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Identification of *GFAT1-L*, a novel splice variant of human glutamine: fructose-6-phosphate amidotransferase (*GFAT1*) that is expressed abundantly in skeletal muscle

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Abstract Glutamine:fructose-6-phosphate amidotransferase (GFAT1) is the rate-limiting enzyme in the hexosamine biosynthetic pathway, which plays an important role in hyperglycemia-induced insulin resistance. To evaluate the role of *GFAT1* expression, we analyzed the expression profiles of *GFAT1* mRNA in various human tissues using reverse transcriptase-polymerase chain reaction. We report here the identification and cDNA cloning of a novel *GFAT1* splice variant expressed abundantly in skeletal muscle and heart. This subtype, designated *GFAT1-L*, contains a 54-bp insertion within the *GFAT1* coding sequence. Recombinant GFAT1-L protein possessed functional GFAT activities and biochemical characteristics similar to GFAT1. Previously, GFAT1 was considered a simplex enzyme. The identification of a novel GFAT1 subtype possessing functional enzymatic activity and tissue-specific expression should provide additional insight into the mechanism of skeletal muscle insulin resistance and diabetes complications.

Key words GFAT1 · NIDDM · Insulin resistance · Skeletal muscle · Tissue-specific gene

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Introduction

Insulin resistance, leading to chronic hyperglycemia, is a cardinal feature of noninsulin-dependent diabetes mellitus (NIDDM) (Rizza et al. 1981). Recent studies have shown that increased activity of the hexosamine biosynthetic pathway might contribute to glucose-induced insulin resistance in skeletal muscle (DeFronzo et al. 1985; Garvey et al. 1987; Rossetti et al. 1990; Marshall et al. 1991; Baron et al. 1995; Robinson et al. 1995; Davidson et al. 1994; Virkamaki et al. 1997), which is a major organ of insulin resistance in NIDDM patients. Specifically, glutamine:fructose-6-phosphate amidotransferase (GFAT1), a key regulator and rate-limiting enzyme in the formation of hexosamine products (Crook et al. 1995; McClain et al. 1996; Daniels et al. 1996; Chen et al. 1997), displays elevated activity in the skeletal muscle of NIDDM patients (Yki-Jarvinen et al. 1996) and in *ob/ob* mice, a rodent model of NIDDM (Buse et al. 1997). Transgenic mice overexpressing the *GFAT1* gene in skeletal muscle and adipose tissue also exhibit insulin resistance, as determined by the hyperinsulinemic euglycemic glucose clump technique (Herbert et al. 1996; Cooksey et al. 1999). On the other hand, in situ hybridization and immunostaining studies show that *GFAT1* expression is normally tightly regulated in both a tissue-specific and a cell-specific manner (Nerlich et al. 1998; Yki-Jarvinen et al. 1999). The recent identification of *GFAT2*, a highly similar gene that exhibits different tissue distribution from *GFAT1*, suggests additional levels of complexity in GFAT activity (Oki et al. 1999).

Through examination of *GFAT1* mRNA expression profiles, we have identified an additional *GFAT1* variant expressed predominantly in skeletal muscle. We present here the cloning and biochemical characterization of a novel splice variant of *GFAT1*, designated *GFAT1-L*.

Materials and methods

Expression profile of *GFAT1* mRNA

Expression profile of *GFAT1* mRNA was analyzed by polymerase chain reaction (PCR) amplification from commercially prepared human tissue cDNA libraries (QUICK-Clone human heart, brain, kidney, skeletal muscle, pancreas, liver, small intestine, and fat libraries; Clontech, Palo Alto, CA, USA) with GFAT1-specific primers (AGC CCTCTGTTGATTGGTGT and TCCATCTGGAGTGT TTGCAC). Amplification was performed using KOD dash polymerase (TOYOBO, Tokyo, Japan) under the following conditions: 40 cycles of 95°C for 30s, 55°C for 30s, and 72°C for 45s, with a final extension at 72°C for 1min. PCR products were analyzed by electrophoresis through a 4% agarose gel.

Quantitative PCR analysis

Quantitative PCR analysis specific for the *GFAT1-L* gene was carried out using SYBR Green I methods (Wittwer et al. 1997; Morrison et al. 1998; Hiratsuka et al. 1999) and an ABI PRISM 7700 sequence detector (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Amplification mixes contained template DNA (described earlier), 10× SYBR Green PCR buffer (Perkin-Elmer Applied Biosystems), 200μM dNTPs, 5mM $MgCl_2$, 1.25 units of AmpliTaq Gold (Perkin-Elmer Applied Biosystems) and 5pM of each primer (AGCTAGGACTCAGATTGGATC and TCCATCTGGAGTGTTTGCAC). The amplification was carried out for 50 cycles under the following conditions: 95°C for 30s, 55°C for 30s, and 72°C for 30s. PCR products were quantified and normalized to human beta 2-microglobulin (beta 2-m) mRNA levels determined in parallel assays for each sample (*GFAT1-L* / *beta 2-m*). The ratios for mean expression levels ±S.D. in each tissue relative to skeletal muscle were shown. Assays were performed in duplicate and repeated four times.

Cloning of full-length GFAT1-L cDNA

A full-length GFAT1-L cDNA was PCR-amplified from human skeletal muscle QUICK-Clone cDNA (Clontech) using the primers TTTTTTTCATATGTGTGGTATATT TGCTTACTTAAACTAC and CCCTCGAGTTACTCT ACAGTCACAGATTTGGC. Reactions were carried out for 40 cycles under the following conditions: 95°C for 30s, 55°C for 30s, and 72°C for 2min, with a final extension at 72°C for 3min. The PCR product was isolated by 0.8% agarose gel electrophoresis, purified, inserted into the pT7Blue T-vector (Novagen, Madison, WI, USA), and sequenced.

Construction of *Escherichia coli* expression vectors for GFAT1 and GFAT1-L cDNAs

To increase expression levels of GFAT1 and GFAT1-L proteins in *E. coli*, we used PCR-based mutagenesis to introduce conservative mutations, converting *E. coli* low-usage codons to high-usage codons within the human *GFAT1* and *GFAT1-L* gene sequences. Reverse transcription was performed using 2μg of human skeletal muscle mRNA, the primer P4 (CCCTCGAGTTACTCTACAGT CACAGATTTGGC), and M-MLV reverse transcriptase (Gibco BRL, Grand Island, NY) for 10min at 37°C followed by a 5-min denaturation step. We performed PCR using the mutagenesis primer P1 GTTCCGCGTACTCGT CGTGAAATCCTGGAGACC (underlined nucleotides denote substitutions in the *GFAT1* sequence), primer P4, and KOD dash polymerase (TOYOBO). The amplification was executed under the following conditions: preheated at 95°C for 1min, 40 cycles at 95°C for 30s, 55°C for 30s, and 72°C for 45s, with a final extension at 72°C for 1min. A second PCR reaction was performed using 5μl of the above PCR product as template, the mutagenesis primer P2 GCTTACCTGAACTACCACGTTCCGCGTACTCGT, and primer P4 under the same conditions. Five microliters of the second PCR product was reamplified using the mutagenesis primer P3 TTTTTTTCATATGTGTGGTATCT TTGCTTACCTGAACTAC and primer P4 under the same conditions. The final PCR product was analyzed by 0.8% agarose gel electrophoresis, purified, inserted into the pET23b vector (Novagen), and sequenced.

Expression and purification of recombinant GFAT1 and GFAT1-L

Vectors containing the full-length *GFAT1* and *GFAT1-L* coding sequences, pET23/GFAT1 and pET23/GFAT1-L, were transformed into the *E. coli* strain BL21-Codon Plus (DE3)-RIL (Stratagene, La Jolla, CA, USA). Cultures were grown in Luria-Bertani medium containing 50mg/l ampicillin at 30°C overnight. Fifty microliters of the culture was added to 1l of the fresh medium and incubated for 3h at 37°C with vigorous agitation. Following the addition of 2ml of 1M isopropyl-β-D-thiogalactopyranoside (Wako Pure Chemicals Industries, Tokyo, Japan), cultures were incubated for 18h at 20°C with vigorous agitation. Cells were harvested from 1l of culture by centrifugation, and the bacteria pellet was washed with cold Dulbecco's PBS (−) (Nissui Pharmaceutical, Tokyo, Japan). The cell pellet was the suspended in sonication buffer (50mM sodium phosphate buffer [pH7.5] containing 20% [v/v] glycerol). After sonication, the lysate was centrifuged at 100,000*g* for 30min at 4°C.

To concentrate the recombinant GFAT1 and GFAT1-L proteins, we slowly added 100% saturated ammonium sulfate solution to the lysates to obtain 55% saturation. After centrifugation at 15,000rpm for 30min, the precipitates were dissolved in buffer G (50mM sodium phosphate buffer [pH7.5] containing 20% [v/v] glycerol and 1mM dithiothreitol) and desalted using a PD-10 gel filtration column (Amersham Pharmacia Biotech, UK). The concentrated sample solution was applied to a DEAE-Sepharose column, which had been preequilibrated with buffer G. The sample was eluted with buffer G containing

0.1 M NaCl. Fractions containing recombinant GFAT1 were applied to a Sulfate-Cellulofine column that had been preequilibrated with buffer G containing 0.1 M NaCl. Proteins were step eluted with buffer G containing 0.1 M, 0.2 M, 0.3 M, 0.4 M, and 0.5 M NaCl. Fractions containing recombinant GFAT1-L were applied to a Heparin-Sepharose CL-6B column that had been preequilibrated with buffer G containing 0.1 M NaCl. Proteins were step eluted with buffer G containing 0.1 M, 0.2 M, and 0.3 M NaCl.

The sample was mixed with sodiumdodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (62.5 mM Tris-HCl [pH6.8] containing 2% [w/v] SDS, 7% [v/v] glycerol, 5% [v/v] 2-mercaptoethanol, and a trace of bromophenol blue) and boiled for 5 min. Samples were fractionated on a 13% SDS polyacrylamide gel and stained with Coomassie Brilliant Blue R-250.

Assays of enzyme activity

GFAT activities were measured using a spectrophotometric assay, as described in Marshall et al. (1991). In a typical assay, 200 µl of substrate buffer (400 mM sodium phosphate buffer [pH7.5], 50 mM KCl, 1.25 mM ethylenediaminetetraacetate, 0.3 mM 3-acetylpyridine adenine dinucleotide [APAD], 6 U/ml glutamate dehydrogenase, 0–6 mM fructose-6-phosphate [F6P]) was incubated with 50 µl of diluted human GFAT1 or GFAT1-L recombinant protein for 60 min at 37°C. The change in absorbance due to reduction of APAD to APADH was monitored spectrophotometrically at 365 nm.

Results and discussion

Expression profiles of *GFAT1*

To investigate the role of *GFAT1* gene expression in insulin resistance, we examined expression profiles in human-tissue cDNA panels (Fig. 1). In the skeletal muscle cDNA library, we observed two distinct PCR products of 323 bp and 377 bp. Sequence analysis identified the smaller product as the previously reported *GFAT1* cDNA sequence (McKnight et al. 1992), whereas the larger product contained a novel 54-bp insertion between nucleotides 685 and 686 of the *GFAT1* cDNA. We have designated this variant *GFAT1-L*. PCR products corresponding to *GFAT1-L* were detected in skeletal muscle and heart, with weak expression observed in brain. On the other hand, PCR products corresponding to *GFAT1* were detected uniformly in all tissues.

To measure the relative levels of *GFAT1-L* mRNA in various tissue types, we performed quantitative PCR analysis (Fig. 2). Data were normalized to human beta 2-microglobulin mRNA levels. The highest levels of *GFAT1-L* expression were seen in skeletal muscle and heart, whereas low levels of expression were seen in brain. *GFAT1-L* expression in skeletal muscle was about 1.4 times higher than in heart and about 3.2 times higher than in brain. In other tissues, only background levels of expression were observed. These findings suggest that expression of *GFAT1-L* is regulated in a tissue-specific manner.

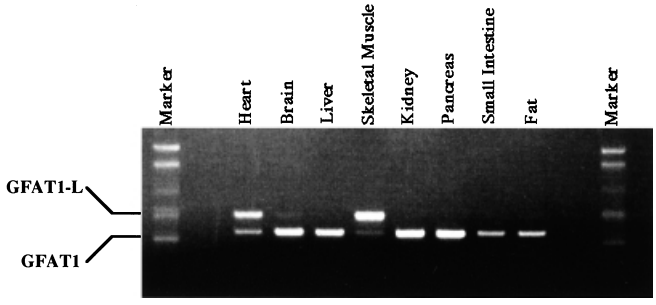

Fig. 1. Detection of two alternative splice transcripts, human *GFAT1* and *GFAT1-L*, by reverse transcriptase-polymerase chain reaction (RT-PCR). PCR was performed using QUICK-clone cDNA panels as templates. *GFAT1-L* transcripts were identified in human heart, brain, and skeletal muscle

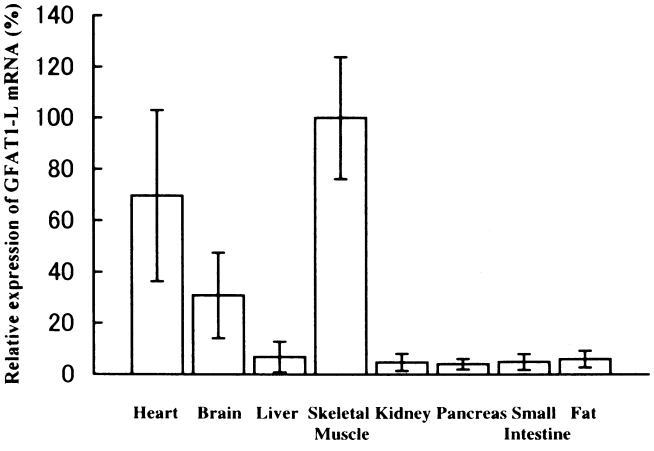

Fig. 2. Quantitative analysis of *GFAT1-L* mRNA in tissues. SYBR Green I quantitative PCR methods were performed in duplicate and repeated four times. Levels of *GFAT1-L* mRNA amplification were normalized by comparison with human beta 2-microglobulin mRNA. Results show the mean ±S.D. expression levels relative to skeletal muscle

Recently, the *GFAT2* gene, which shares high homology with *GFAT1*, was isolated (Oki et al. 1999). Northern blot analysis showed differences in tissue distribution between *GFAT1* and *GFAT2*. The highest levels of *GFAT2* gene expression were observed in heart, placenta, and throughout the central nervous system. In contrast, *GFAT1-L* is expressed most abundantly in skeletal muscle, the most important tissue of insulin resistance in NIDDM (Rossetti et al. 1990; DeFronzo et al. 1985). In light of the known role of GFAT1 activation in insulin resistance, it is possible that GFAT1-L is also involved in the mechanism of skeletal muscle insulin resistance.

Cloning of a novel *GFAT1* splice variant

To confirm the presence of complete transcripts coding for the putative *GFAT1-L* open reading frame, we isolated a

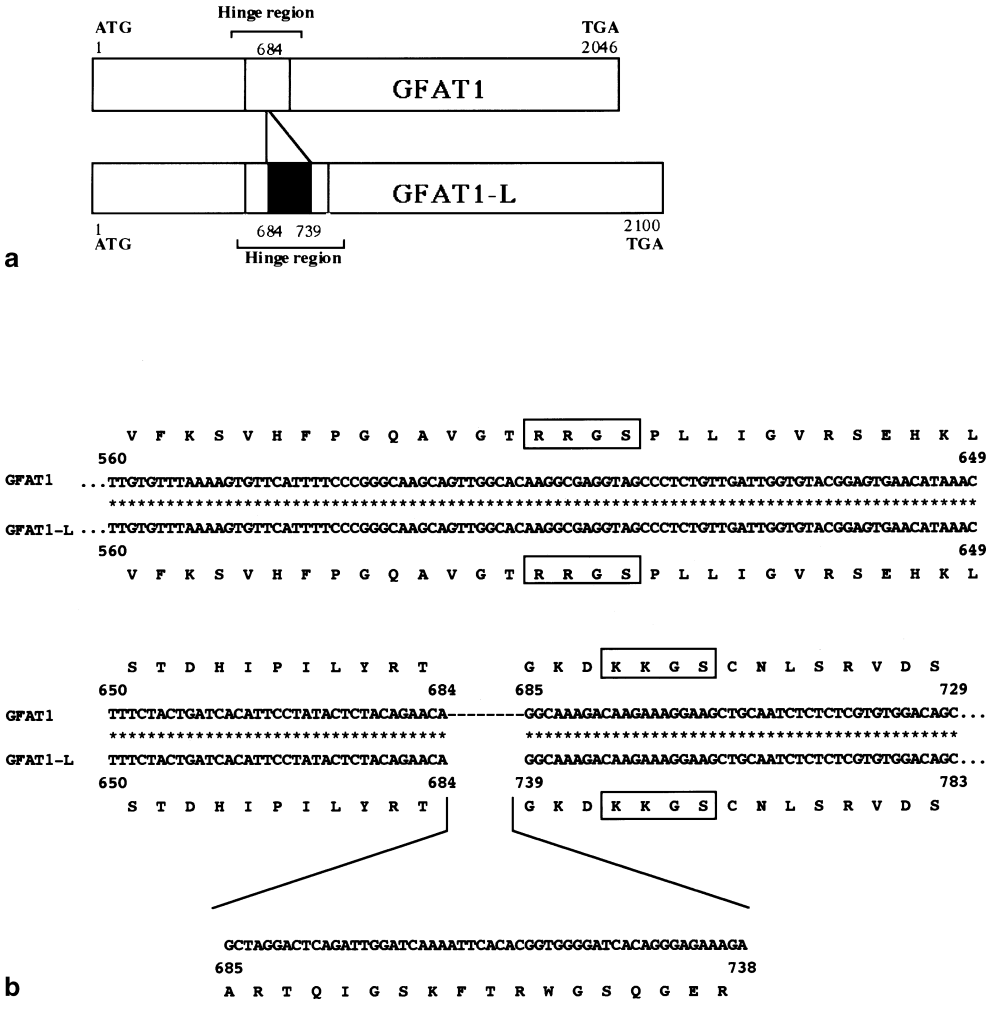

Fig. 3.a,b Comparison of human GFAT1 and GFAT1-L sequences. **a** Schematic diagram of *GFAT1* and *GFAT1-L* showing the GFAT1-L 54-bp insertion sequence. **b** Nucleotide and amino acid sequences of the hinge regions of human GFAT1 and GFAT1-L. Amino acid sequences of GFAT1 and GFAT1-L are indicated *below* and *above* the nucleotide sequences, respectively. *Asterisks* indicate nucleotide identity between GFAT1 and GFAT1-L. A putative protein kinase A phosphorylation site is *boxed*

full-length cDNA clone from the human skeletal muscle cDNA library. DNA and amino acid sequences of GFAT1 and GFAT1-L, along with schematic representations of GFAT1 and GFAT-1L proteins, are presented in Fig. 3a,b. Aside from the insertion, the sequences of *GFAT1* and *GFAT1-L* were identical. The *GFAT1-L* open reading frame consists of 2097 nucleotides, encoding a 699-amino-acid protein with a predicted molecular mass of 78790.38 Da (GFAT1: 2043 bp, 681 amino acids, and 76743.22 Da). The GFAT1-L insertion occurs within a sequence corresponding to a hinge region in the GFAT1 protein (Zhou et al. 1998).

Expression of GFAT1 and GFAT1-L protein and GFAT activity assay

To produce recombinant GFAT1 and GFAT1-L proteins, we subcloned full-length cDNAs for these variants into *E. coli* expression vector pET23 vector and introduced them into *E. coli* strain BL21-Codon Plus (DE3)-RIL. As shown in Fig. 4, the approximately 77- and 79-kDa proteins were purified GFAT1 and GFAT1-L, respectively, which is in reasonable agreement with the molecular masses predicted.

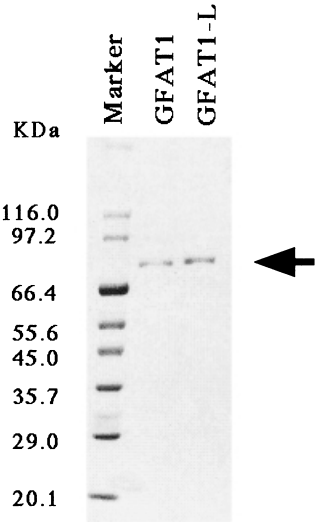

Fig. 4. Expression of recombinant GFAT1 and GFAT1-L proteins. *Lane 1*, molecular weight standards; *lane 2*, GFAT1; *lane 3*, GFAT1-L. Proteins were solubilized in the sample buffer, and proteins were fractionated on a 13% sodium dodecyl sulfate polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. *Arrow* at *right* denote recombinant GFAT1 and GFAT1-L proteins

To determine if the GFAT1-L protein being expressed was functional, GFAT enzymatic activities were measured

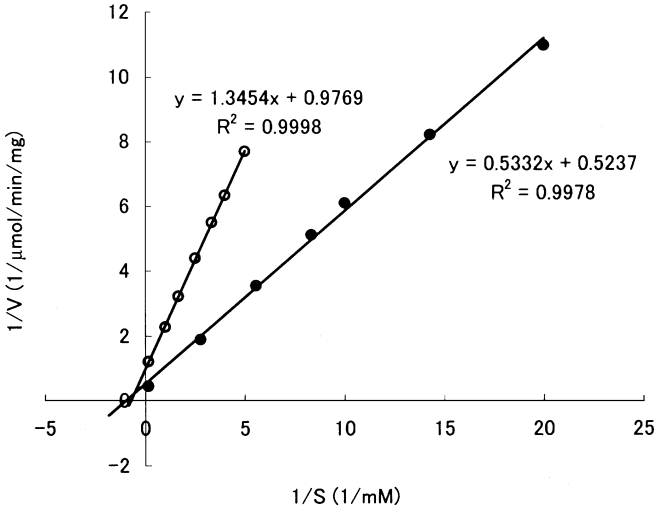

Fig. 5. Kinetic analysis of GFAT1 (*solid circles*) and GFAT1-L (*open circles*). GFAT enzyme activities were assayed at varying concentrations of fructose-6-phosphate. The reaction velocity was monitored spectrophotometrically (A_{365}). The dependence of the initial reaction velocity on substrate concentration was plotted in a linear fashion (Lineweaver-Burk plots). Each data point represents the mean of duplicate samples

(Fig. 5). As can be seen in Fig. 5, GFAT enzymatic activities were shown in recombinant GFAT1-L proteins to be the same as recombinant GFAT1 proteins. The *Km* values of GFAT1 and GFAT1-L were derived from Lineweaver-Burk plots of activities using F6P as a substrate (Fig. 5). The *Km* values of GFAT1 and GFAT1-L for F6P were calculated to be 1.02 mM and 1.38 mM respectively. *Vmax* values of GFAT1 and GFAT1-L for F6P were calculated to be 1.92 μmol/min/mg and 1.02 μmol/min/mg, respectively. Some differences were shown in kinetic data between GFAT1 and GFAT1-L. The *Vmax* value showed two fold differences. Further investigation will be needed to understand whether these differences are significant in biochemical function.

We have described the identification of *GFAT1-L*, a novel splice variant of human *GFAT1*. *GFAT1-L* contains a 54-bp insertion in the *GFAT1* open reading frame, corresponding to an 18-amino-acid insertion in the predicted hinge region of the GFAT1 protein. Moreover, recombinant GFAT1-L proteins display functional GFAT enzymatic activities in vitro. These findings strongly suggest the existence of a functional variant of GFAT1 in vivo, previously considered to be a simplex enzyme. Also, the unique tissue distribution makes GFAT1-L a very interesting target for further research on the tissue-specific regulation of hexosamine generation and the potential link to insulin resistance.

Zhou et al. (1998) have shown that GFAT1 activity is regulated through phosphorylation by cAMP-dependent protein kinase A (PKA). Human GFAT1 contains two potential PKA phosphorylation sites. Using mass spectroscopy, Chang et al. (2000) recently mapped the PKA phosphorylation sites, demonstrating that GFAT1 is stoichiometrically phosphorylated at serine 205 and phosphorylated to a lesser extent at serine 235. This region, called the GFAT hinge region, is thought to be the regulatory element for GFAT activity. It is likely that the 18-amino-acid insertion into this hinge region in GFAT1-L results in altered substrate binding or phosphorylation. Further investigation is required to confirm the potential effects of this insertion on substrate binding or phosphorylation in vitro and in vivo.

It will be interesting to compare the contributions of GFAT1-L to skeletal muscle insulin resistance with the contributions of GFAT1, as defined by the studies of GFAT1 transgenic mice (Hebert et al. 1996; Cooksey et al. 1999) and GFAT1 overexpression in fibroblast cells (Crook et al. 1995, Crook et al. 2000). Also, immunostaining and in situ hybridization studies of GFAT should be revisited, as it will be possible to distinguish between GFAT1, GFAT1-L, and GFAT2. The identification of GFAT1-L has the potential to open new avenues of research into the mechanism of insulin resistance.

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