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Binding of Sulfosuccinimidyl Fatty Acids to Adipocyte Membrane Proteins: Isolation and Amino-Terminal Sequence of an 88-kD Protein Implicated in Transport of Long-Chain Fatty Acids

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Summary. We recently reported (Harmon et al., *J. Membrane Biol.* **124**:261–268, 1991) that sulfo-*N*-succinimidyl derivatives of long-chain fatty acids (SS-FA) specifically inhibited transport of oleate by rat adipocytes. These compounds bound to an 85–90 kD membrane protein which was also labeled by another inhibitor of FA transport [^{3}H]DIDS (4,4′-diisothiocyanostilbene-2-2′-sulfonate). These results indicated that the protein was a strong candidate as the transporter for long-chain fatty acids. In this report we determined that the apparent size of the protein is 88 kD and its isoelectric point is 6.9. We used [^{3}H]SS-oleate (SSO), which specifically labels the 88-kD protein, to isolate it from rat adipocyte plasma membranes. Identification of 15 amino acids at the N-terminus region revealed strong sequence homology with two previously described membrane glycoproteins: CD36, a ubiquitous protein originally identified in platelets and PAS IV, a protein that is enriched in the apical membranes of lipid-secreting mammary cells during lactation. Antibody against PAS IV cross-reacted with the adipocyte protein. This, together with the N-terminal sequence homology, suggested that the adipocyte protein belongs to a family of related intrinsic membrane proteins which include CD36 and PAS IV.

Key Words fatty acids · transport · adipocyte · plasma membrane · proteins · sulfosuccinimidyl fatty acid

Introduction

Long-chain fatty acids (FA) are important energy substrates for most cells. In addition, they are implicated in multiple processes such as inflammation (Stanislawski & Hornedeck, 1988; Burton, 1989), immune responses (Robinson et al., 1986; Calder, Bevan & Newsholme, 1992) atherosclerosis (Calder et al., 1990) and thrombosis (Higazi et al., 1992). FA also modulate activity of various enzymes and membrane carriers (El Touny, Khan & Hannun, 1990; Ordway, Singer & Walsh, 1991; Henderson & Chappell, 1992) and competitively inhibit thyroid hormone binding to its nuclear receptor in vitro and in vivo (Inoue et al., 1989; Van der Klis, Wiersinga & de Vijlder, 1989). Recently FA were also shown to regulate expression of selected genes which include those for the FA-binding protein and the FA-acyl-CoA synthase (Amri et al., 1991; Abumrad et al., 1991; Tiwari et al., 1991; Grimaldi et al., 1992). Given their multiple roles in cell function, it is important to understand the mechanisms which mediate cellular uptake of FA.

Membrane permeation of long chain FA in adipocytes has been reported to exhibit the characteristics of facilitated diffusion suggesting it is a protein-mediated event (Abumrad et al., 1981; Abumrad, Park & Park, 1984; Schwieterman et al., 1988). A similar mechanism has been described for hepatocytes (Stremmel, Strohmeyer and Berk, 1986) and for cultured adipocytes (Abumrad et al., 1991; Storch, Schulman & Kleinfeld, 1991). Recently we reported that sulfo-*N*-succinimidyl derivatives of long-chain fatty acids inhibited transport of oleate (~70%) in rat adipocytes. An apparent 85–90 kD plasma membrane protein was covalently and specifically labeled by one of the derivatives [^{3}H]-sulfosuccinimidyl-oleate, SSO, (Harmon et al., 1991). The same protein band was one of the bands labelled by 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS), another irreversible inhibitor of FA transport (Abumrad et al., 1984; Harmon et al., 1991).

In this study [^{3}H]sulfosuccinimidyl-oleate ([^{3}H]SSO) was used to label and isolate the 85–90 kD rat adipocyte plasma membrane protein. N-terminal sequence was obtained and is reported.

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Materials and Methods

Synthesis of Sulfo-*N*-Succinimidyl-Oleate

The synthesis of the radiolabeled sulfosuccinimidyl derivative of oleate (SSO) was accomplished as previously described (Harmon et al., 1991). Briefly, oleate (0.25 mmol, Sigma), $HOSu(SO_3)Na$ (0.25 mmol, Pierce Chemical) and dicyclohexylcarbodiimide (DCC) (0.275 mmol) were dissolved in 0.5 ml of dry *N,N,*-dimethyl formamide (DMF) and stirred at room temperature overnight. The precipitated dicyclohexylurea was removed by filtration and the filtrate was maintained at 3°C for 4 hr. Eight volumes of ethyl acetate were added and the precipitated product was collected by filtration (Ultipor $Nylon_{66}$, 45 μm pore size) under nitrogen in a Glove Bag (Instruments for Research and Industry) and stored in a vacuumed desiccator over phosphorous pentoxide. Purity of the product was established as greater than 95% by high performance liquid chromatography and by mass spectroscopy as previously described (Harmon et al., 1991).

Preparation of Rat Adipocytes

Male Sprague-Dawley rats, 170–200 g, (Harlan Industries) fed *ad libitum* were sacrificed by decapitation. The epididymal fat pads were removed and adipocytes were isolated following methodology previously detailed (Abumrad, Park & Whitesell, 1986). The washed isolated cells were suspended (30%, vol/vol) in Krebs-Ringer HEPES buffer (KRH) containing 0.2% fatty acid-free bovine serum albumin (BSA; Sigma, Fraction V) and glucose (2 mM).

Adipocytes were treated with [^{3}H]SSO as previously described (Harmon et al., 1991). Stock solutions of [^{3}H]SSO were made by dissolving the compounds in dimethyl sulfoxide (DMSO) and few microliters were added to adipocyte suspensions to a final concentration of 200 μM. DMSO concentration never exceeded 0.05% and it was included in control cell preparations. The cells were incubated in a metabolic shaker at 37°C for 25 min. Free [^{3}H]SSO was removed by four washes (each 2 × v) with buffer containing 2.0% and then 0.2% FA-free BSA (two washes with each buffer).

Preparation of Adipocyte Plasma Membranes

Adipocyte plasma membrane fractions were prepared from adipocytes treated with [^{3}H]SSO according to a modification of the technique of Kono, Robinson and Sarver (1975) as previously detailed by Abumrad et al. (1984). Membrane pellets were stored at −70°C until use. In these experiments greater than 95% of particulate-associated [^{3}H]SSO radioactivity was recovered in the plasma membrane fraction.

Electrophoresis of Plasma Membranes and Elution of [^{3}H]SSO-Labeled Protein

In order to isolate the protein radiolabeled by SSO, we first explored the use of anion exchange chromatography for separating plasma membrane proteins. Plasma membrane fractions from cells treated with [^{3}H]SSO were solubilized in 0.4% triton and were subjected to anion exchange chromatography on DEAE-Sephacel. Radioactive proteins were retained by the column and were eluted by a salt gradient. However, further processing of the relatively large-volume eluate to concentrate and isolate the labeled protein(s) resulted in significant losses in protein and label. For these reasons, alternate procedures requiring minimal sample processing steps were explored and subsequently used. Adipocyte membranes were subjected to SDS-PAGE (Laemmli, 1970). Prestained molecular weight standards (Sigma and BioRad) which included a standard of 84 kD were run simultaneously in lanes adjacent to the plasma membrane lane. Thin (1 mm) gel slices were taken from the lane containing the plasma membrane sample in the location of the radioactive band (between 80–90 kD). Individual slices were homogenized (Kontes Potter-Elvehjem tissue grinder, 1 ml capacity, code 0019) and proteins were passively eluted in 1% SDS, 1 mM EDTA buffer at 23°C for approximately 12 hr. Other methods were used to extract the radiolabeled proteins from gel slices. These included passive elution using Triton X100 and Tween 20 to solubilize the protein or electrolution (Centrilutor, Amicon). However, slice homogenization and passive elution in SDS-EDTA buffer resulted in the highest yields and was adopted for routine use. At the end of the 12-hr period, the elution mixture was passed over a filter column (Econocolumn, BioRad) to retain gel bits and column eluates were tested for radioactivity. The eluate with the most radioactivity was then concentrated using a microconcentrator with a 30-kD size exclusion (Centricon 30, Amicon). The sample was diluted and recentrifuged several times in order to exchange the buffer for either buffer A: 0.0625 M Tris-HCL pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol for SDS-PAGE, or for buffer B: 9.5 M urea, 2% Nonidet P-40 (NP-40), 2% ampholytes and 5% β-mercaptoethanol for isoelectric focusing. Protein content was measured using the BCA assay (Pierce Chemical).

Isoelectric Focusing of Partially Purified SSO-Labeled Protein

The radiolabeled protein eluted from gel slices as described above were further characterized by isoelectric focusing. Cylindrical gels were used (2.0 ml 30% acrylamide/1.8% bis acrylamide, 0.75 ml ampholine pH 3.5 to 10, 3 ml 10% Nonidet P-40 (NP-40), 3.3 ml H_2O, 10 μl N,N,N′,N′-tetramethylethylenediamine, and 10 μl 10% ammonium persulfate). The gels were overlayed with 10 μl of 9 M urea, 5% NP-40, 2% ampholine pH 3.5 to 10, and 5% β-mercaptoethanol and prerun for 2 hr at 200 V. The top buffer contained 10 mM NaOH (degassed) and the bottom buffer 10 mM H_3PO_4. The radioactive samples were dialyzed using the Centricon 30 microconcentrator (Amicon), vacuum-dried (Speed-Vac, Savant) and reconstituted in buffer B. Samples were electrophoresed for 12 hr at 600 V and then at 800 V for 1 hr. Gels were fixed for 1 hr in 30% MeOH, 10% trichloroacetic acid and 5% sulphosalicylic acid, stained with 0.1% Coomassie Brilliant Blue R-250 in 25% MeOH/8% acetic acid and destained with 25% MeOH/8% acetic acid solution. A duplicate tube gel was not stained but cut into 0.5 cm pieces, soaked overnight in 2 ml H_2O for determination of the pH gradient.

N-Terminal Sequence of [^{3}H]SSO-Labeled Protein

Radioactive protein eluates concentrated and reconstituted in buffer A as described earlier were subjected to SDS-PAGE on a 7.5% acrylamide. A Tris-glycine buffer was used with thioglycolic

acid (11 mg/ml) in the upper tank buffer. The proteins were then electroblotted (30 V for 3 hr) to a PVDF (polyvinylidene difluoride) membrane (ProBlott®, Applied Biosystems). The electroblotting buffer contained 10 mM CAPS (3-cyclohexylamino-1-propane-sulfonic acid) and 10% methanol. Following transfer the membrane was stained with a Coomassie Brilliant Blue (BioRad) solution (0.1% Coomassie Blue R-250 in 1% acetic acid/40% MeOH) and destained in 50% MeOH. The radiolabeled stained band was excised, washed in deionized water, and subjected to N-terminal sequence analysis via automated Edman degradations using an Applied Biosystems model 475A protein sequencer with an on-line Model 120A phenylthiohydration analyzer.

Immunoblots of Plasma Membranes

Plasma membranes as well as radioactive proteins eluted from SDS-PAGE gel slices were separated by SDS-PAGE in 7.5% (w/v)-acrylamide slab gels as described by Laemmli (1970). Proteins were then electrophoretically transferred to a nitrocellulose membrane (Schleicher and Schuell). The filter was incubated 2 hr in TTBS buffer (20 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl and 0.025% Tween 20) and 3% gelatin (Sigma) 1% BSA (Sigma) at 37°C and then for 2 hr with rabbit polyclonal antibody to bovine PAS IV (generously supplied by Dr. L. Leung). The antibody was diluted 1 : 100 in TTBS with 1% Gelatin. The filter was then washed 3 times in TTBS (5 min each), incubated 1 hr with peroxidase-conjugated goat anti-rabbit IgG (Vectastain Kit) and then washed three times with TTBS. Bands were visualized using 3,3'-diaminobenzedine tetrahydrochloride or 4-chloro-1-naphthol and hydrogen peroxide.

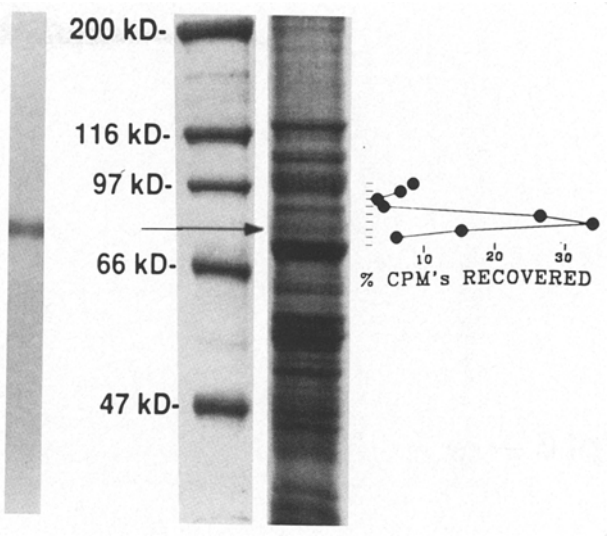

Fig. 1. Identification of a Coomassie-stained protein band labeled by [^{3}H]SSO. Rat adipocyte plasma membranes (right lane) prepared from cells treated with [^{3}H]SSO as described in Materials and Methods were subjected to SDS-PAGE (7.5% acrylamide w/v) followed by Coomassie staining. The gel lane containing the plasma membranes was cut into 1-mm slices between the 80–95 kD molecular weight markers, run in an adjacent lane (middle lane). Slices were soaked for 12 hr in Protosol to solubilize the proteins and determine slice-associated radioactivity (*shown on the right*). Alternatively, the stained gel was processed for autoradiography and a representative autoradiograph is shown in the left lane.

Results

Characterization of the Radioactive Protein Eluates

The exact size on SDS-PAGE of the membrane protein labeled by [^{3}H]-SS-FA was determined. Adipocyte membrane preparations (about 50 μg protein) from [^{3}H]SSO treated cells were subjected to separation by SDS-PAGE gradients (5–7.5% acrylamide gradient) and stained with Coomassie. Gels were dried and subjected to autoradiography as described above. We had previously shown that labeled SSO binds specifically to one protein band with an apparent size of 85–90 kD (Harmon et al., 1991). Direct comparison of Coomassie-stainable protein bands on the gel with the radioactive band on the autoradiograph identified a distinctly stained band at 88 kD (Fig. 1, arrow) which corresponded to the radioactive band on the autoradiographs (Fig. 1, left lane). A second approach, which yielded a similar size estimate, entailed cutting thin slices of the Coomassie stained gel (not dried) between 80–90 kD based on molecular weight standards and determining slice-associated radioactivity after solubilization overnight in Protosol (NEN Research Products). There was a clear peak of radioactivity (Fig. 1) from a distinct 88-kD Coomassie-stained protein band. Finally, electrophoresis of proteins eluted from the gel, based on the highest recovery of radioactivity confirmed the presence of a dominant 88-kD protein band.

Proteins in the gel slice eluate with the highest radioactivity were characterized by isoelectric focusing (Fig. 2). A prominent focused protein band with a pI of about 6.9 was observed. Few faint bands with a more acidic pI were also apparent. All bands, as well as blank gel regions, were cut from the gel and soaked in Protosol for determination of radioactivity. A clear radioactive peak was associated with the protein band with the pI of 6.9 while radioactivity in the more faint bands was not significant from the background level. It must be noted that the [^{3}H]oleate covalently linked to the protein (via [^{3}H]SSO) might alter the native pI of the 88-kD protein. For example, the faintly stained bands in the more acidic region of the tube gel could be the 88-kD protein with different pI's reflecting [^{3}H]-oleate linking to different amino acid residues. However, the relative concentration of these bands appeared very low and the sample was judged suitable for sequence determination.

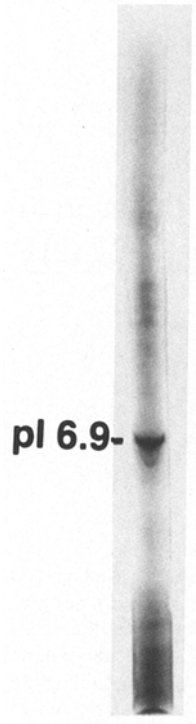

Fig. 2. Isoelectric focusing (IEF) of partially purified 88-kD adipocyte membrane protein labeled by [^{3}]SSO. The protein eluates obtained as described under Materials and Methods and having the highest radioactivity were concentrated, lyophilized and resuspended in isoelectric focusing buffer. Isoelectric focusing was conducted in an IEF tube gel which was subsequently stained with Coomassie Blue. A duplicate gel was simultaneously run, sliced and soaked in deionized water to determine the pH gradient. A prominent protein band is seen at pI ~6.9.

Amino Terminal Sequence of SSO-Labeled 88-kD Protein

Gel eluates containing the highest amount of radioactivity were dialyzed using a Centricon 30 microconcentrator and subjected to a second SDS-PAGE separation which was followed by electroblot transfer to a PVDF membrane and Coomassie staining (as described in Materials and Methods). About one-fourth of the band identified at 88 kD was excised, extracted in 1% Triton, 2% SDS for detection of radioactivity. The band was confirmed to be radioactive while no radioactivity was associated with two adjacent bands recovered on the PVDF membrane. The remaining PVDF strip with the 88-kD radioactive band was subjected to N-terminal amino acid sequencing. The yield of amino acids after the first cycle of Edman degradation averaged repetitively about 90% of the amount of protein sample loaded on the sequencing gel. This indicated that the sequence

FAT 88	GXDRNXGLITGAVIG
CD36 a	GCDRNCGLIAGAVIGAVLAVFGGILMPV
CD36 b	GXDRNXGLIAGAVIGAVLAVFGGILMPV
CD36 c	XXNRNXGLIAGAVIGAVLAVF
PAS-IV a	XXNRNXGLIAGAVIGAVLAVFGGILMPV
PAS-IV b	XXDXNXGLIAGAVIGAVLAVFGGILMPV

Fig. 3. Amino-terminal sequence of the 88-kD (FAT 88) protein. Sequence is compared to that for two homologous proteins identified from the Swiss-Prot data bank; CD36 from human endothelium (*a*), human platelets (*b*) and bovine endothelium (*c*); and PAS IV from bovine (*a*) and human (*b*) endothelia.

obtained corresponded to the major protein band present in the sample. The resulting sequence was then compared to matching sequences for other known membrane proteins (Fig. 3).

A search of the Swiss-Prot 15 database (FastDB, Intelligenetics) revealed near identity of the N-terminal sequence obtained for the 88-kD rat adipocyte membrane protein with those of two integral membrane proteins; glycoprotein (GP) IV (also termed GPIIIb or CD36) and PAS IV. GP IV is a ubiquitous membrane protein, first identified in platelets (Asch et al., 1987) but later also described in leukocytes (referred to as CD36 or OMK5 antigen) Ockenhouse, Magowan & Chulay, 1989; Lisby et al., 1990), endothelial cells (Oquendo et al., 1989), skin keratinocytes (Lisby et al., 1990), and several neoplasms including sebaceous carcinoma of the skin (Asch et al., 1987; Zhao, Chen & He, 1992) and lipomas (Mechtersheimer, 1991). PAS IV was identified in mammary secretory epithelial cells where it is concentrated in the apical surface (Greenwalt & Mather, 1985). However, it is probably widely expressed since antibodies against the protein reacted with various tissues (Greenwalt, Johnson & Mather, 1985). The first 15 amino acids sequenced at the N-terminus of the rat adipocyte 88-kD protein demonstrated only one substitution, threonine for alanine, at amino acid #10 as compared to N-terminal sequence for human PAS IV and to the sequence deduced from the cDNA for human placenta CD36 and to amino terminal sequence of GP IV from human platelets.

Immunoblot Analysis Using Polyclonal Antibody to Bovine PAS IV

PAS IV is concentrated in mammary epithelial cells during lipid secretion. Thus, it was of interest to test whether the mammary protein and the 88-kD

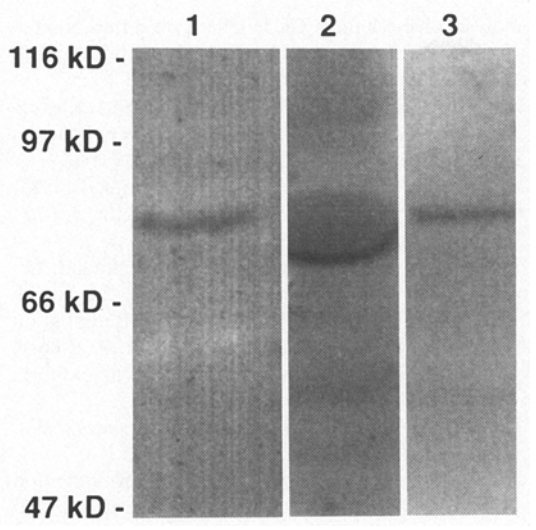

Fig. 4. Reaction of antibody for PAS IV with the 88-kD protein. Antibody was reacted at 1 : 100 dilution with adipocyte plasma membranes or with the partially purified 88 kD, eluted following SDS-PAGE and concentrated, as described in the text. Lane *1*: platelets, Lane *2*: rat adipocytes, Lane *3*: gel eluate containing partially pure 88-kD protein.

adipocyte protein which shared near common N-terminal sequence were also related immunologically. Plasma membrane preparations from rat adipocytes were reacted with an affinity-purified polyclonal antibody to bovine PAS IV. The results are shown in Fig. 4. In human platelets, included as a positive control (lane *1*), an apparent 88-kD reactive band was identified, presumably corresponding to platelet CD36. Plasma membranes from rat adipocytes (lane *2*) exhibited cross-reactivity in a broad band in the 80–90 kD range. Other reactive bands of a higher molecular weight (about 160 and 200 kD) were also observed. However, these additional bands were visible with the second anti-rabbit IgG antibody alone in the absence of PAS IV antibody (or with nonimmune sera) and were thus considered nonspecific. Gel eluates containing partially purified rat adipocyte plasma membrane protein labeled with [^{3}H]SSO (lane *3*) yielded a single band at the expected molecular size, 88 kD. Bands in the same size range were cross-reactive in membranes from human adipocytes and from cultured BFC-1 adipocytes (*data not shown*).

Discussion

Radiolabeled sulfo-*N*-succinimidyl oleate has been shown to specifically label an 88-kD rat plasma membrane protein under conditions where long-chain fatty acid uptake was inhibited by about 75% (Harmon et al., 1991). This and previous data obtained with another inhibitor of FA transport, DIDS, suggested a role for the labeled protein in the transport process (Abumrad et al., 1984). In this study we have used [^{3}H]SSO to isolate the 88-kD protein from rat adipocyte plasma membranes which allowed the determination of its N-terminal amino acid sequence. The sequence obtained was found similar to that of two other integral plasma membrane proteins; glycoprotein-IV (CD36) and PAS IV. Other lines of evidence also supported similarities between the three proteins. First, the 88-kD protein cross-reacted with antibody against PAS IV. Although the antibody data do not link the 88-kD protein and PAS IV in a definitive manner, they are consistent with our more recent results. Partial sequence for a cDNA clone we recently isolated from a rat fat cDNA library, using an oligonucleotide based on the N-terminal sequence of the 88-kD protein, yielded 75% nucleotide sequence similarity with CD36 (Abumrad et al., *unpublished observations*). The different isoelectric point of the 88-kD protein, 6.9, as compared with the 4.5–5.5 value reported for human platelet GP IV (Tandon et al., 1989) might reflect different tissue-specific post-translational modifications of the two proteins. For example, there is evidence that CD36 and PAS IV, although sharing almost identical amino-terminal sequences, have different biochemical characteristics as they exhibit different peptide maps and immunological properties (Catimel et al., 1991). Similarly, CD36 from bovine heart endothelium and CD36 from human platelets also demonstrate distinct structural, functional and antigenic differences (Greenwalt et al., 1990).

The identification of a glycoprotein-IV/CD36-related plasma membrane protein in rat adipocytes is consistent with the immunohistochemical findings of Mechtersheimer (1991) who reported strong cross-reactivity of human adipose tissue with monoclonal antibody (OKM5) for CD36. Recent evidence indicates that CD36 (GP IV) is a ubiquitous protein (Borne et al., 1989), although it has been most extensively studied in human platelets (Tandon et al., 1989). Its function, however, remains uncertain. It has been reported to function as a receptor for collagen (Tandon et al., 1989), for thrombospondin (Asch et al., 1987; McGregor et al., 1989), for malaria-infected erythrocytes (Ockenhouse et al., 1989) and for a 37-kD agglutination protein (PAP p37) found in the serum of patients with thrombotic thrombocytopenic purpura (Lian et al., 1991). GP IV might also mediate the cell-cell interactions which are part of inflammation, thrombosis and atherosclerosis (Silverstein, Asch & Nachman, 1989) or function in transmembrane signaling (Ockenhouse et al., 1989;

Alessio et al., 1991). The protein is also physically associated with several src family tyrosine kinases suggesting that ligand interaction may activate signaling pathways (Huang et al., 1991).

Our findings provide the first link between a CD36-like protein in adipocytes and the binding and/or transport of long-chain fatty acids. This interpretation, however, might be supported by multiple indirect lines of evidence. For example, PAS IV is concentrated at the apical membrane of mammary cells and is highly enriched in membrane envelopes encircling the lipid secreted in milk. It is possible to speculate that this protein might facilitate release of FA hydrolyzed within the lipid droplets. A survey of what is known about the tissue distribution of both CD36 and PAS IV generally documents strong expression in tissues with high metabolic requirements for long chain FA or with activity in lipid secretion. The list includes adipocytes, cardiac and microvascular endothelia, keratinocytes, sebaceous glands, lipid secreting mammary tissue, neoplasms such as lipomas, liposarcomas and sebaceous carcinomas (Borne et al., 1988; Mechtersheimer, 1991; Zhao et al., 1992). Hematopoeitic cells that express CD36 such as lymphocytes and macrophages, also have high FA requirements for their function in inflammation and immune responses (Calder et al., 1990). In addition, similar cellular effects have been attributed to CD36 and to FA indirectly linking them. For example, both CD36 (Ockenhouse et al., 1989) and FA (Calder et al., 1990) have been implicated in macrophage adhesion and consequently in the pathogenesis of atherosclerosis. Finally, differentiation of preadipocytes in culture is associated with both, a strong induction of the mRNA complementary to CD36 (Grimaldi & Abumrad, *unpublished observations*) and with a large increase in activity of the membrane transport for long-chain FA (Abumrad et al., 1991).

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