitro expression studies was described previously (18). Cells were maintained in a DMEM (Invitrogen) containing 4.5 g/liter r-glucose, 1.5 g/liter NaHCO3, 25 mM HEPES, 4 mM l-glutamine, and 110 mg/liter sodium pyruvate in a humidified atmosphere of 95% air-5% CO2 at 37°C. Medium was supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (Invitrogen). Each cell line was grown on a 100-mm culture dish (Nunclon; Nalgene Nunc, Mississauga, Ontario, Canada). Cells were seeded on six-well plates to achieve 60% confluence and transfected using calcium phosphate precipitation with one of the three PPARγ/Luc reporter constructs. For each transfection, 2 μg pSV-β-galactosidase vector was cotransfected according to manufacturer’s instructions (Promega). Cells were harvested 48 h post transfection. Transfection experiments were repeated in triplicate, three times on separate days for each cell line. Luciferase activity was assayed according to the manufacturer’s instructions (Promega), and luminescence was determined using a Lumat LB9507 luminometer (Berthold Systems, Pittsburgh, PA). Luciferase activities were normalized for transfection efficiencies using reporter luciferase units (RLU) per unit of activity of β-galactosidase (B-Gal). The β-galactosidase enzyme assay system was used according to manufacturer’s directions (Promega).

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 PPARγ4 isoform
In the genome of the proband, we found a heterozygous nucleotide substitution in PPARγ intron B, −14A>G upstream of exon 1 within the PPARγ4 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 PPARγ4 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.

PPARγ4 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 PPARγ 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 PPARγ4. 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 PPARγ are associated with partial lipodystrophy and implicate the PPARγ4 isoform as being potentially important in adipocyte biology. Because the mutation is in PPARγ, this patient and father have FPLD3.

Differential splicing of PPARγ gives rise to four distinct
FIG. 3. In vitro expression studies of wild-type and mutant PPARγ promoter. Normalized RLU/B-Gal are shown for three sets of triplicate experiments for two cell lines: 3T3 (A) and 3T3-L1 (B). Adjusted (least squares) means ± se values are shown for luciferase expression studies of wild-type and mutant PPARγ promoter. A negative control construct made by cloning of wild-type promoter 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 RORα1, 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 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...
consistent with observations in the small numbers of PPARγ-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, severely affected, with respect to anthropometry, hypertension, and dyslipidemia 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 PPAR gene had severe insulin resistance (22). In an analogous manner, therefore, it is possible that the haploinsufficiency resulting from the PPARG4 – 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 PPARG4 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 PPARγ deficiency is a cause of partial lipodystrophy with associated metabolic disturbances. Finally, the findings implicate the PPARG4 isoform as being potentially important in adipocyte biology.

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References


| TABLE 1. Clinical and biochemical features in FPLD3 subjects with PPARG mutations |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                | PPARG F388L     | PPARG R425C     | PPARG V290M     | PPARG P467L     | PPARG γ4        |
| No.                            | 4               | 1               | 1               | 2               | 2               |
| Age (yr)                        | 44.3 ± 6.1      | 1 subject       | 1 subject       | 1 subject       | 21 and 56       |
| % Female                        | 2 of 4 subjects | Hispanic        | N. European     | N. European     | N. European     |
| Ethnicity                       | N. European     | Hispanic        | N. European     | N. European     | N. European     |
| Limb fat                        | Present         | Absent, mainly distal | Decreased       | Decreased       | Decreased       |
| Facial fat                      | Present         | Absent, mainly distal | Decreased       | Decreased       | Decreased       |
| Gluteal fat                     | Present         | Increased       | Increased       | Increased       | Increased       |
| Trunca1 fat                     | Present         | Increased       | Increased       | Increased       | Increased       |
| Lipodystrophy onset             | Females: –15; males: unknown  | Mid-adult       | Early adult     | Mid-adult       | Early adult     |
| Diabetes onset (yr)             | 45.5 ± 6.7      | 32              | 17              | 30.0 ± 4.0      | 20              |
| Hypertension                    | Present         | Present         | Severe          | Severe          | Absent          |
| Acanthosis nigricans            | 2/4             | Absent          | Present         | Present         | Present         |
| Hirsutism                       | Absent          | Present         | Present         | Present         | Absent          |
| Hepatic steatosis               | Absent          | Present         | Present         | Dysmenorrhea    | Dysmenorrhea    |
| Polycystic ovaries              | 1 of 2 women    | Absent          | Present         | Absent          | Present         |
| Body mass index                 | 1.0-fold        | 9.0-fold        | 1.1-fold        | 1.0-fold        | 1.0-fold        |
| Insulin                         | 2.7-fold        | 1.6-fold        | 6.4-fold        | 4.8-fold        | 1.5-fold        |
| MetS criteria met               | Yes, all four   | Yes             | Yes             | Yes, both       | Yes, both       |

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.
15. Al-Shali et al. • PPARγ4 Promoter Mutation in Lipodystrophy

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