厚生労働科学研究補助金

難治性疾患克服研究事業

リポジストロフィーとミオパチーを合併する新規遺伝性疾患についての疾患概念の確立と治療法の開発に向けた研究

平成２１年度　総括・分担研究報告書

研究代表者　　林　由起子

平成 22 年（2010 年）3 月

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Ⅰ．　総括研究報告

厚生労働科学研究費補助金（難治性疾患克服研究事業）
（総括・分担）研究報告書

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研究代表者　　林　由起子
国立精神・神経センター神経研究所疾病研究第一部　室長

研究要旨

我々は*PTRF*遺伝子変異によるリポジストロフィーとミオパチーを合併する新規遺伝性疾患（cavinopathy）を見いだした（文献添付）。本研究では、この新規疾患の新たな患者を発見し、その頻度ならびに臨床的特徴を明らかにすることによって、疾患概念を確立することを目的に、全国アンケート調査を実施した。その結果、これまでに我々の見いだした５例に加え、確定例1例、疑い例1例の存在が明らかになった。現在さらに疑い例２０例について情報収集を行っており、その結果を踏まえ、本疾患の疾患概念を確立したい。また細胞生物学的解析からは、カベオラの構造形成並びに機能にPTRFを含むキャビンファミリーの相互作用の重要性を示唆する知見がえられている。

研究分担者：　西野　一三
国立精神・神経センター神経研究所
疾病研究第一部　部長

A. 研究目的

我々は、カベオラ構成タンパク質PTRF の欠損がミオパチーとリポジストロフィーを合併する遺伝性疾患を引き起こすことを見いだした。本研究では、この新規疾患について全国規模の調査を行い、その頻度を明らかにするとともに、詳細な臨床病理学的、生理・生化学的解析を行い、本疾患の病態解明を進めるとともに、カベオラの細胞生物学的機能に迫ることを目的とする。さ、カベオラの機能に注目し、新たな治療法の開発を目指す。

B．研究方法

１）疾患の実態把握を目的とした全国アンケート調査

PTRF 欠損症の実態を明らかにするこ

とを目的に、幅広くアンケート調査を行った。

①対象：

・日本糖尿病学会会員（学会より会員 16,158 名分の会員番号・住所・氏名を記載した宛名シールを購入し、アンケート用紙を個別に郵送）

・日本神経学会会員（学会の了承を得て、学会ホームページに掲載してある会員名簿を用いて、各会員にアンケート用紙を個別に郵送）

・日本小児科学会（研修指定施設の住所の宛名シールを学会より購入し、アンケート用紙を施設宛に郵送；526 件）

・日本小児神経学会（学会の了承を得て、会員名簿を用いてアンケート用紙を個別に郵送）

・日本小児内分泌代謝学会（学会のホームページに掲載

・日本内科学会（内科専門医会メーリングリストを用いて調査;約 2,150 人）

②アンケートの結果解析及び第二次、第三次アンケート調査の実施、およびそのデータ解析

２）疑い例の *PTRF* 変異解析

臨床的、病理学的に本疾患の疑われる症例について、ゲノム DNA を用いて PTRF 変異の有無を解析した。

３）細胞生物学的解析

患者骨格筋、マウス骨格筋および培養細胞を用いて、下記の解析を行った。

・患者骨格筋におけるキャビンファミリータンパク質の発現解析

・カベオリン－３欠損マウス骨格筋ならびに培養細胞を用いた PTRF 関連分子の発現解析

・患者骨格筋及びマウス培養細胞を用いた代謝関連分子の発現解析

（倫理面への配慮）

本研究において使用する全ての臨床情報は、国立精神・神経センター倫理委員会で承認された所定の承諾書を用いて、患者あるいはその親権者から遺伝子解析を含む研究使用に対する検体の使用許可（インフォームドコンセント）を得たものを用いる。遺伝子解析に関しては「ヒトゲノム解析研究に関する共通指針」を遵守した上で、施行される。これらの情報を使用するに当たっては、プライバシーを尊重し、連結可能匿名化した上で使用する。得られた研究結果は公開する。すべての組み換え DNA 実験は、カルタヘナ議定書に基づく「遺伝子組み換え生物等の使用等の規制による生物の多様性の確保に関する法律」と関係省令を遵守し、国立精神・神経センター神経研究所組み換え DNA 実験安全委員会の審査・承認を得る。

C. 研究結果

1）全国アンケート調査

全国アンケート調査の結果は別紙に示した。

第一次アンケート調査の結果、本疾患の臨床的特徴を呈する947件について第二次アンケート調査を、さらに本症を考慮する必要のある 22 件について、第三次アンケート調査を行った。その結果、平成 22 年 2 月時点で 1 例の確定例が神戸大学小児科より報告された。また 1 例、きわめて本例である可能性が高い症例が存在したが、すでに経過観察されておらず、臨床情報は得ることができたが、遺伝子解析は不可能であった。現在、その他の症例について追加情報の取得ならびに遺伝子解析の可否について主治医と連携を取り検討中である。

2）臨床・筋病理学的に本疾患の疑われる下人不明の筋疾患患者 14 例、ならびに京都大学 海老原先生との共同研究で、原因不明のリポジストロフィー患者 8 例の変異解析を行ったが、*PTRF*変異例は見いだせなかった。

3）細胞生物学的解析

①キャビンファミリータンパク質の発現

本疾患の報告後、キャビンに少なくとも4つのファミリータンパク質;本疾患の原因遺伝子産物である PTRF (cavin-1)、SDR (cavin-2)、SRBC (cavin-3)、MURC (cavin-4)が存在することが報告された(Bastiani M, et al. J Cell Biol 2009)。そのうち心筋・骨格筋に強く発現している MURC (cavin-4)の研究を精力的に続けている京都大学 上山先生のグループとの共同研究を開始し、PTRF と MURC の骨格筋における機能と疾患との関連について現在研究を進めている。 これまでのところ、PTRF 欠損筋、およびカベオリン－3欠損マウス筋の細胞膜ではカベオリン－3 の異常とともに SDR および MURC の筋細胞膜での発現が低下しており、これらのタンパク質の相互用がカベオラの形成及び機能に重要であることが示唆されている（論文投稿準備中)。

②骨格筋におけるエネルギー代謝とキャビン・カベオラに関する研究

骨格筋は肝臓とともにエネルギー代謝に重要な役割を担っている。特にカベオラは脂質代謝に重要であることが示唆されている。そこで骨格筋における代謝関連分子の変化を患者筋とコントロールで比較し,興味深い結果がでつつある。今後さらに検討を進めていく予定である。

D. 考察

アンケート調査、ならびに疑い例の変異解析の結果、本疾患は極めて稀な

疾患であることが明らかになった。しかしながら、アンケート調査を幅広く行うことによって、本疾患の存在についての理解を深めることが可能となったと考えられる。

現在、アンケート調査で見いだされた新たな疑い例について、さらなる患者情報の集積中である。本研究の最終目標である「疾患概念の確立」には未だ至っていないが、新たな患者の発見並びに基礎医学的知見も徐々に得られつつある。病態の解明、治療法の開発には、主治医との連携を密にし、継続的な臨床情報の収集に努めるとともに、基礎医学的見地からの病態解明へむけた更なる取り組みを積極的に進めていく予定である。

E. 結論

リポジストロフィーと筋ジストロフィーを合併するcavinopathyは極めて稀な疾患であるが、臨床的重症度やその症状を考慮すると、社会医学的には極めて重要な疾患であると考える。今後も症例の集積を積極的に進めていくことで、診断基準の確立を図っていきたいと考えている。

また、基礎医学的解析から、細胞生物学的に重要な役割を担っているカベオラの機能について、さらなる知見を積み重ねていく予定である。

F. 健康危機情報

特になし

G. 研究発表

1. 論文発表

なし

2. 学会発表

・Hayashi YK, et al: A Novel Muscular Dystrophy Associated with Secondary Deficiency of Caveolin. 9th Annual Asian and Oceanian Myology Center Scientific Meeting, Seoul, Korea, 3.25, 2010 (予定)

・Hayashi YK, et al: *PTRF* mutations cause lipodystrophy with muscular dystrophy. 14th International Congress of Endocrinology Official Satellite Symposium; Obesity and Metaboloc Syndrome, Kyoto, Japan, 3.30-31, 2010 (予定)

・林　由起子、他：第 107 回日本内科学会講演会,東京,2010 年 4 月 9 日(予定)

・林　由起子、他：第51回日本神経学会総会,東京,2010年5月20-22日（予定)

・Hayashi YK, et al: PTRF Mutations Causes A Novel Disease Associated With Muscular Dystrophy And Lipodystorphy.

XII International Congress of Nueromuscular Diseases, Naples, Iatly, Jul 17-22, 2010 (予定)

・Hayashi YK, et al: PTRF Mutations Causes A Novel Disease Associated With Muscular Dystrophy And Lipodystorphy. 15th International Congress of the World Muscle Society, Kumamoto, Japan, Oct 12-16, 2010 (予定)

H. 知的財産の出願・登録状況

特になし

Human *PTRF* mutations cause secondary deficiency of caveolins resulting in muscular dystrophy with generalized lipodystrophy

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Caveolae are invaginations of the plasma membrane involved in many cellular processes, including clathrin-independent endocytosis, cholesterol transport, and signal transduction. They are characterized by the presence of caveolin proteins. Mutations that cause deficiency in caveolin-3, which is expressed exclusively in skeletal and cardiac muscle, have been linked to muscular dystrophy. Polymerase I and transcript release factor (PTRF; also known as cavin) is a caveolar-associated protein suggested to play an essential role in the formation of caveolae and the stabilization of caveolins. Here, we identified *PTRF* mutations in 5 nonconsanguineous patients who presented with both generalized lipodystrophy and muscular dystrophy. Muscle hypertrophy, muscle mounding, mild metabolic complications, and elevated serum creatine kinase levels were observed in these patients. Skeletal muscle biopsies revealed chronic dystrophic changes, deficiency and mislocalization of all 3 caveolin family members, and reduction of caveolae structure. We generated expression constructs recapitulating the human mutations; upon overexpression in myoblasts, these mutations resulted in PTRF mislocalization and disrupted physical interaction with caveolins. Our data confirm that PTRF is essential for formation of caveolae and proper localization of caveolins in human cells and suggest that clinical features observed in the patients with *PTRF* mutations are associated with a secondary deficiency of caveolins.

Introduction

Caveolae are specific invaginations of the plasma membrane characterized by the presence of the protein caveolin. To date, 3 caveolin family members have been identified. Caveolin-1 and -2 are coexpressed in many cell types, such as endothelial cells, smooth muscle cells, fibroblasts, and adipocytes, and form a hetero-oligomeric complex (1). In contrast, caveolin-3 is expressed exclusively in skeletal and cardiac muscles (2). Caveolae are involved in several important cellular processes, including clathrin-independent endocytosis, regulation and transport of cellular cholesterol, and signal transduction (3, 4).

Polymerase I and transcript release factor (PTRF; also known as cavin) is a highly abundant caveolae component and is suggested to have an essential role in caveolar formation. In both mammalian cells and zebrafish, knockdown of PTRF leads to a reduction in caveolae density (5). Mice lacking PTRF do not have morphologically detectable caveolae, in addition to a markedly diminished protein expression of all 3 caveolin isoforms (6). Interestingly, PTRF-knockout mice mimic lipodystrophy in humans, demonstrating considerably reduced adipose tissue mass, high circulating triglyceride levels, glucose intolerance, and hyperinsulinemia (6).

Here we report that mutations in *PTRF* (GenBank accession no. 284119) caused a disorder presenting as generalized lipodystrophy and muscular dystrophy. We demonstrate that this condition was associated with deficiency and mislocalization of all 3 caveolin family members and reduction of caveolae structure.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* **119**:2623–2633 (2009). doi:10.1172/JCI38660.

Results

Identification of PTRF mutations. Deficiency of caveolin-3 as a result of *CAV3* gene mutations is known to cause muscular dystrophy (7). We found 5 nonconsanguineous Japanese patients whose muscle showed caveolin-3 deficiency but without *CAV3* mutation among 2,745 muscular dystrophy specimens kept in the muscle repository of the National Center of Neurology and Psychiatry. Importantly, all 5 patients also had congenital generalized lipodystrophy (CGL; also known as Berardinelli-Seip syndrome). From the findings observed in lacking cells and animal models lacking PTRF (5, 6), we screened for *PTRF* mutations.

We identified 2 different frameshift mutations in all 5 patients examined: patients 1–4 (P1–P4) had the same homozygous c.696_697insC (p.K233fs) mutation in exon 2, and P5 harbored a compound heterozygous mutation of the same c.696_697insC and c.525delG (p.E176fs) in exon 2 (Figure 1A). The c.525delG mutation changes the last 275 amino acids to an unrelated 98-amino acid sequence, whereas c.696_697insC substitutes the last 158 amino acids with an unrelated 191-amino acid sequence (Figure 1B). Both mutations were not identified in the chromosomes of 200 Japanese control subjects.

In order to determine whether the common c.696_697insC mutation has the same haplotype, we examined 6 sets of single nucleotide polymorphisms (SNPs) within *PTRF*: rs2062213, rs8070945, rs963988, rs963987, rs963986, and rs9252. All 5 patients had the same haplotype for all 6 SNPs, which occurred homozygously (Table 1). During mutation screening, we found a novel 9-bp insertion polymorphism in the 3′ noncoding region

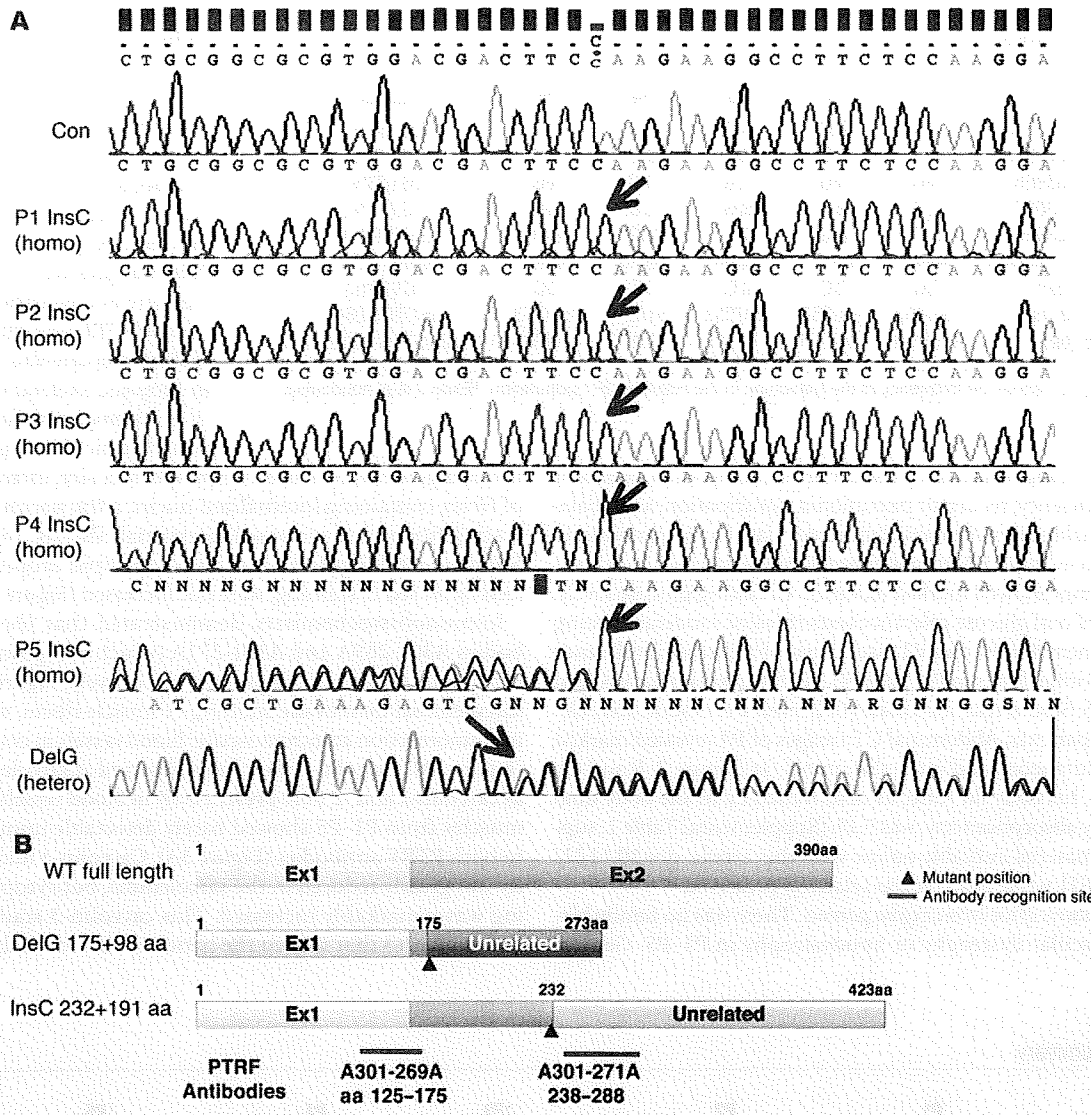

Figure 1

Mutations in *PTRF*. (**A**) All 5 patients had a homozygous or compound heterozygous mutation in *PTRF* (shown by arrows). P1–P4 had the same homozygous insertion mutation of c.696_697insC (InsC) in exon 2, whereas P5 had a compound heterozygous mutation of the same c.696_697insC insertion mutation and a deletion mutation of c.525delG (DelG) in exon 2. (**B**) Schema of the position of mutations in *PTRF*, putative proteins produced by mutations, and antibody recognition sites. The c.525delG mutant changes the last 275 amino acids to an unrelated 98–amino acid sequence, while the c.696_697insC mutant substitutes the last 158 amino acids with an unrelated 191–amino acid sequence.

of *PTRF* (c.1235_1236insTCTCGGCTC). This 9-bp insertion was found heterozygously in 26% and homozygously in 2% of Japanese control individuals. In P1–P5, none had this 9-bp insertion. We also examined 2 microsatellite markers (STS-W93348 and D17S1185) close to *PTRF* and found heterozygosity in the patients (Table 1). From these results, a founder effect may not be likely, although we could not completely rule out the possibility.

Mutation screening of the other genes associated with lipodystrophy and muscular dystrophy. From the clinical and pathological findings, we performed mutation screening for the genes associated with muscular dystrophy and lipodystrophy, including *CAV3*, *LMNA*, *AGPAT2*, *BSCL2*, *CAV1*, *PPARG*, *AKT2*, and *ZMPSTE24*. We found a heterozygous nucleotide change of c.1138G>A (p.D380N) in *BSCL2* in P1. This substitution was also identified heterozygously in 16% of Japanese control individuals, and we believe this to be a novel nonsynonymous SNP. For all the other genes examined, no other mutation was identified in P1–P5.

Clinical features of the patients with PTRF mutations. Clinical information for P1–P5 is summarized in Table 2. Common to all patients was the presence of muscular dystrophy and generalized lipodystrophy. However, despite having the same mutation, the patients' additional symptoms were variable. Generalized loss of subcutaneous adipose tissue in several areas, including the face, was noticed in infancy or early childhood. Hepatosplenomegaly, acromegaloid features, and umbilical prominence were often observed in the patients. No patient showed intellectual deficit or acanthosis nigricans. Patients presented with mild muscle weakness, but with hypertrophy of muscles (Figure 2A). Electrically silent percussion-induced

Table 1
Haplotype analysis

	P1	P2	P3	P4	P5	Control[A]
Intron 1, rs2062213	C/C	C/C	C/C	C/C	C/C	C/C (53%)
Intron 1, rs8070945	C/C	C/C	C/C	C/C	C/C	C/C (78%)
Intron 1, rs963988	G/G	G/G	G/G	G/G	G/G	G/G (33%)
Intron 1, rs963987	G/G	G/G	G/G	G/G	G/G	G/G (31%)
Intron 1, rs963986	G/G	G/G	G/G	G/G	G/G	G/G (34%)
Exon 2, 9-bp insertion[B]	no/no	no/no	no/no	no/no	no/no	no/no (72%)
Exon 2, rs9252[B]	C/C	C/C	C/C	C/C	C/C	C/C (78%)
STS-W93348 (bp)	251/253	251/253	251/253	251/253	251/253	251/253/264
D17S1185 (bp)	219/219	170/219	170/219	170/170	170/203	170/203/215/219/225/237

[A]Percentages denote the frequency of the haplotype in the HapMap JPT population. [B]Exon 2 is 3′ noncoding.

muscle mounding was characteristic. Cardiac arrhythmia, transient immunodeficiency, recurrent pneumonia, constipation, and chalasia were variably seen. Available laboratory data in the patients are summarized in Table 3. Metabolic complication was mild, and none of our patients showed marked elevation of fasting glucose levels. The result of oral glucose tolerance tests revealed moderate fasting hyperinsulinemia in P1 and P2 associated with glucose intolerance in P2, but normal levels in P4 (Table 4). High triglyceremia was seen in P4 and P5. Serum creatine kinase (CK) levels were moderately elevated in all patients. Abdominal CT images of P4 revealed marked loss of subcutaneous and intra-abdominal fat (Figure 2, B and C). In addition, his body fat ratio, as determined by whole body dual energy X-ray absorptiometry, was 7.1% (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI38660DS1), while head fat was relatively preserved.

Clinical features of the heterozygous parents. There was no family history of muscular dystrophy or lipodystrophy in P1–P5. Genetic analysis revealed a heterozygous c.696_698insC mutation in both parents of P4. Clinically, both father and mother had hypertension requiring medication, whereas P4 was normotensive. Mild lipid metabolism abnormality and borderline glucose intolerance was also seen (Supplemental Table 2). DNA samples from the other parents were not available.

Loss of PTRF with deficiency or mislocalization of caveolins in skeletal muscle. Biopsied skeletal muscles from P1–P5 showed consistent findings, with chronic dystrophic changes including marked variation in muscle fiber size, increased number of fibers containing internalized nuclei, a few necrotic and regenerating fibers, and increased interstitial fibrosis (Figure 2D and Supplemental Figure 1). Intramuscular lipid droplets, as visualized by oil red O staining, were not increased (Figure 2D).

Immunohistochemistry demonstrated that the PTRF antibodies A301-269A and A301-271A (which recognize the N- and C-terminal regions of the protein, respectively; Figure 1B) showed sarcolemmal membrane staining of muscle fibers, with stronger immunoreaction at intramuscular blood vessels in control muscles (Figure 3A). Caveolin-3 was clearly observed at sarcolemma, whereas caveolin-1 and -2 were present only in blood vessels. In contrast, muscles from P1–P5 showed barely detectable immunoreaction to both PTRF antibodies (Figure 3A). Caveolin-3 immunoreactivity was greatly reduced in the sarcolemma, but cytoplasmic staining was remarkably increased. This caveolin-3 staining pattern was similar to that seen in the patients with muscular dystrophy

Table 2
Clinical summary

	P1	P2	P3	P4	P5
Age/sex	8-yr-old female	14-yr-old female	10-yr-old male	27-yr-old male	24-yr-old male
Height, body weight	124 cm, 21.3 kg	149 cm, 40.5 kg	NA	164 cm, 49.0 kg	152 cm, 40 kg
Lipodystrophy	Generalized	Generalized	Generalized	Generalized	Generalized
Mental retardation	No	No	No	No	No
Acanthosis nigricans	No	No	No	No	No
Liver/spleen	Hepatosplenomegaly	Fatty liver	NA	Hepatosplenomegaly	No
Endocrine abnormalities	Reduced growth hormone secretion	NA	NA	Accelerated bone age, acromegaloid features, no androgynism	Acromegaloid features, no androgynism
Muscle weakness	Distal dominant	No	No	Generalized	Distal dominant
Muscle mounding	Positive	NA	NA	Positive	Positive
Other muscle symptoms	Muscle hypertrophy	Myalgia, muscle stiffness	NA	Muscle hypertrophy	Muscle hypertrophy
Cardiac symptoms	Arrhythmia	No	No	Atrial fibrillation	No
Skeletal abnormalities	Lordosis, Contractures (ankles, shoulders, fingers)	No	No	Scoliosis, contractures (ankles)	Scoliosis
Other symptoms	Constipation	Transient IgA deficiency, recurrent pneumonia	Nephrosis	Umbilical prominence, renal stones	Recurrent pneumonia, chalasia, constipation

NA, not available.

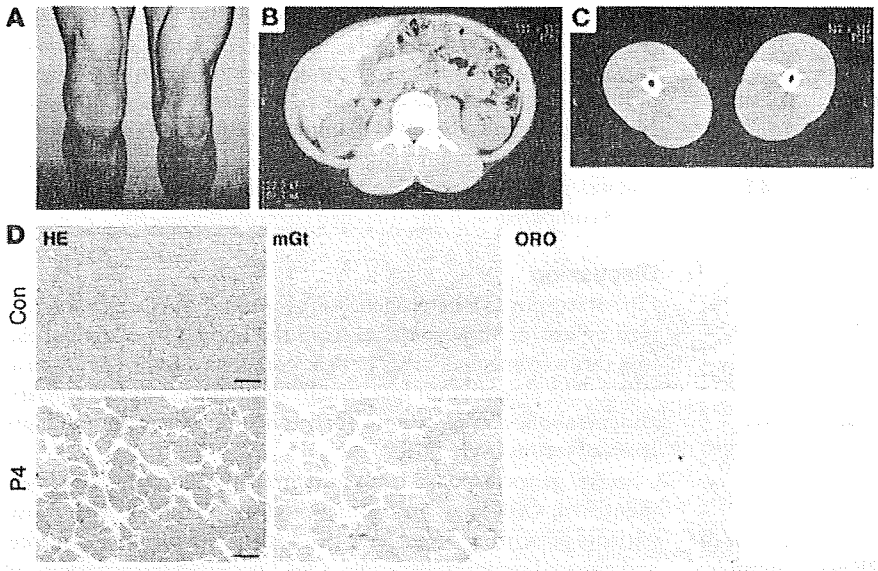

Figure 2
Muscle hypertrophy and dystrophic changes. (**A**) Prominent musculature feature of legs in P5. (**B** and **C**) CT images from P4 showed hypertrophy of paravertebral and thigh muscles with minimal subcutaneous and intra-abdominal fat tissue. (**D**) H&E stain of biopsied skeletal muscle from P4 showed dystrophic changes, including marked variation in fiber size, enlarged fibers with internalized nuclei, endomysial fibrosis, and few necrotic and regenerating fibers. Intramuscular lipid droplets were not increased compared with control. mGt, modified Gomori trichrome; ORO, oil red O. Scale bars: 50 μm.

caused by *CAV3* mutations (data not shown). Similarly, dysferlin was decreased in the sarcolemma and mislocalized into the cytoplasm (data not shown), and the same pattern is also seen in muscles of individuals with *CAV3* mutations (8). Immunoreactivity to caveolin-1 and caveolin-2 in blood vessels was barely detectable in P1–P5 (Figure 3A). Other antibodies related to muscular dystrophy, including dystrophin, sarcoglycans, dystroglycans, emerin, merosin, and collagen VI, showed normal immunostaining patterns (data not shown).

Immunoblotting showed detection of PTRF as an approximately 50-kDa band in control muscles and 3T3 cells, which were used as a positive control. No band was detected in the muscle of P1–P5 (Figure 3B). Caveolin-3 was detected in all samples examined, but relative protein amount, determined using densitometry and normalized by myosin heavy chain (MHC), decreased in P1–P5 compared with control subjects (Figure 3C). The band for caveolin-2 was observed in control muscles and 3T3 cells, but was barely detectable in the muscles of P1–P5 (Figure 3B).

In order to determine mRNA expression of PTRF, RT-PCR was performed using total RNA extracted from biopsied skeletal muscles. Using primers designed to amplify whole coding region of mRNA, PTRF was amplified as a single transcript in control muscles. In contrast, no PCR product was amplified in P1–P5 (Figure 4A). To compare mRNA levels for caveolins, we performed quantitative RT-PCR and normalized results to GAPDH expression. The mRNA amounts of all 3 caveolin families in the patients' muscles were variable, but not markedly decreased, compared with control muscles (Figure 4, B and C). Preserved mRNA levels, but decreased protein amounts of caveolins, suggested destabilization of caveolin proteins when PTRF is lacking, as previously reported (9).

Loss of PTRF causes reduced caveolae formation in human muscles. Greatly reduced caveolae formation was previously reported in PTRF knockdown mammalian cells, zebrafish, and knockout mice (5, 9). Decreased caveolae number was also reported in skeletal muscle from limb girdle muscular dystrophy type 1C (LGMD1C) patients with *CAV3* mutations (10). We therefore examined muscle caveolae in P2 and P3 using electron microscopy. Plasma membrane of muscle fibers from both patients was nearly flat, and caveolae density was notably reduced, compared with control muscle (Figure 5). Caveolae formation in the intramuscular vascular smooth muscle cells was also remarkably reduced (data not shown).

Altered localization of mutant PTRF and reduced interaction with caveolins in transfected cells. In order to determine the intracellular localization of mutant PTRF, FLAG-tagged WT or 2 mutants (c.525delG and c.696_697insC) and T7-tagged caveolin-3 or -1 were cotransfected in C2C12 myoblasts and COS-7 cells. In C2C12 cells, WT PTRF was detected at the cell membrane and colocalized with caveolin-3 (Figure 6A). Interestingly, c.525delG was detected as intranuclear aggregations and was not observed at the cell membrane (Figure 6, A and B). Caveolin-3 was present only in cytoplasm, and did not merge with PTRF (Figure 6A). The c.696_697insC mutant was observed as microtubular filament network in cytoplasm and colocalized with β-tubulin (Figure 6B). This finding is consistent with the localization of the truncated $PTRF_{1-322}$, as described previously (9). Similar mislocalization and/or aggregation of transfected mutant PTRF was observed in COS-7 cells (data not shown).

Table 3
Laboratory data

Measurement	Reference range	P1	P2	P3	P4	P5
CK (IU/l)	56–244	1,374	542–2,253	2,000	554–1,545	645–2,630
Fasting glucose (mg/dl)	70–109	75	99	NA	93–116	102
HbA1c (%)	4.3–5.8	NA	NA	NA	5.0–5.4	NA
Total cholesterol (mg/dl)	130–220	164	NA	NA	185–267	218
Triglyceride (mg/dl)	50–150	93	NA	NA	143–450	359
LDL-C (mg/dl)	70–139	NA	NA	NA	188	NA
Leptin (ng/ml)	0.9–13.0	NA	NA	NA	0.6	NA
Adiponectin (μg/ml)	None	NA	NA	NA	1.05	NA

NA, not available.

Table 4
Oral glucose tolerance test of P1, P2, and P4

	Pre	30 min	60 min	120 min
P1				
Glucose (mg/dl)	75	98	69	62
IRI (μU/ml)	22.8	141.6	64.7	23.8
P2				
Glucose (mg/dl)	99	127	160	172
IRI (μU/ml)	20	53	65	80
P4				
Glucose (mg/dl)	93	124	140	70
CPR (ng/ml)[A]	2.8	5.9	8.3	5.5
IRI (μU/ml)	1.0	22.3	32.9	6.2

IRI, immunoreactive insulin; CPR, C-peptide immunoreactivity. [A]Reference range, 0.7–2.2 ng/ml.

We performed immunoprecipitation assay in order to examine the binding ability of PTRF and caveolins. WT PTRF was coimmunoprecipitated by anti-T7 antibody, and vice versa (Figure 6, C and D). The c.525delG mutant showed smaller molecular weight (estimated 30 kDa; Figure 1B), and no immunoprecipitated protein was detected by FLAG and T7 antibodies. The c.696_697insC mutant showed slightly larger molecular weight, and coimmunoprecipitated proteins were greatly reduced (Figure 6, C and D). These results suggest that mutant PTRFs cannot localize properly and lose their binding ability to caveolins even if they are produced.

Activation of myostatin and Akt signaling pathways in PTRF-deficient skeletal muscles. Caveolin-3 is suggested to have an important role for suppression of myostatin-mediated signaling in skeletal muscle (11). In order to determine the functions of mislocalized caveolin-3 in *PTRF* mutated cells, we performed quantitative RT-PCR for myostatin and immunoblotting analysis to examine phosphorylation status of Mad homolog 2/3 (p-Smad2/3), an intracellular effector of myostatin in skeletal muscles. In P1–P5, increased amounts of p-Smad2/3$^{S423/425}$ were observed in skeletal muscles, while myostatin mRNA levels were variable (Figure 7, A–C). Positive immunoreaction to p-Smad2/3 was detected in few myonuclei from muscle of patients with *PTRF* or *CAV3* mutations, but not in those from muscle of control subjects (data not shown). These results suggest that myostatin signaling is also activated in P1–P5.

Despite the activation of myostatin, a negative regulator of muscle growth, the patients showed hypertrophy of muscles. Since Akt (also known as protein kinase B) is known as the key molecule to regulate muscle mass (12), we examined p-Akt^{T308} and p-Akt^{S473} by immunoblotting analysis. p-Akt was elevated in the muscle of P1–P5 compared with controls, except for p-Akt^{S473} in P2 (Figure 7, D–F). This result suggests that Akt pathway is activated, probably through an as-yet-unidentified mechanism, and could contribute to the muscle hypertrophy observed in P1–P5.

Neuronal NOS activity is variable and mildly increased in PTRF-deficient skeletal muscles. Caveolin-3 is known to interact with and negatively regulate the catalytic activity of neuronal NOS (nNOS) in skeletal muscle (13); this notion is supported by the finding of increased nNOS activity in muscle of transgenic mice expressing mutant caveolin-3 (14). We thus examined nNOS expression and its activity in muscles from patients with mutations in *PTRF* or *CAV3* compared with those from age-matched controls. The immunoreactivity of nNOS was seen in sarcolemma and cytoplasm of each muscle fiber with variable intensity, but no obvious difference was seen between patients and controls (Figure 3A). Immunoblotting analysis also revealed comparable amounts of nNOS (Figure 3, B and D). In order to examine nNOS activity of each muscle fiber, we performed NADPH diaphorase (NDP) activity assay. The intensity of NDP staining appeared variable among muscle fibers and was slightly increased in patients with mutations in *PTRF* or *CAV3* compared with age-matched controls (Figure 8).

Discussion

Lipodystrophy is a heterogeneous group of disorders characterized by loss of adipose tissue from the body. The degree of fat loss varies from small areas to near-complete absence of adipose tissue. The extent of fat loss usually determines clinical severity and metabolic complications, such as insulin resistance and high levels of serum triglycerides.

Several genes responsible for inherited lipodystrophy have been identified. CGL is an autosomal-recessive disorder, with most patients presenting soon after birth with severe insulin resistance and elevated serum triglycerides. CGL1 is caused by mutations in *AGPAT2* on chromosome 9q34, which encodes 1-acylglycerol-3-phospate-O-acyltransferase 2, an enzyme involved in the biosynthesis of triacylglycerol and glycerophospholipids (15). CGL2 is caused by mutations in *BSCL2* on chromosome 11q13, which encodes a functionally unknown protein named seipin (16). Recently, mutations in *CAV1* on chromosome 7q31 have been reported to cause generalized (i.e., CGL3) and partial lipodystrophy (17, 18).

Several causative genes for autosomal-dominant familial partial lipodystrophy are known: *LMNA* on chromosome 1q21 (19), *ZMPSTE24* on chromosome 1p34 (20), *AKT2* on chromosome 19q13 (21), *PPARG* on chromosome 3p25 (22), and *LMNB2* on chromosome 19q1 (23). Nevertheless, many patients clinically diagnosed with lipodystrophy carry no mutation in the known genes, suggesting the presence of other causative genes.

Here we conclude that *PTRF* mutations can cause CGL. In our series, patients showed generalized loss of adipose tissue from infancy or early childhood. Because PTRF is reported to colocalize with hormone-sensitive lipase and translocate to the nucleus in the presence of insulin in adipocytes (24), it could be surmised that PTRF plays an important role in lipid metabolism and insulin-regulated gene expression. Interestingly, metabolic complications were milder in patients with *PTRF* mutations than in patients with CGL1 and CGL2, and these were observed only in the elder patients. Although we could not examine the status of caveolae and caveolins in adipose tissues, the secondary deficiency of caveolins might have an important role in the process of lipodystrophy, since *CAV1* mutation can cause lipodystrophy in both humans and mice (17, 18, 25). Notably, the heterozygous parents had mild metabolic disorders, but a robust conclusion could not be reached, as a limited number of the heterozygous carriers of the *PTRF* mutation were available to us. Further investigation is needed to determine the effect of haploinsufficiency of PTRF.

Skeletal muscle symptoms with serum CK elevation represent another common symptom in patients with *PTRF* mutations. The clinical and pathological findings are very similar to those observed in patients with *CAV3* mutation (7, 26–28), although P1–P5 had no *CAV3* mutations. The secondary loss of caveolin-3 in the sarcolemma may contribute to the muscle phenotype. Moreover, serum CK elevation may be a good laboratory marker for diagnosis of lipodystrophy patients with *PTRF* mutations.

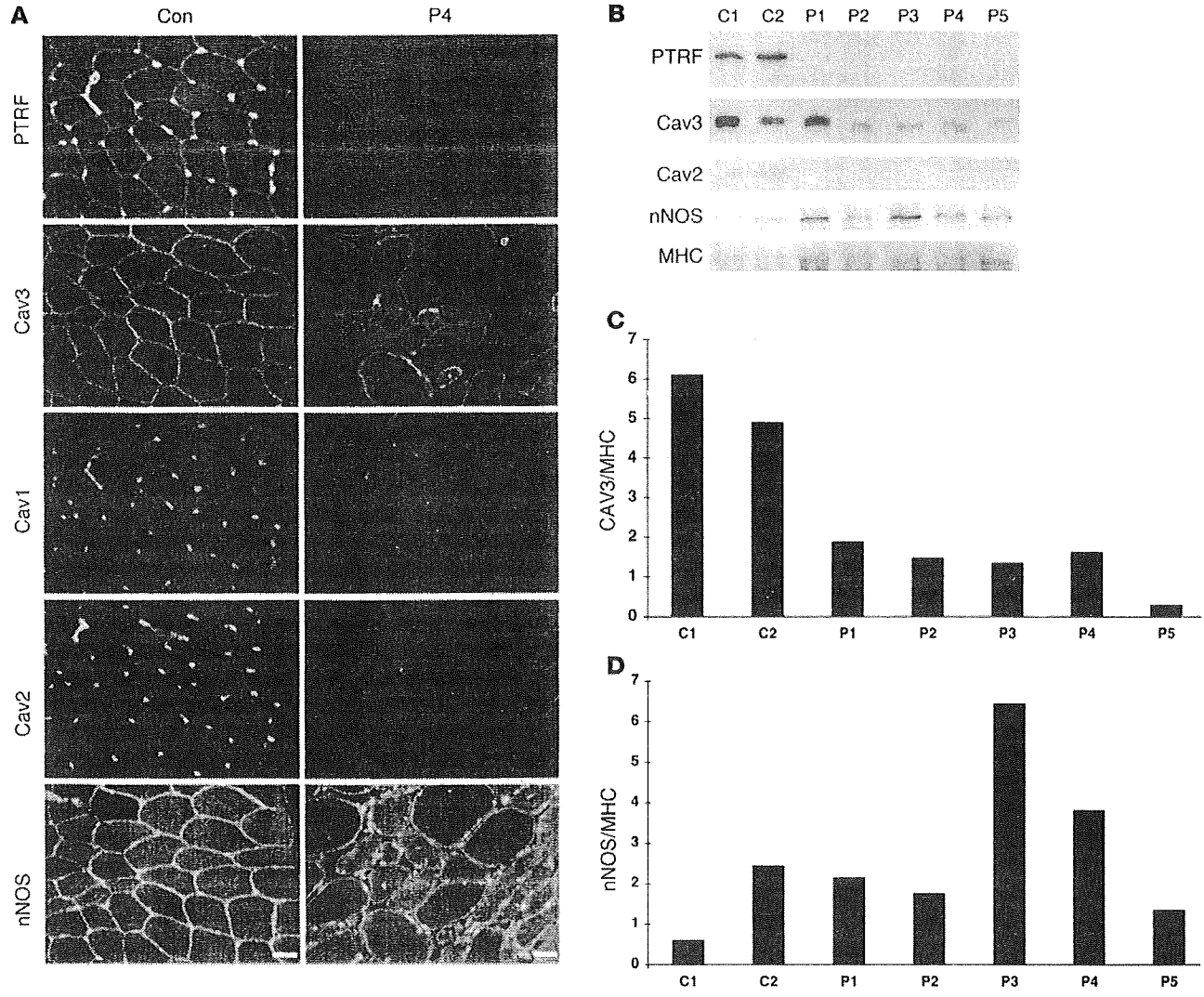

Figure 3

Loss of PTRF is associated with deficiency and mislocalization of caveolins in muscle. (**A**) In control muscle, PTRF was clearly seen in sarcolemma as strongly staining blood vessels. Caveolin-3 (Cav3) was clearly visible at sarcolemma, and caveion-1 and -2 stained intramuscular blood vessels. The muscle of P4 was negative for PTRF. Membrane staining of caveolin-3 was reduced with increased cytoplasmic staining, and caveolin-1 and -2 were barely detectable. Immunoreactivity of nNOS varied between muscle fibers, but was not markedly different between control and patient muscle. Scale bar: 50 μm. (**B**) Immunoblotting analysis of skeletal muscles. 3T3 cells were used as a positive control. PTRF and caveolin-2 were seen only in the muscles of 2 control subjects and in 3T3 cells, and were barely detectable in the muscles of P1–P5. The bands for caveolin-3 and nNOS were variably seen. (**C** and **D**) Quantification of immunoreactive bands was performed by densitometric analysis and normalized with MHC. In P1–P5, relative amounts of caveolin-3 decreased compared with control subjects (**C**), whereas nNOS amounts varied (**D**).

Caveolin-3 was previously reported to have an important role in inhibition of myostatin signaling by suppressing activation of its type I receptor. In mutant *Cav3* transgenic mice, loss of caveolin-3 causes muscular atrophy with increased p-Smad2, and this muscle atrophy can be rescued by myostatin inhibition (11). Consistent with the secondary reduction of caveolin-3, skeletal muscles from P1–P5 showed increased amounts of p-Smad2/3. Unexpectedly, however, muscle hypertrophy was seen in these patients.

The Akt pathway, when activated, is known to promote protein synthesis, stimulate muscle hypertrophy, and inhibit atrophy-related gene expression by phosphorylating FoxO transcription factors (12). This pathway is also known to play a pivotal role in the regulation of glucose transport and glycogen synthesis in skeletal muscle cells. Akt is activated by insulin, various growth factors, nutrients, and exercise, whereas it is negatively regulated by myostatin and cytokines. Akt is phosphorylated at T308 by phosphoinositide-dependent kinase and at S473 by mammalian target of rapamycin in association with rictor. The increase in phosphorylated Akt in the muscle of P1–P5 may explain, at least in part, the muscle hypertrophy observed. Akt pathway activation might be associated with the metabolic complications observed in P1–P5. However, the upregulation of myostatin observed is contradictory to the established knowledge on muscle hypertrophy. This would be worthwhile to investigate in future studies, in order to

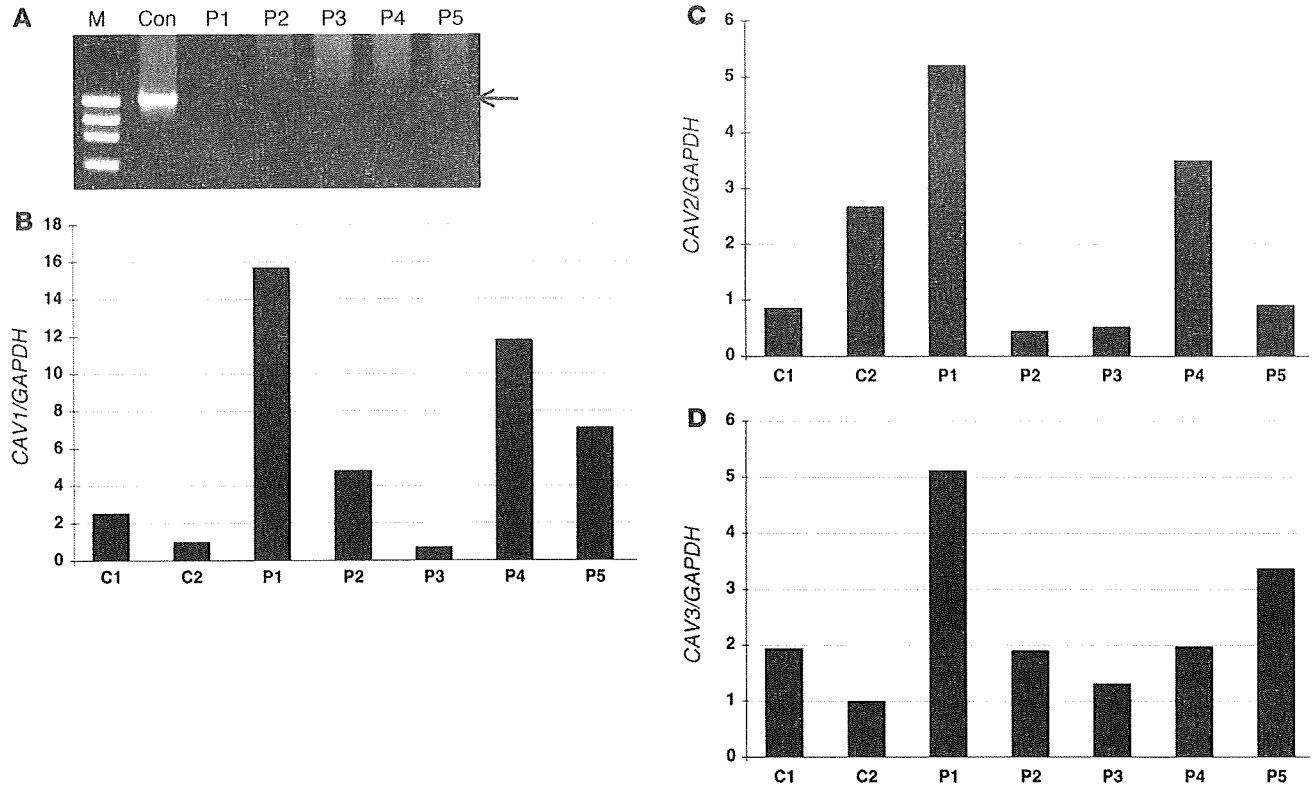

Figure 4
mRNA expression of PTRF in skeletal muscle, and quantitative RT-PCR of mRNAs for caveolins. (**A**) RT-PCR analysis revealed a single band for *PTRF* mRNA (arrow) in a control subject, but no detectable product was seen in P1–P5. M, marker. (**B–D**) By quantitative RT-PCR, mRNA for *CAV1*, *CAV2*, and *CAV3* normalized with *GAPDH* expression was not decreased in P1–P5.

elucidate the role of PTRF deficiency in muscle hypertrophy and related signaling pathway.

In addition to lipodystrophy and muscular dystrophy, P1–P5 had various other symptoms, whose association to *PTRF* mutation might be difficult to ascertain at this time. For example, 2 of 5 patients had arrhythmia. Although we could not examine the expression of caveolins in cardiac muscle, this cardiac abnormality may be caused by secondary deficiency of caveolins in heart, as cardiac involvement was previously reported in patients with *CAV3* mutations and in mutant mice with double knockout of *Cav1* and *Cav3* (29–33).

Remarkable reduction in expression of caveolin-1 and -2 with decreased caveolae density was observed in vascular endothelial cells in P1–P5. There was no obvious symptom related to vascular endothelial blood vessels in the patients; however, further careful investigation is necessary in order to determine the involvement of endothelial cells, which was observed in *Cav1* knockout mice (34). The severe constipation and esophageal dilatation observed in the patients might be associated with dysfunction of caveolin-1 in smooth muscle cells, as *Cav1* knockout mice had alteration of smooth muscles and interstitial cells of Cajal, the pacemaker cells of the muscle layers of the gastrointestinal tract (35).

Caveolae was previously suggested to have a role in the internalization of growth hormone in vitro (36). The acromegaloid features, accelerated bone age, or abnormal growth hormone activity observed in 3 patients in the present study might be associated with reduced caveolae formation. Recurrent pneumonia and transient immunodeficiency observed in 2 patients were also noted, although the pathomechanisms are still unknown. Further detailed studies are needed to elucidate the roles of PTRF; however,

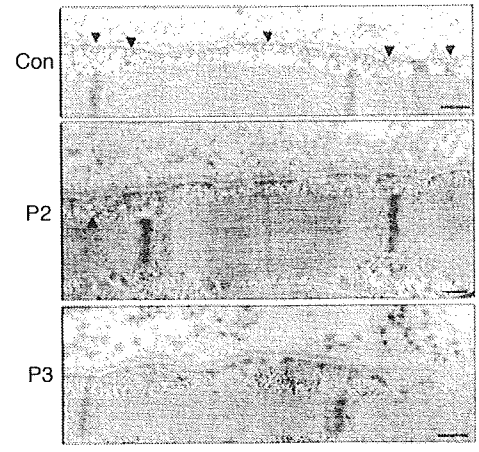

Figure 5
Reduced caveolae formation in skeletal muscle, as assessed by electron microscopy. In control muscle, an abundance of caveolae (arrowheads) was observed close to the plasma membrane. Plasma membrane of muscle fibers from P2 and P3 was nearly flat, and caveolae density was greatly reduced compared with that of control muscle. Only a few caveolae were seen in P2. Scale bars: 200 nm.

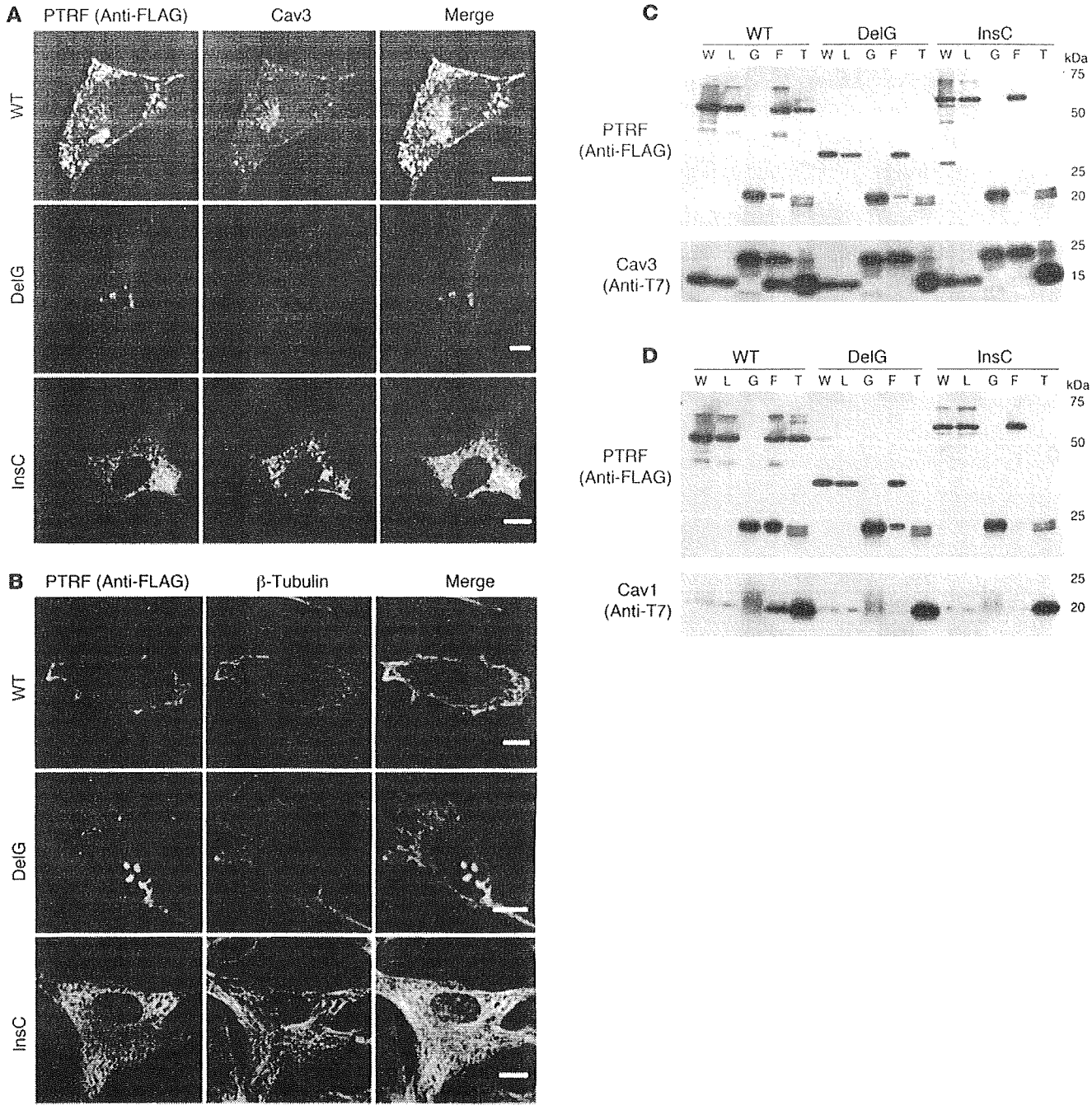

Figure 6

Altered localization of mutant PTRF in C2C12 cells and reduced binding ability to caveolins. C2C12 myoblasts were cotransfected with FLAG-tagged WT or mutant (c.525delG or c.696_697insC) PTRF cDNA and T7-tagged human caveolin-3. (**A** and **B**) WT PTRF stained by anti-FLAG antibody colocalized with caveolin-3 at the cell membrane. The deletion mutant accumulated in the nucleus, and the insertion mutant was seen in cytoplasm. (**A**) Membrane staining of caveolin-3 was decreased and was not colocalized with mutant PTRF. (**B**) The PTRF insertion mutant clearly colocalized with β-tubulin. Scale bars: 10 μm. (**C** and **D**) COS-7 cells were cotransfected with FLAG-tagged WT or mutant PTRF cDNA and T7-tagged human caveolin-3 (**C**) or caveolin-1 (**D**). The PTRF deletion mutant showed smaller molecular weight (estimated 30 kDa), and no immunoprecipitated protein was detected for FLAG or T7 antibodies. The PTRF insertion mutant showed slightly larger molecular weight, and amounts of coimmunoprecipitated proteins were greatly reduced. W, whole homogenate; L, cell lysate, G, control IgG; F, anti-FLAG; T, anti-T7.

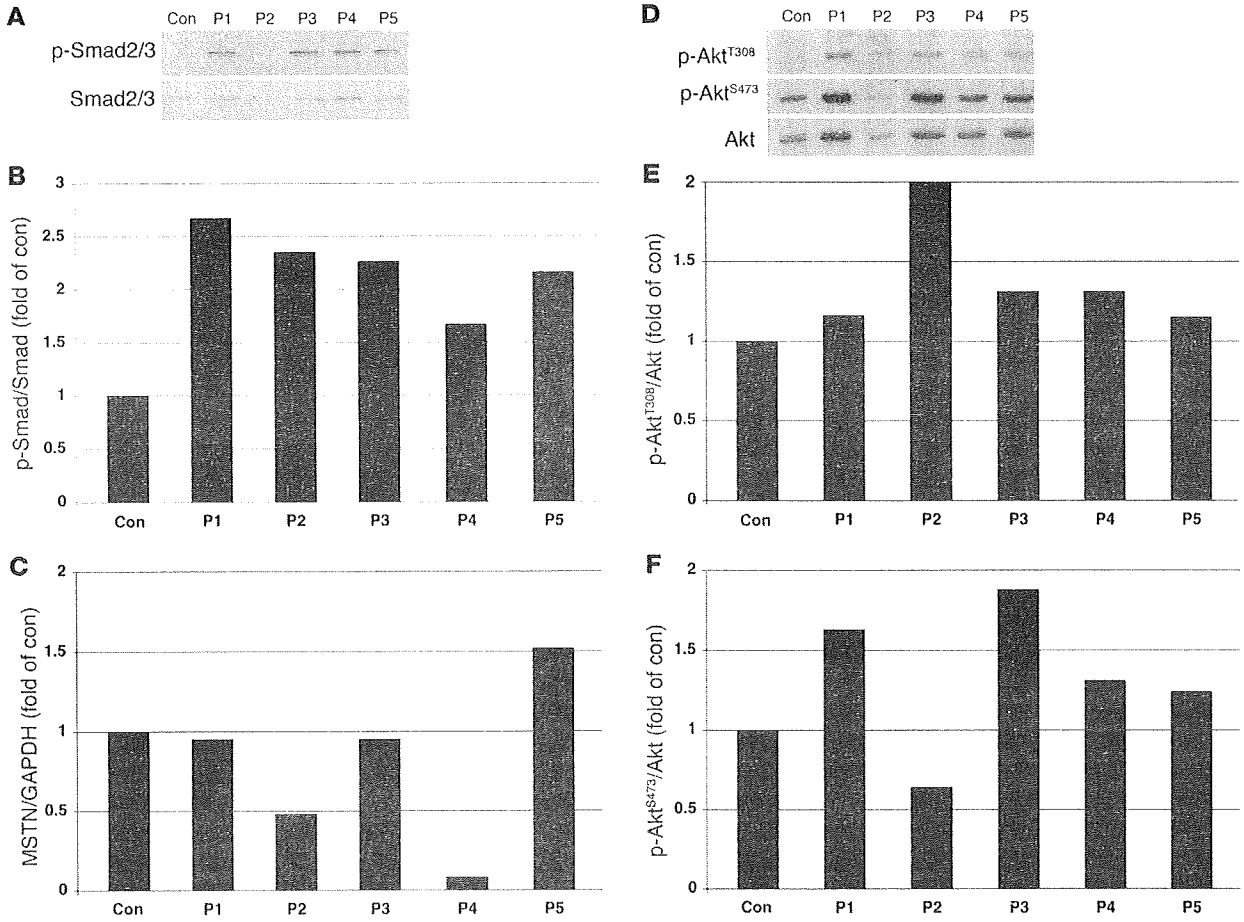

Figure 7
Increased p-Smad2 and p-Akt in P1–P5 skeletal muscle. (**A**–**C**) Immunoblotting analysis of Smad2/3 and p-Smad2/3$^{S423/425}$ (**A**) and densitometric analysis (**B**) showed increased p-Smad2/3 in P1–P5 compared with control muscle, with variable mRNA expression levels of myostatin (MSTN; **C**). (**D**–**F**) Immunoblotting analysis of p-Akt^{T308} and p-Akt^{S473}. Total Akt (**D**) and densitometric analysis (**E** and **F**) showed increased amounts of p-Akt in all patients except for p-Akt^{S473} in P2.

most clinical features observed in P1–P5 are likely to be explained by secondary reduction of caveolae and deficiency of caveolins.

Previously, Rajab et al. reported 10 of 17 patients with congenital generalized lipodystrophy unlinked to the loci of known CGL genes (37). The patients showed reduced exercise tolerance, percussion myoedema, cardiac hypertrophy, and arrhythmias. None of these patients had insulin resistance or early endocrine abnormalities (37). Ghanem also reported myoedema in a patient with Berardinelli-Seip lipodystrophy (38). Very recently, Simha et al. described CGL patients with muscle weakness and cervical spine instability (39). Because muscle involvement of these patients is similar to that of P1–P5 in the present study, *PTRF* mutations may not be rare in CGL patients.

This entity of generalized lipodystrophy with muscular dystrophy — which we believe to be novel — seems to represent a complicated disorder, as the occurrence of other symptoms could not readily be explained. Collection of detailed clinical information would therefore be essential in order to understand the precise function of PTRF.

Methods

Clinical materials. All clinical materials used in this study were obtained for diagnostic purposes and with informed consent. Subjects were selected from 2,745 muscular dystrophy specimens kept in the muscle repository of the National Center of Neurology and Psychiatry. The present studies were approved by the Ethical Committee at National Center of Neurology and Psychiatry.

Mutation screening and haplotype analysis. Genomic DNA was isolated from peripheral lymphocytes or muscles using standard techniques. All exons and their flanking intronic regions of *PTRF*, *CAV3*, *LMNA*, *AGPAT2*, *BSCL2*, *CAV1*, *PPARG*, *AKT2*, and *ZMPSTE24* were directly sequenced using genomic DNA from all patients using an ABI PRISM 3100 automated sequencer (Applied Biosystems). Primer sequences are listed in Supplemental Table 3. To confirm the compound heterozygosity in P5, the PCR product was cloned and sequenced. In order to determine the frequency of the mutations in *PTRF*, we performed enzyme digestion of PCR products from 200 Japanese control subjects using *Hpy*188III (New England Biolabs) for c.696_697insC and *Taq*I (New England Biolabs) for c.525delG. *Mbo*II (New England Biolabs) was used for enzyme digestion of PCR products to detect the c.1138G>A substitution in *BSCL2*.

For haplotype analysis, we used 6 SNPs (rs2062213, rs8070945, rs963988, rs963987, rs963986, and rs9252) within *PTRF*. PCR products were analyzed by direct sequencing or enzyme digestion using *Mae*III (Boehringer Mannheim). We also identified a novel 9-bp insertion polymorphism at the

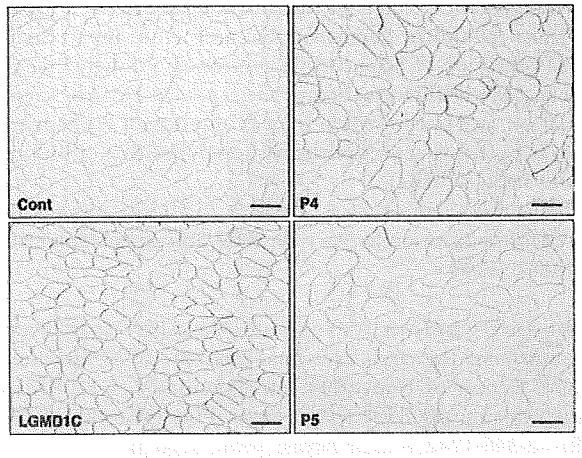

Figure 8
NDP activity assay. NDP activity was variable between muscle fibers, and was slightly increased in the muscle of P4, P5, and a LGMD1C patient with *CAV3* mutation compared with an age-matched control subject. Scale bars: 100 μm.

3′ noncoding region, and its frequency was calculated by PCR amplification using 50 Japanese control individuals. We also examined 2 microsatellite markers, STS-W93348 and D17S1185, the closest markers to *PTRF*. PCR product size was analyzed by GeneMapper using ABI 310 automated sequencer (Applied Biosystems).

Histochemical analysis. Biopsied muscle specimens were flash frozen with isopentane cooled in liquid nitrogen. Serial 10-μm-thick frozen sections were analyzed with 20 kinds of histochemical staining, including H&E, modified Gomori trichrome, NADH-tetrazolium reductase, and oil red O. The NDP activity assay was performed to examine nNOS activity of each muscle fiber, as described previously (40). In brief, 10-μm-thick frozen sections were fixed with 4% paraformaldehyde in PBS for 2 hours at 4°C. After a brief rinse with PBS, sections were incubated with 0.2% Triton X-100 in PBS for 20 minutes at 37°C. The reaction was performed for 1 hour in a dark, humidified chamber at 37°C in 0.2% Triton X-100, 0.2 mM NADPH, and 0.16 mg/ml nitro blue tetrazolium. The reaction was terminated by washing with water. We examined 6 age-matched controls and 2 LGMD1C patients with *CAV3* mutations (p.R27G and p.E33K).

Immunohistochemical analysis. Immunostaining was performed using standard methods. Serial 6-μm-thick frozen muscle sections were fixed in cold acetone for 5 minutes. After blocking with normal goat serum, sections were incubated with the primary antibodies for 2 hours at 37°C. We used antibodies against PTRF (A301-269A and A301-271A; BETHYL Laboratories), caveolin-1 (BD Biosciences), caveolin-2 (Sigma-Aldrich), caveolin-3 (BD Biosciences), and nNOS (BD Biosciences). Rabbit anti-PTRF antibody of A301-269A recognizes residue from 125 to 175, and A301-271A was raised against residue 238 and 288 of human PTRF (Figure 1B). In order to exclude other diagnosable muscular dystrophies, we used antibodies for dystrophin (DYS1, DYS2, and DYS3; Novocastra); α-, β-, γ-, and δ-sarcoglycans (Novocastra); α-dystroglycan (Upstate Biotech); β-dystroglycan (Novocastra); dysferlin (Novocastra); emerin (Novocastra); merosin (Chemicon); and collagen VI (ICN Biomedicals). After 6 rinses with PBS, sections were incubated with secondary antibodies of Alexa Fluor 488– or Alexa Fluor 568–labeled goat anti-mouse or -rabbit antibodies at room temperature for 45 minutes.

Immunoblotting analysis. Immunoblotting analysis was performed according to standard methods. Frozen muscle specimens were homogenized in SDS sample buffer and centrifuged at 15,000 *g* for 5 minutes. Protein (20 μg) from each sample was loaded on 12% SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore). The membranes were blocked with 5% skim milk in PBS and immunoreacted with antibodies to PTRF (A301-269A and A301-271A), caveolin-2, caveolin-3, nNOS, Smad2/3 (Cell Signaling Technology), p-Smad2/3$^{S423/425}$ (Santa Cruz Biotechnology Inc.), Akt (Cell Signaling Technology), p-Akt^{T308} (Cell Signaling Technology), and p-Akt^{S473} (Cell Signaling Technology) overnight at 4°C. After washing in PBS containing 0.1% Tween-20, the membrane was incubated with horseradish peroxidase–labeled secondary antibody and visualized with ECL (Amersham Pharmacia Biotech). Data were analyzed using LAS-1000 chemiluminescence imaging system (Fujifilm). Quantification of immunoreactive bands was performed by densitometric analysis using Quantity One (PDI), and protein amounts for caveolin-3 and nNOS were normalized by the intensity of MHC. The ratio of p-Smad2/3 and p-Akt, to Smad and Akt, respectively, was also calculated.

Electron microscopy. Muscle specimens were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer. After shaking with a mixture of 4% osmium tetroxide, 1.5% lanthanum nitrate, and 0.2 M s-collidine for 2–3 hours, samples were embedded in epoxy resin. Semithin sections (1 μm thick) were stained with toluidine blue. Ultrathin sections 50 nm thick were stained with uranyl acetate and lead citrate, then examined under H-600 transmission electron microscope (Hitachi) at 75 kV.

RT-PCR. Total RNA was extracted from biopsied skeletal muscles using TRIzol (Invitrogen), and RT-PCR was performed using SuperScript III (Invitrogen) with random hexamer according to the manufacturer's instructions. Primers for each gene were located on different exons or directly spanning exon-exon boundaries of the genomic sequence in order to minimize amplification from any contaminating genomic DNA. After performing preliminary gradient PCR assays, the optimal annealing temperature for all the primer pairs was determined in order to generate the lowest Ct value as well as a sharp melting peak, with no amplification of nonspecific products or primer-dimer artifacts. Quantitative RT-PCR was performed to compare the mRNA expression of caveoin-1, caveolin-2, caveolin-3, and myostatin using Rotor-Gene 6000 according to the manufacturer's instructions (Corbett Life Science). The reactions were performed in reference to the GAPDH. We used 4 points consisting of 10-fold serial dilution using each primer set to build the standard curve. The PCR reaction (50 cycles) was followed by a melting curve analysis, ranging from 72°C to 95°C, with temperature increasing steps of 0.5°C every 10 seconds. Baseline and threshold values were automatically determined and analyzed. R^2 values exceeded 0.97. The 2–standard curve method was used to determine the relative expression ratio of the target gene in the patient samples versus the control sample, with reference to GAPDH expression.

Cell culture and transfection. COS-7 and C2C12 cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum. Full-length PTRF and caveolin-1 and -3 were amplified using total RNA from control human muscle and cloned into the pGEM-T-easy vector (Promega). The PTRF mutants c.525delG and c.696_697insC were generated using appropriate primers. All primer sequences are shown in Supplemental Table 3.

Immunocytochemistry. COS-7 and C2C12 myoblasts were cotransfected with FLAG-tagged WT or mutant (c.525delG or c.696_697insC) PTRF cDNA and T7-tagged human caveolin-3 using FuGENE6 (Roche). Transfectants were fixed for 30 minutes in 2% paraformaldehyde or 100% methanol, then permeabilized in 0.1% Triton X-100 for 10 minutes. Polyclonal antibodies to FLAG (Sigma-Aldrich) with caveolin-3 (BD Biosciences) or FLAG with β-tubulin (Calbiochem) were applied for double staining.

Immunoprecipitation. COS-7 cells were cotransfected with FLAG-tagged WT or mutant (c.525delG or c.696_697insC) PTRF cDNA and T7-tagged human caveolin-1 or caveolin-3 using FuGENE6 (Roche). The sequences of all constructs were verified with DNA sequencing using ABI PRISM 310 (Applied Biosystems). After 48 hours, the lysates from transfectants were

solubilized with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 50 mM EDTA, 1% Triton X-100, and Complete-mini EDTA-free proteinase inhibitors (Roche) (9). The solubilized lysate precleared with Protein G Sepharose (GE Healthcare) was incubated with anti-FLAG (M2; Sigma-Aldrich) and anti-T7 (Novagen) antibodies. Immunoprecipitated proteins were dissociated from beads by boiling in sample buffer and were resolved by SDS-PAGE. Immunoblotting was performed using standard techniques.

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