Cloning of a Rat Adipocyte Membrane Protein Implicated in Binding or Transport of Long-chain Fatty Acids That Is Induced during Preadipocyte Differentiation

HOMOLOGY WITH HUMAN CD36

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Nada A. Abumrad§§, M. Raafat El-Maghrabi, Ez-Zoubir Amri, Ellen Lopez, and Paul A. Grimaldi¶

From the Department of Physiology and Biophysics, State University of New York, Stony Brook, New York 11794 and l'Institut de la Sante et de la Recherche Scientifique, UMR-134, Universite de Nice Sophia-Antipolis, Parc Valrose 06108, Nice Cedex 2, France

A cDNA for an adipocyte membrane protein, implicated in the transport of long-chain fatty acids, was isolated by screening with a synthetic oligonucleotide derived from the amino terminal sequence of the protein. The 88-kDa adipocyte membrane protein was previously identified by covalent labeling with N-sulfo-N-succinimidyl esters of long-chain fatty acids which irreversibly inhibited fatty acid transport by 75% (Harmon, C. M., and Abumrad, N. A. (1993) J. Membr. Biol. 124, 261–268). The cDNA (FAT, 2432 base pairs (bp)) contained 70 bp of 5'-untranslated sequence, an open reading frame encoding a 472-amino acid protein with a predicted molecular mass of 52466, and 940 bp of 3'-untranslated sequence with two polyadenylation signal sequences but with no polyadenylation tail. The deduced protein sequence predicted two membrane segments and 10 potential N-linked glycosylation sites. Extensive glycosylation most likely explains why the molecular mass of the isolated protein (88 kDa) is different from that deduced from the cDNA sequence (53 kDa). The sequence of FAT is 85% homologous with that of glycoprotein IV (CD36) identified in human platelets and in lactating mammary epithelium. Consistent with this, a polyclonal antibody against CD36 reacted with adipocyte plasma membranes and detected a single band at 88 kDa. Northern blot analysis of RNA obtained from rat adipose tissue and probed with the cDNA identified two major transcripts of 4.8 and 2.9 kilobases which were abundant in heart, intestine, fat, muscle, and testis. The mRNAs were not detectable in cultured adipose cell lines (Ob1771, 3T3F442A) at the fibroblastic stage but were strongly induced during the differentiation process and by treatment of preadipocytes with dexamethasone, conditions that were also associated with an increase in oleate transport. In contrast, the fibroblastic cell lines 3T3-C2 and L929, which do not differentiate, did not express the mRNAs at all stages of culture. The data suggest that FAT and CD36 belong to a family of proteins that bind/transport long-chain fatty acids or function as regulators of these processes.

A significant body of biochemical evidence supports the existence of a high affinity membrane transport system for long-chain fatty acids (FA) in adipocytes (1–8). We recently identified an 88-kDa adipocyte membrane protein which was specifically labeled by [3H]-sulfo-N-succinimidyl-FA derivatives of long-chain fatty acids (FA), most notably oleate (4). The same protein was one of those identified by labeling with DIDS (2). Both labeling conditions caused a strong inhibition of FA transport by adipocytes, suggesting a role of the protein in the transport process. The isolated sulfo-N-succinimidyl-FA-labeled protein had an amino-terminal sequence (5) similar to that of glycoprotein IV or CD36 (6,7) a membrane protein expressed in lactating mammary epithelium, in human platelets and in vascular endothelium (6–8). In this study we have used a synthetic oligonucleotide deduced from the amino-terminal sequence of the 88-kDa protein to isolate a cDNA clone from a rat adipocyte cDNA library in Igt11. The complete protein sequence deduced from the isolated cDNA shows a high degree of homology with CD36. Tissue distribution of the corresponding mRNA and induction of its expression in cultured preadipocytes by differentiation and dexamethasone are reported.

EXPERIMENTAL PROCEDURES

Materials—The random-primer cDNA labeling kit, the [α-32P]dCTP, the [α-32P]dATP, and the nylon filters (Hybond N+) used for plaque lifts were from Amersham Corp. The DNA sequencing kits (SequenaseTM and the 7-deaza-dGTP Sequencing Kit) and Thq DyeDeoxy Terminator Cycle Sequencing Kit) were from U. S. Biochemical Corp. and Applied Biosystems, respectively. Enzymes for DNA and RNA manipulation were from Boehringer Mannheim or New England Biolabs. [3H]Oleate was from Du Pont-New England Nuclear.

cDNA Library Screening—A rat adipose tissue cDNA expression library in Igt11 (a gift from Dr. A. Kimmel at the National Institutes of Health) was plated at a density of about 5 x 10⁶ plaque-forming units/150-mm Petri dish on a layer of Escherichia coli Y1090. Filters were washed sequentially in a denaturing solution (0.5 M NaOH, 1.5 M NaCl), a neutralizing solution (0.5 M Tris, 1.5 M NaCl) and then in 3 x SSC (0.45 M NaCl, 0.05 M sodium citrate). They were prehybridized in a solution consisting of 10 x Denhardt’s (2 g/liter Ficoll, 2 g/liter polyvinylpyrrolidone, 2 g/liter BSA) and 5 x SSPE (0.75 M NaCl, 0.05 M NaH₂PO₄, 5 mM EDTA, pH 7.4) and hybridized at 42 °C with a 32-base mixed oligonucleotide (ATGGGCCGCTGTCAGGGAAACCTGGTGCGCTCATACGTAC), (synthesized using Applied Biosystems model 381A) deduced from the amino-terminal sequence of the adipocyte protein (5), and 32P-end-labeled with polynucleotide kinase and [γ-32P]ATP. Filters were washed 4 times with 2 x SSC containing 0.1% SDS, dried, and exposed to film for 20 h at −70 °C with an intensifying screen. Positive clones

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† To whom correspondence should be addressed: Dept. of Physiology and Biophysics, State University of New York, Stony Brook, NY 11794-8661.

‡ The abbreviations used are: FA, fatty acid(s); DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonate; BSA, bovine serum albumin; kb, kilobase(s); LDL, low density lipoprotein.
were isolated and purified by three successive screening rounds. DNA sequencing was done according to the method of Sanger et al. (9), either manually with Sequenase™ or on an Applied Biosystems Automated Sequencer (model 373A) with Taq DNA polymerase. Initially, universal M13 forward and reverse primers were used, followed later by synthetic oligonucleotides primers derived from obtained sequence.

Cell Culture and Assays of Fatty Acid Transport—Ob1771 (10) 3T3-F442A, 3T3-C2 (11), and L929 cells (American Type Cell Culture Collection) were plated at a density of 2 x 10⁶/cm² and were grown and differentiated, in the case of 3T3-F442A and Ob1771, as previously described (12). Before the assays, the cells were washed in serum-free Dulbecco’s modified Eagle’s medium for 1 h at 37 °C. The medium was then switched (30 min) to a Krebs-Ringer solution, buffered with 25 mM Hepes, pH 7.5 (KRH), and containing 0.5% fatty acid-free bovine serum albumin (BSA) and 2 mM glucose. The cells were then washed three times with KRH lacking BSA before addition of transport buffer. Transport assays were conducted as detailed elsewhere (3). Controls for extracellularly trapped FA were routinely subtracted from uptake values and were obtained by adding cold buffer to the cells before addition of isotopes. Uptake was expressed per 10⁶ cells or per pg of DNA (13).

RNA Preparation and Probing—Total RNA was prepared (14), separated on 1% agarose/formaldehyde gels, and transferred to nylon membranes in the presence of 50% formamide (15), then washed with 1 × SSC three times before exposure to film. To quantitate the mRNA, films from 10⁶ plaque-forming units (PFU) were used, followed later by synthetic oligonucleotides primers derived from obtained sequence.

RESULTS AND DISCUSSION

Cloning of the Adipocyte Membrane Protein—Screening of the rat adipose tissue cDNA library yielded eight positive clones from 10⁶ plaque-forming units. These were purified by three successive screenings. The cDNA inserts, excised by digestion with EcoRI, yielded fragments ranging in size from 900 to 2400 kb. Sequencing and restriction mapping indicated that all the clones represented different lengths of the same mRNA. The largest inserts, 1.4 and 2.4 kb, were sequenced in their entirety. The larger clone contained all the sequence of the 1.4-kb insert and about 1 kb more at the 3' end. The 2.4-kb cDNA (FAT) contained an open reading frame, starting at base 75, that encodes a 472-amino acid protein with a calculated mass of about 53 kDa. The two inserts contained two putative polyadenylation sequences and five destabilization consensus sequences (ATTTA). The absence of a polyadenylation tail suggested that the complete message is longer than 2.4 kb. The nucleotide and deduced amino acid sequences are shown in Fig. 1. The deduced protein sequence for FAT revealed 10 potential glycosylation sites (N-X-S/N-X-T). Glycosylation at some or all of these sites would account for the difference between the mass of the deduced protein and the molecular weight of the adipocyte protein (88 kDa). Consistent with this, an antipeptide against the deduced carboxyl-terminal protein sequence immunoreacted with an 88-kDa protein band in plasma membranes from isolated adipocytes or from Ob1771 cells (data not shown).

A search of data base nucleotide sequences revealed 79% similarity (75% identity) of the sequence obtained with that of the human placenta cell surface antigen, glycoprotein IV, or CD36 (6). The deduced protein sequence for CD36 also consists of a 472-amino acid protein and a molecular mass that is significantly less than the apparent size determined by gel electrophoresis (83 kDa). Glycosylation explains this difference (6, 16). Eight of the glycosylation sequences are strictly conserved between FAT and CD36, and one (at 103) exhibits substitution of 1 residue. Other significant features that are conserved between FAT and CD36 are the number and position of cysteines and the carboxyl-terminal sequence, CXCX₅₉, postulated to mediate interaction of CD36 with other proteins.
with tyrosine kinases (reviewed in Ref. 16). Consistent with the strong similarity of the FAT and CD36 proteins, the polyclonal antibody against human CD36 (7) reacted with plasma membranes from rat adipocytes or from differentiated cultured adipocytes giving one sharp band at about 88 kDa (data not shown).

Hydropathy analysis of the deduced amino acid sequence of FAT predicts two major hydrophobic domains, one close to the amino-terminal end (residues 8–36) and another close to the carboxyl-terminal end (residues 437–464). This suggests, as postulated for CD36 (16), the presence of two membrane-spanning domains, one at each end, possibly forming a hairpin-like configuration with most of the protein being extracellular. The extramembranous segment of FAT exhibits multiple stretches of hydrophobic amino acids, suggesting that some protein domains (for example, residues 118–129, 137–147, 186–200, and 285–302) although too short to span the membrane, might be embedded in it or might form small hydrophobic pockets. In the case of CD36, residues 184–204 are thought to be membrane-associated. The configuration predicted for the FAT protein is unlike that of well known membrane carriers which generally have many more (6–12) transmembrane segments (17, 18).

However, proteins with single transmembrane domains and multiple glycosylation sites have been recently cloned, and transport of dibasic and neutral amino acids (21, 22). As shown in Fig. 4, a similar progressive increase in oleate transport was measured in Ob1771 cells, beginning at about 3 days after confluence. This increase paralleled induction of FAT mRNA, which first became detectable starting at 2 days after confluence (Fig. 4), and which followed appearance of the mRNA for lipoprotein lipase and preceded that for the fatty acid-binding protein, aP2 (data not shown). Under the same conditions, mRNA for two enzymes important for FA esterification; fatty acid-CoA ligase and glycerol phosphate dehydrogenase, were not detectable until after 6 days post-confluence (data not shown). In line with this, microscopic examination of Ob1771 cells, demonstrated no evidence of intracellular lipid vacuoles before about day 6 following confluence.

Since treatment of preadipocytes with dexamethasone induced accumulation of FAT mRNAs (Fig. 3B), it was of interest to examine transport activity under those conditions. As shown in Fig. 5, an increase in FA transport was observed with 24- and 48-h dexamethasone treatment of Ob1771 preadipocytes (day +1), which paralleled accumulation of FAT mRNAs. There was no induction of the mRNA for aP2 or fatty acid-CoA ligase under the same conditions.

In summary, we report the isolation of a cDNA that encodes an adipocyte membrane protein which has been implicated in the binding or transport of long-chain FA. The cDNA was obtained by screening with a probe derived from the amino-terminal sequence of a rat adipocyte membrane protein (88 kDa) identified by labeling with [3H]N-sulfosuccinimidyloleate. This derivative, synthesized by reacting [3H]oleate with N-hydroxysuccinimide, produced a 75% inhibition of FA transport (4). The protein was also among those labeled by another irreversible inhibitor of FA transport, [3H]DIDS (2) since preincubation with DIDS reduced subsequent labeling with [3H]N-

![Fig. 2. Tissue distribution of FAT mRNA. RNA (20 μg/lane) was analyzed by Northern blot and probed with 32P-labeled FAT cDNA. mRNA for glyceraldehyde phosphate dehydrogenase (GAPDH) is shown as an internal control. A, adipose tissue; B, kidney; C, liver; D, heart; F, testis; E, spleen; G, intestine; H, skeletal muscle.](image)

![Fig. 3. Induction of FAT mRNA during differentiation of preadipocytes in culture and by dexamethasone. Panel A, FAT mRNA in 3T3F442A, Ob1771, 3T3-C2, and L929 cells. Lanes A, preconfluent stage; lanes B, at 10 days following confluence (day +10). Glyceraldehyde phosphate dehydrogenase mRNA is included as a control. Panel B, induction of FAT mRNA in Ob1771 preadipocytes (day +1) by treatment with 1 μM dexamethasone for 24 h.](image)
expression has also been reported in sebaceous glands, lipomas, and liposarcomas (30, 31). Although CD36 has been hypothesized to be a collagen or a thrombospondin receptor or to function in some form of signal transduction, its definitive role remains uncertain (15, 32). Its similarity to FAT, which binds reactive FA esters and is a marker of predipose differentiation, strongly suggests that it might function as a FA acceptor and that FA binding might initiate some of the cellular events linked with both FA (33, 34) and CD36 (7, 16) in platelets. A recent report has suggested that CD36 might bind oxidized but not acetylated or native low density lipoprotein (LDL) (35). Oxidized LDL are formed consequent to peroxidation of LDL fatty acids and attachment of peroxidation products to the apoprotein moiety. It is possible that CD36 is recognizing the FA peroxidation products.

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REFERENCES
31. Madrid and CD36 antibody.

Cloning of an Adipocyte Membrane Protein

It is possible that the FAT protein is part of a functional membrane-spanning complex comprised of multiple similar or dissimilar units. The FAT protein might also constitute one part of a vescular transport system comprising several proteins such as the one postulated for FA transport into E. coli (28, 29). Alternatively, FAT might function in sequestering FA within hydrophobic pockets formed in its extracellular segment, allowing the formation of high concentrations of free FA at the membrane vicinity, and promoting FA partitioning in the bilayer since this is not favored at the low physiological molar ratios of FA/Palmitol (1, 2). In line with this, binding of sulfosuccinimidyl-FA to the protein in intact adipocytes appeared to be a function of the long hydrophobic hydrocarbon chain since the protein was not labeled by the propionic acid derivative (4). Finally, it is possible that the protein modulates rather than mediates the transport of FA, although the labeling data suggested a more direct interaction. Studies are under way to test these various possibilities.

The strong similarity between FAT and CD36 suggests that FAT is the rat homologue of human CD36. The primary protein sequence appears to be highly conserved; however, tissue-specific glycosylation might confer different biochemical characteristics to the two proteins. FAT has a different isoelectric point from CD36 (4) and is not recognized by the CD36 monoclonal antibody, OKM5 (data not shown). CD36 has only been cloned from a human placental library (6) and, based on immunohistochemical data, is thought to be expressed mainly in vascular cells and in lipid-secreting mammary epithelium (16). Strong

sulfosuccinimidylolate.\(^2\)

FIG. 4. Time course of FAT mRNA abundance and oleate uptake activity during adipose differentiation. Data shown are from three separate experiments and are presented as percent of maximal values measured at day 18. At that time oleate uptake was 55 pmol/min/μg DNA. FAT mRNA, ○, oleate uptake, •. Uptake was determined in quadruplicate in each experiment.

FIG. 5. Parallel induction of FAT mRNA and of oleate transport activity in preadipocytes by dexamethasone. Data shown are from three separate experiments. FAT mRNA (○) and oleate uptake (●) were determined in the absence (□) or presence (●) of dexamethasone (1 μM).

\(^2\) C. M. Harmon and N. A. Abumrad, unpublished observations.