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Identification of novel putative membrane proteins selectively expressed during adipose conversion of 3T3-L1 cells

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Abstract

Fat tissue plays a critical role in the regulation of energy metabolism. Here we report the proteomic identification of a novel fat tissue-specific low molecular weight protein (Falp) which responds to insulin. Falp is preferentially expressed in adipocytes but not in preadipocytes, as shown by two-dimensional gel electrophoresis. Northern blot analysis shows that the *Falp* gene is predominantly expressed in brown and white fat tissues, but not in any other tissues examined. Human homologs of mouse Falp are found to exist as two alternatively spliced isoforms, which share the same N-terminus but have different C-termini. Both human and mouse Falp contain a conserved putative transmembrane domain. Immunofluorescent analyses of 3T3-L1 adipocytes show that Falp protein strictly localizes at a compact perinuclear membrane compartment. Treatment of cells with insulin induces the redistribution of Falp into numerous discrete spotty structures spreading throughout the cytoplasm. Whereas the function of Falp is currently unclear, its tissue specific expression and the responsiveness to insulin suggest that Falp might be involved in a process specifically restricted to adipose tissue function, such as vesicular transport and protein secretion. © 2002 Elsevier Science (USA). All rights reserved.

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Adipose tissue has recently been shown to play an important role in the regulation of energy metabolism, and its malfunction is one of the major causes of insulin resistance and its associated complications, such as type 2 diabetes and cardiovascular diseases [1,2]. A close correlation exists between changes in fat mass and insulin sensitivity [3]. Insulin resistance and hyperinsulinemia are present in obese as well as in lipodystrophic individuals [4,5]. The pivotal role of adipocytes in the regulation of insulin sensitivity is further supported by two recent independent genetic studies on fat-ablated mice, which have severe insulin resistance and hyperglycemia [6,7]. Furthermore, insulin-sensitizing drugs that target transcriptional regulation of adipocytes also link insulin sensitivity to adipocyte function [8].

Studies of adipocytes became experimentally feasible with the availability of immortal cell lines that differentiate in vitro into white adipocytes [9]. When cultured in defined media, 3T3-L1 preadipocytes deposit triglyceride in cytoplasmic lipid droplets and express genes that are also expressed in adipocytes in vivo. Genetically based approaches have led to the identification of key transcription factors in the complex transcriptional cascade that is activated during adipose conversion [10,11]. These factors include peroxisome proliferator-activated receptor γ (PPAR γ), CCAAT/enhancer binding protein (C/EBP), and adipocyte differentiation and determination factor 1 (ADD1)-sterol regulatory element binding protein 1c (SREBP1c). Sequential activation of these transcription factors induces expression of adipocyte-specific genes, including enzymes, structural proteins, hormone receptors, and a variety of secreted factors involved in paracrine and endocrine functions [12,13]. Some of the adipocyte-specific

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proteins, such as β_3 adrenergic receptor, uncoupling protein, and PPAR γ , are now being developed as pharmaceutical targets for the treatment of metabolic disorders [14,15].

The molecular characterization of adipocyte-specific genes has previously been arisen from genetically based approaches, such as differential hybridization, subtractive cloning, shotgun techniques, and microarray analysis [16–18]. Our laboratory has used two dimensional gel electrophoresis based proteomic techniques to identify proteins differentially expressed and/or modified during adipose conversion of 3T3-L1 preadipocyte cell lines. The present study describes the molecular identification and cloning of a novel, putative integral membrane protein selectively expressed during adipose conversion. The function of this protein is so far unknown and we have termed it Falp (for fat tissue specific low MW proteins). Human homologs of Falp are found to exist as two alternatively spliced isoforms. A sequence homology search reveals that Falp does not share sequence identity with any genes of known functions. Given the specific adipose tissue expression and its responsiveness to insulin, Falp could represent a novel class of proteins involved in adipose tissue-mediated regulation of energy metabolism.

Materials and methods

Differentiation of 3T3-L1 cells. 3T3-L1 preadipocytes were maintained as subconfluent cultures in DMEM supplemented with 10% fetal calf serum. Differentiation of postconfluent cells was initiated by treatment with 0.25 μ M dexamethasone, 0.5 mM IBMX, and 10 μ g/ml insulin for two days. This was followed by incubation with 10 μ g/ml insulin for two days. The cells were then maintained in DMEM with 10% fetal calf serum for another four days. Staining with red Oil O revealed that over 90% of cells exhibited typical morphology of adipocytes.

Two-dimensional gel electrophoresis (2-DE). 3T3-L1 preadipocytes or adipocytes (day 8 after differentiation) were solubilized in a lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 10 mM DTT, 1% pharmalyte 3–10. Equal amounts of proteins from these samples were then analyzed by two-dimensional gel electrophoresis as described previously [19], using pH 4–7 immobilized Drystrips (Amersham Pharmacia) for the first dimensional focussing. The separated proteins were visualized with either silver or Coomassie Brilliant Blue R250 (CBB) staining. The differentially expressed proteins were identified by Melany 2 software (Bio-Rad).

In-gel trypsin digestion, reversed phase high performance liquid chromatography (RP HPLC), and amino acid sequencing. Proteins of interest separated by 2-DE gels were excised, and gel pieces were subjected to in-gel trypsin digestion as described previously [20]. The extracted tryptic peptide mixtures were fractionated by RP HPLC on a Jupiter 5 μ C18 column (250 \times 2.00 mm, Phenomenex). The pre-warmed column (37 $^{\circ}$ C) was washed for 7 min with 0.1% trifluoroacetic acid (v/v) followed by elution using a 50 min linear gradient from 8% to 36% acetonitrile at a flow rate of 200 μ l/min. The well-resolved fractions were chosen for amino acid sequencing using the Edman degradation method with a Perkin–Elmer (Procise, Model 492) protein sequencer.

Cloning of human and mouse Falp. Total RNA was purified from mouse 3T3-L1 adipocytes or human fat pads using Trizol reagent according to the manufacturer's instructions (Invitrogen). The

oligo-dT primed cDNA from the total RNA was used as a template for degenerate PCR analysis. The two degenerate primers, which were designed according to the amino acid sequences of the two tryptic peptides of mouse Falp, are 5'-ATGGCNAAYGGNACNGAYGCN AGY-3' and 5'-YTGNGTNAAYCCAYTGRTCTNGTNCNGC-3'. The two 'guessers' used for amplification of human *FALP* are hFALP/US: 5'-ATCGGGATCCATGGCCAACGGGACCAAC-3' (sense) and hFALP/DS: 5'-GTACGAATTCCTCATCTGCGGGGAGGC-3' (antisense). The full-length cDNAs of mouse and human *FALP* were obtained using 3'- and 5'-rapid amplification of cDNA end (RACE) according to the manufacturer's instructions (Invitrogen). The DNA fragments were then inserted into the pGEMT-easy vector (Promega) for DNA sequence verification.

The vector for mammalian expression of mouse Falp was generated by cDNA amplification using 5'-ATCGGGATCCATGGCCAACGG GACCGACGCC-3' as the sense primer and 5'-ATCGGAATTCTCA CTTGTCATCGTCGCTCTTGTAGTCGGGGAGAGCCAGGGGC C-3' as the antisense primer. Following the digestion of the PCR product with *Bam*HI/*Eco*RI, the fragment was inserted into the pcDNA3.1 vector (Invitrogen) to produce pcDNA-Falp-F, which encodes full-length Falp with an FLAG epitope tagged at its C-terminus.

Transient expression of mouse Falp and immunocytochemistry. The mammalian expression vector encoding FLAG-tagged Falp was transfected into COS-7 cells or 3T3-L1 adipocytes using FuGENE 6 transfection reagent (Roche). The cells were allowed to grow for 24 or 48 h, fixed using methanol/acetone, incubated with anti-FLAG monoclonal antibody (Sigma), and then stained with cy3-conjugated goat anti-mouse polyclonal antibody (Sigma). The specimens were then analyzed using a ZEISS Axioskop 2 plus microscopy equipped with a digitized camera.

Northern blot analysis. Ten micrograms of total RNA purified from either 3T3-L1 cells or FVB/N mouse adipose tissue was separated on a 1.2% formaldehyde-denaturing agarose gel and transferred to Nylon membranes (Amersham Pharmacia). Hybridization was carried out as described previously [21]. The membranes were visualized using a phosphorimager and quantitated using MacBAS software (Fujifilm).

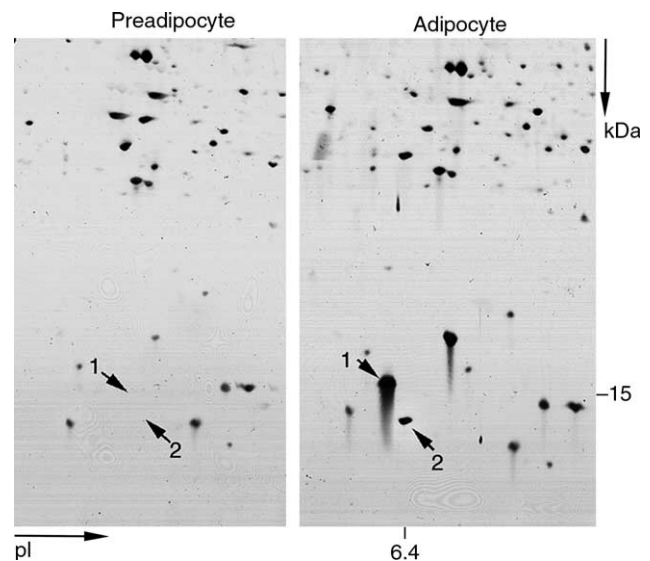


Fig. 1. 2-DE separation of low molecular weight proteins induced after conversion of 3T3-L1 preadipocytes to adipocytes. Equal amount of proteins from preadipocytes or adipocytes (day 8 after differentiation) was separated by 2-DE and stained with silver. The arrows denote the proteins which are only expressed in adipocytes and not in preadipocytes.

Results and discussion

Characterization of Falp, a novel protein expressed during adipose conversion

To identify novel proteins that are differentially expressed during adipose conversion, proteins from 3T3-L1 preadipocytes and adipocytes were analyzed by 2-DE. Within the low molecular weight region of the gels, we found two proteins which were selectively expressed in adipocytes (day 8 after differentiation), but not in preadipocytes (Fig. 1).

To identify the nature of these two proteins, the spots were excised from multiple preparative two-dimensional gels and *in gel* digested by trypsin. The tryptic peptide mixture of each protein was separated by RP HPLC and the well-resolved fractions were analyzed by amino acid sequencing. Database searching revealed that a sequenced peptide (FDETTAD) derived from spot 1 matched exclusively with mouse epidermal fatty acid-binding protein (Swissprot accession number P55053). The amino acid sequences of the tryptic peptides (MANGTDASVPLT and AGTDQWLTQQSPS) de-

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agccgggtgattctgatagaattagtaagcaatcttctctctgggctc
acggccagctgtgggtgcgagcctctgacagacactggaaaaagccacagtc
atg gcc aac ggg acc gac gcc tct gtc ccg ctc acc agc 39
M A N G T D A S V P L T S 13
tat gag tat tac ctg gac tac ata gac ctc att cct gtg 78
Y E Y Y L D Y I D L I P V 26
gac gag aag aag ctg aaa gcc aac aag cat tcc att gtc 117
D E K K L K A N K H S I V 39
ctc ttt ctc atc atc gcc ctg tgg ttg agc ctg gct acc 156
I A L W L S L A T F V V L 52
ttc gtg gtg ctc ctg ctc tac atg tcc tgg tgc ggc tcc 195
L F L I L L Y M S W S G S 65
cca cag atg agg cac agt ccc caa ccc cag cca ata tgt 234
P Q M R H S P Q P Q P I C 78
tca tgg act cac agc ttc aac ctc cct ctg tgc ctc cgg 273
S W T H S F N L P L C L R 91
agg gcc tcc ctg cag aca aca gag gag cca gga agg aga 312
R A S L Q T T E E P G R R 104
gct ggc act gac cag tgg tta acg cag cag agt cct tct 351
A G T D Q W L T Q Q S P S 117
gcc tca gcc ccg ggg ecc ctg gct ctc ccc tag gaccaggtcca
A S A P G P L A L P * 127
ggatggagggtcccagggtcagctggcctcacactcaagCagtgggtgagcctgg
agacagagcgtctcaactgtagaacggatgatgcccagagagccagctcgggctcaa
gcaaacgggtgaactccaaccaaccggggcagctacgtcttttttagggcgttta
caatggccttgaatatagcaggaaactgacgggacaaaaccaagtttacaaga
ggaccatcacacacattgatagtgacagctaggatgcaggagctgccttggacaca
gctgtctctgttgagcaagcttagcctgcttctgcttaccatttcttgggggt
acacaggaaaataaaatgtgaattaggataaaaaaaaaaaaaa
    
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Fig. 2. Nucleotide and amino acid sequences of mouse Falp (GenBank accession number AY079153). The amino acid sequences for the two tryptic peptides that were determined by amino acid sequencing are underlined. The highlighted amino acid residues between 38 and 61 represent a hydrophobic transmembrane domain, as predicted by the TMpred program. The stop codon and putative polyadenylation signal are indicated in bold letters. Note that the start codon was also confirmed by N-terminal amino acid sequencing.

rived from ‘spot’ 2 did not show significant homology with any known proteins present in the available database. Further N-terminal amino acid sequencing analysis of spot 2 blotted onto PVDF membrane also obtained the sequence MANGTDASVPLT, suggesting that the methionine at this fragment is encoded by the start codon of the gene.

The partial cDNA sequence of this protein was obtained by degenerate RT PCR using the primers designed according to the amino acid sequence of the tryptic peptides. The entire coding cDNA sequence was obtained by 3'- and 5'-RACE PCR strategy (Fig. 2). The DNA sequence of this gene was deposited in GenBank (accession number AY079153). Blastn search of the nucleic acid database at the National Center for Biotechnology Information revealed that the cDNA

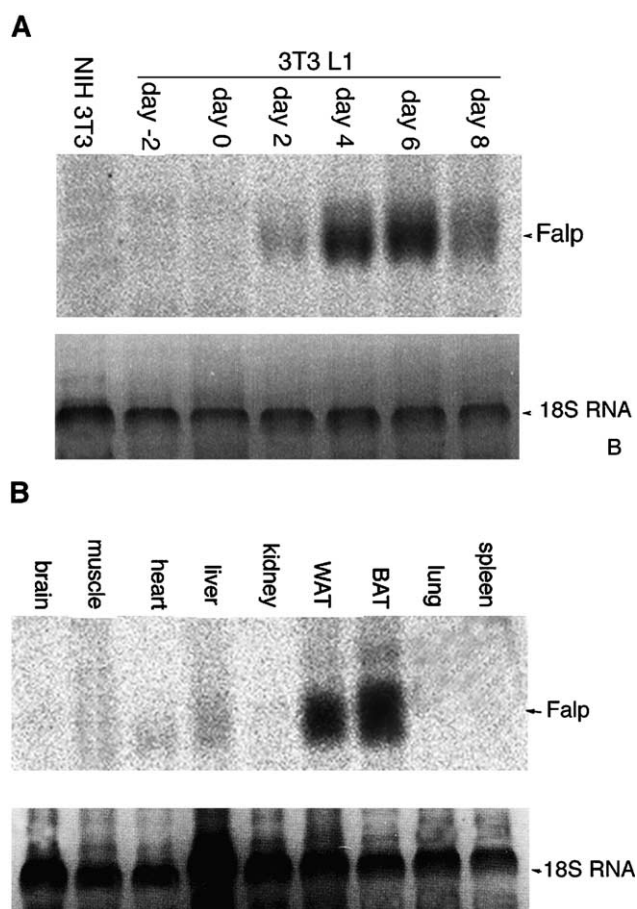


Fig. 3. Northern blot analysis of mouse Falp mRNA expression. (A) Time dependent expression of FALP during differentiation induction of 3T3-L1 cell lines. Cells were grown in DMEM with 10% FCS. Confluent preadipocytes were exposed to a differentiation mixture as described in the Methods. Ten micrograms of total RNA prepared from NIH 3T3 cells, preconfluent 3T3-L1 cells (day 2), or 3T3-L1 cells at the indicated time points of differentiation was subjected to Northern blot analysis for mouse FALP and 18 S RNA. (B) Tissue distribution of FALP mRNA. Northern blot analysis was performed with total RNA derived from a variety of mouse tissues as indicated. WAT: white adipocyte tissue; BAT: brown adipocyte tissue.

Human homologs of Falp exist as two alternatively spliced isoforms

To identify the human homologs of mouse Falp, its amino acid sequence was used to search against human genomic sequences. This analysis found that two DNA fragments within chromosome 21q22.1 region, which are intercepted by 7670 base pairs, encodes a potential polypeptide which has over 85% identity with the N-terminal 69 amino acid residues of mouse Falp. To confirm the existence of this potential human *FALP* homolog in adipose tissue, two ‘guessmers’ were designed based on the potential sequence of the human *FALP* gene, as described in the Methods. Reverse transcription PCR analysis using the two ‘guessmers’ revealed a band with the expected size (~180 bp) in human fat tissue (Lane 2, Fig. 4). DNA sequence analysis verified the expression of this putative gene. Further analysis using 3' rapid amplification of cDNA end found that the gene encoding human FALP existed as two distinct isoforms, which we have termed human FALP α and FALP β , respectively (Figs. 4 and 5). The DNA sequences of these two genes were deposited in the GenBank (accession number: AY078152 and AF4835 49). These two isoforms share a common N-terminus, but have distinct C-termini. The conceptual human FALP α and β proteins are composed of 173 and 102 amino acid residues, with the predicted molecular masses of 19 and 11 kDa, respectively. The gene structure of human FALP consists of three exons, with exons I and II encoding the NH₂-terminal 68 amino acid residues which are shared by both isoforms (Fig. 5). Exons III and III' are alternatively spliced and encode COOH-terminal 105 amino acid residues of α

isoform and 33 amino acid residues of the β isoform, respectively.

Subcellular distribution of mouse Falp

Sequence analysis of human and mouse FALPs using the program TMpred [23], identified a conserved single transmembrane domain spanning 23 amino acid residues, indicating that Falp might be an integral membrane protein. In order to investigate the intracellular localization of Falp, a COOH-terminal FLAG epitope-tagged mouse FALP construct (pcDNA-Falp-F) was introduced into either COS 7 cells or 3T3-L1 adipocytes by transient transfection. Analysis using an immunofluorescent microscopy revealed that a majority of FLAG-tagged Falp accumulated at the perinuclear region, where it formed a compact patch-like structure (Figs. 6A and B). Treatment of 3T3-L1 adipocytes with 50 nM insulin caused the redistribution of mouse Falp protein from the compact perinuclear compartment into numerous discrete spotty structures spreading throughout the cytoplasm (Fig. 6C). A few spotty structures could also be observed at the peripheral region of the cells, implicating its presence at the plasma membrane. These results suggest that FALP is localized at a dynamic compartment and its distribution is regulated by insulin. Insulin treatment did not cause redistribution of Falp in COS 7 cells (data not shown), perhaps due to the lack of an insulin receptor or other signaling components in this cell line.

Insulin-dependent intracellular trafficking of membrane organelles has been implicated in many metabolic functions of adipose tissue [24]. A recent study suggested the existence of at least three different insulin-regulated

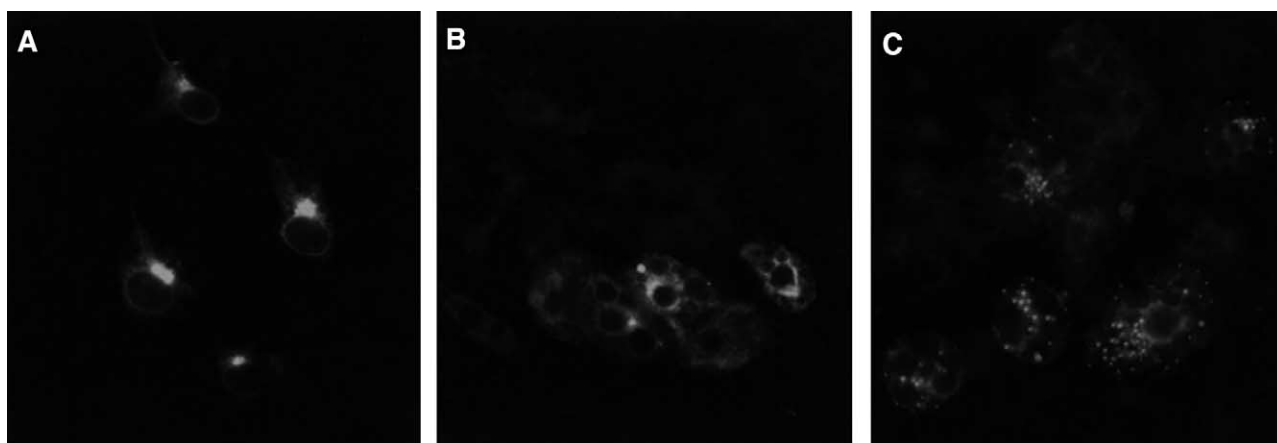


Fig. 6. Immunolocalization of FLAG-tagged mouse Falp in basal and insulin-treated cells. COS 7 cells (A) or 3T3-L1 adipocytes at day 6 after differentiation induction (B and C) were transfected with plasmid pcDNA-Falp-F using Fugene 6, and grown in DMEM containing 0.1% FBS for 48 h. The cells were then treated with 50 nM insulin for 15 min (C), or untreated (A and B). After fixation with methanol/acetone for 2 min, the specimen was sequentially stained with anti-FLAG monoclonal antibody (27 μ g/ml) and cy3-conjugated goat anti-mouse polyclonal antibody (1:500), and then visualized under fluorescent microscopy. Note that expression of FALP was mainly restricted to a compact structure at the perinuclear region (A and B), and was redistributed into numerous discrete spots throughout cytoplasm following treatment of 3T3-L1 adipocytes with insulin (C).

vesicular trafficking pathways in this tissue [25]. A typical example is insulin-induced intracellular trafficking of GLUT4, a mammalian facilitative glucose transporter that is highly expressed in adipose tissue and striated muscle [24]. In the basal state, GLUT4 appears to be targeted to an intracellular storage compartment at the perinuclear region. Insulin induces the plasma membrane translocation of GLUT4 through activation of at least two parallel signaling pathways, including the PI 3 kinase/Akt/PKC ξ/λ and the Cbl/CAP pathways [24]. This insulin-regulated trafficking process also involves multiple intracellular organelles and a variety of protein components [26].

Adipose tissue has also recently been recognized as an important endocrine organ which secretes a variety of proteins (adipocytokines), such as leptin, adiponectin, adipisin, TNF α , and resistin [27,28]. Studies both in vivo and in vitro have demonstrated that secretion of adipocytokines from adipose cells occurs via two compartments, constitutive, and regulatory [29]. The secretion of these adipocytokines, such as leptin [30], adiponectin [31], and adipisin [32], could also be enhanced by insulin. However, the intracellular storage compartments of these adipocytokines, and their vesicular trafficking pathways are distinct from those of GLUT4 [25].

The physiological role of Falp is currently unclear. Given its adipose tissue specific expression, its localization at a dynamic intracellular membrane compartment and its responsiveness to insulin, it is highly possible that the function of Falp is related to the intracellular trafficking pathways of adipose cells, such as GLUT4 translocation and hormone secretion. These possibilities are currently under investigation in our laboratory.

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