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Reversible Binding of Long-chain Fatty Acids to Purified FAT, the Adipose CD36 Homolog

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Abstract. Transport of long-chain fatty acids into rat adipocytes was previously shown to be inhibited by the reactive derivative sulfosuccinimidyl oleate consequent to its binding to a membrane protein FAT, which is homologous to CD36. In this report, the ability of the purified protein to bind native fatty acids was investigated. CD36 was isolated from rat adipocytes by phase partitioning into Triton X-114 followed by chromatography on DEAE and then on wheat germ agglutinin. Fatty acid binding was determined by incubating CD36, solubilized in buffer containing 0.1 Triton X-100, with fatty acids at 37°C, and then by adsorbing the unbound ligand with Lipidex 1,000 at 0°C. Bovine serum albumin was used as a positive control and gelatin, a protein that does not bind fatty acids, as a negative control. Measurements with albumin yielded reproducible binding values which were not altered by the presence of 0.1% Triton X-100. Under the same conditions, gelatin yielded reproducibly negative measurements that did not differ significantly from zero.

CD36 bound various long-chain fatty acids at low ligand to protein ratios. Warming the protein-FA-Lipidex mixture to 37°C removed the FA off the protein. Thus, binding was reversible and distinct from the palmitoylation of the protein known to occur on an extracellular domain. Comparison of the predicted secondary sequence of CD36 with that of human muscle fatty acid binding protein suggested that a potential binding site for the fatty acid on CD36 may exist in its extracellular segment between residues 127 and 279.

Key words: Fatty acids—Binding—CD36—Adipocytes

Previous work has shown that permeation of long chain fatty acids (FA) across the adipocyte plasma membrane has characteristics of facilitated diffusion (Abumrad et al., 1981; Abumrad et al., 1984; Schwieterman et al., 1988). Permeation was also inhibited by several protein modifiers: phloretin; pronase; sulfo-N-succinimidyl (SS) derivatives of long-chain FA; and by 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS). Both SS-oleate (SSO) and DIDS bound to one common membrane protein with an apparent molecular weight of 88kD (Abumrad et al., 1984; Harmon et al., 1991). The protein, termed FAT, was isolated (Harmon & Abumrad, 1993) and was found to have an amino-terminal sequence similar to human CD36 and glycoprotein IV (Oquendo et al., 1989; Tandon et al., 1989). The complete protein sequence deduced from the cDNA showed 85% homology with CD36 (Abumrad et al., 1993). Multiple lines of evidence are consistent with a role for CD36 in the uptake of FA. CD36 mRNA was strongly induced during adipocyte differentiation (Abumrad et al., 1993), coinciding with an increase in cellular FA uptake (Abumrad et al., 1991). In heart tissue, CD36 mRNA exhibited a cellular distribution similar to that of the cytosolic FA-binding protein. Upregulation of expression was also observed during heart development when FA utilization increases (Van Nieuwenhoven et al., 1995). Although all the above findings have implicated CD36 in FA binding and transport, there are no data documenting its ability to reversibly bind native FA. In this report, we have purified CD36 from rat adipocytes and examined its ability to bind long-chain FA using the Lipidex assay (Glatz and Veerkamp, 1983). This assay takes advantage of the property of the Lipidex resin to quantitatively bind all FA at 37°C but only protein-free FA at 0–4°C.

Materials and Methods

Experimental Procedures

Adipocyte Membrane Isolation and Protein Purification

Adipocytes were isolated from the epididymal fat pads of male Sprague-Dawley rats (Charles River, Wilmington, MA) according to the

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procedure of Rodbell (1964) as described previously (Abumrad, Park & Whitesell, 1986). In preliminary experiments, labeled preparations of adipocyte membranes were used to validate and refine the purification procedure. Tritium-labeled SSO ([^{3}H]SSO) was synthesized according to Harmon et al. (1991). Adipocytes were labeled with [^{3}H]SSO by incubation in KRH buffer (including 0.1% BSA; 2 mM glucose) with 10 μM [^{3}H]SSO for 20 min at 37°C. After the incubation, the cells were centrifuged gently, washed several times with KRH buffer containing 1% BSA, and once with homogenization buffer 1 (HB1: 20 mM Tris, 250 mM sucrose, 1.2 mM EGTA, pH 7.5). In all preparations, washed adipocytes were diluted 1:1 with HB1 and 5ml aliquots were homogenized (Kontes glass homogenizer with a clearance B pestle) at room temperature. The homogenate was immediately centrifuged for 3 min at 2,200 × *g* at 4°C to remove the fat cake and then for 75 min at 35,000 × *g*, 4°C. The pellet obtained was solubilized (60 min at 0–4°C with regular mixing), in 2ml homogenization buffer 2 (HB2: 20 Tris, 250 sucrose, 5 EGTA, 2 EDTA, 1% Triton X-114, pH 7.5). This was followed by centrifugation (80,000 × *g*, 4°C, 30 min) to remove non-solubilized material.

The CD36 protein was extracted, from the supernatant, using a modification of two previously published procedures; Greenwalt et al. (1990) and Jochen & Hays (1993). Hydrophobic membrane proteins were, first, selectively concentrated (Bordier, 1981) by incorporation into Triton X-114 micelles (10 min at 37°C). The incubation mixture, turned cloudy from micelle formation, was centrifuged (5 min, 1,000 × *g*) and the supernatant aspirated. The bottom, Triton-rich, layer was diluted (1:4) with 10 mM TRIS, pH 8.0, and dialyzed overnight against TT buffer (10 mM TRIS, 0.1% Triton X-100, pH 8.0), at 4°C.

Chromatography

Anion exchange chromatography was carried out at room temperature with DEAE-Sephacel (Pharmacia). First, a 4-ml aliquot of the dialyzed sample was loaded onto a 10 ml DEAE column equilibrated in TT buffer. The column was washed with TT buffer (about 30 ml) until absorbance at 260 nm dropped and stabilized indicating that all residual Triton X-114 had been removed. Proteins were eluted (1ml fractions) with 0.1–-0.4M NaCl in TT buffer, and localized by absorbance at 260 nm and/or by scintillation counting of [^{3}H]SSO-labeled protein. The column was recycled by washing with 0.5 M NaOH and re-equilibration with TT buffer. The fractions (10–15 ml) containing the protein peak were pooled, dialyzed overnight at 4°C against TT buffer, and concentrated by reloading onto a second small (2ml) DEAE column. The column was washed with 10–15 ml of TT buffer then proteins were eluted with increasing concentrations of NaCl (0–0.4 M) in TT buffer. The pooled protein-containing fractions (1–1.5ml) were loaded onto a wheat germ agglutinin column (1.5 ml, equilibrated with 10 mM TRIS, pH 8.0; 150 mM NaCl; 0.1% Triton X-100), washed extensively with the equilibration buffer, and then eluted with 300 mM, N-acetyl-glucosamine. Eluted fractions were monitored by SDS-PAGE and those containing protein were pooled and dialyzed against TT buffer. Before the binding assays, some protein samples were further concentrated by ultrafiltration using Centricon-50 tubes (Amicon). Purity of the protein samples was checked by silver staining following SDS-PAGE and by Western Blot analysis using a monoclonal antibody against CD36.

Fatty Acid Binding Assay

Fatty acid binding was determined using Lipidex 1000 (Packard) according to Glatz & Veerkamp (1983) and Vork et al. (1990). The Lipidex was washed thoroughly with phosphate buffer (10 mM, pH 7.4) and then equilibrated with TT buffer. The assay was conducted in 0.6 ml siliconized micro-centrifuge tubes (USA Scientific Plastics). To each tube was added 75 μl TT buffer, 25 μl protein (or TT buffer for blanks) and 25 μl of a radioactively labeled fatty acid (0.1–20 μM, 50–350 × 10^6 cpm/mmole). Samples were incubated at 37°C for 15 min to allow binding. A 25 μl aliquot was taken for total counts and the tubes were chilled. Ice-cold Lipidex suspension (25 μl) was added. The tubes were left on ice for 30 min, with vortexing every 10 min, during which time the Lipidex binds any FA not already bound to protein. The tubes were then centrifuged (10,000 × *g*, 4°C, 2 min) and aliquots (50 μl) of the supernatant were taken for measurement of the protein-bound fatty acid.

Binding was calculated as moles of FA bound per mole of protein assayed, and was plotted against the concentration of FA in the assay mixture. The data was then fitted to the Michaelis-Menten equation using a curve-fitting program (SigmaPlot, Jandel Scientific).

Homology Searches

The homology search procedure used has been described in detail by Argos (1987). Briefly, oligopeptides are compared by using two scoring procedures sensitive to distance sequence relationships: The first is the Dayhoff relatedness odds matrix (Dayhoff, Barker & Hunt, 1983) which weighs how well amino acids substitute for one another in aligned sequences of protein families. The second procedure is a calculation of the mean correlation coefficient taking into consideration five physical characteristics of amino acid residues that are important for protein folding (hydrophobicity, turn preference, bulk, anti-parallel strand preference and refractive index). Scores from both procedures are combined and placed in a search matrix that is expanded from the first to the last residues of the oligopeptides compared. The standard deviation of the search matrix is calculated and values are set as the number of deviations above the mean matrix value. Higher fractional deviation is allowed when overlap occurs.

Results

Protein Preparation

Following sample loading on the DEAE column, the initial 260 nm absorbance was very high but decreased rapidly as Triton X-114 was washed off. SSO-labeled protein was eluted in a single peak, immediately upon addition of 0.1M NaCl (Fig. 1, Panel *A*). Concentration of the protein peak using a second DEAE separation produced a very similar profile. Fractions, where protein and radioactivity coeluted, were pooled and dialyzed using a 50,000 molecular weight cutoff membrane. As shown in Fig. 1, Panel *B*, samples prepared in this manner contained relatively pure CD36 protein and showed only minor contaminating bands on a silver stained gel. Secondary purification using a wheat germ agglutinin column reproducibly resulted in very pure CD36 preparations as the protein was the only one retained by the column. Identity of CD36 was confirmed by reaction with three different antibodies raised against rat or human CD36 (Fig. 1, Panel *C*). As shown in the figure, in fractions eluted off the DEAE column the antibody reacted with the CD36 protein (88 kD band) and with

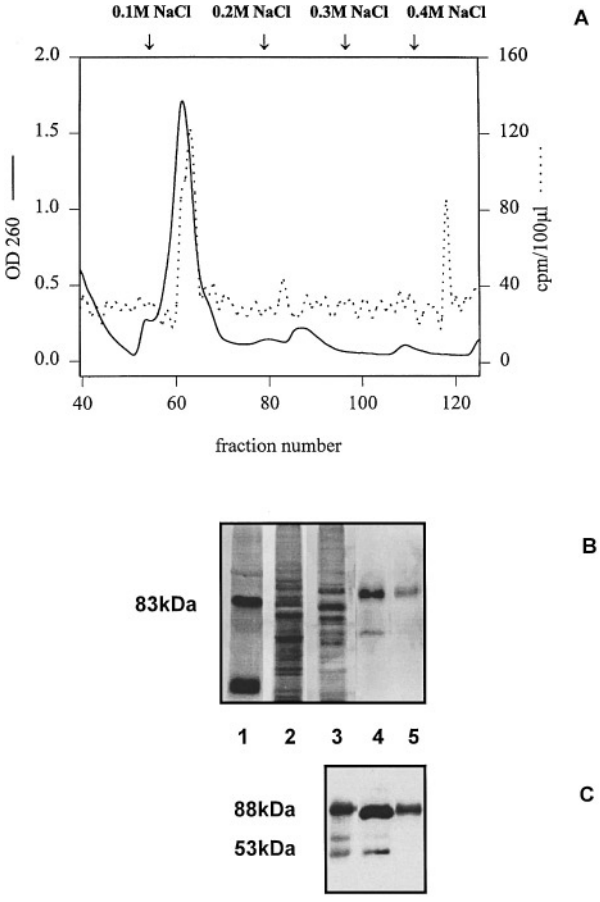

Fig. 1. Isolation of CD36 from rat adipocytes: Panel *A:* DEAE chromatography of Triton X-114 concentrated membrane proteins from cells treated with [^{3}H]SSO. Samples were loaded onto DEAE-Sephacel and washed thoroughly before eluting with 0.1–0.4 M NaCl in TT buffer. The solid line represents the protein concentration from OD at 260 nm with the dotted line showing the radioactivity. Panel *B:* SDS electrophoresis and silver staining of proteins recovered following chromatography on DEAE and then on wheat germ agglutinin. Lane 1: Molecular weight standards, Lane 2: plasma membranes, Lane 3: Triton X-114 membrane extracts, Lane 4: Protein eluted off the DEAE column and Lane 5: Protein eluted off the wheat germ agglutinin column. Panel *C:* Western blot of fractions (lanes 3–5) shown in panel B. The primary antibody used was a monoclonal antibody for human CD36. A polyclonal antibody against rat adipose CD36 was used with similar results.

another protein that is similar in size (about 53 kD) to that encoded by the CD36 cDNA. The 53 kD protein was not retained by the wheat germ agglutinin column while the 88 kD CD36 protein was. This suggested that the lower size form was the nonglycosylated CD36 protein.

Fatty Acid Binding: Assay Validation

One of the advantages of the Lipidex assay is its compatibility with Triton X-100. Vork et al. (1990) recommended using the detergent at a concentration of 0.005% as a means to improving the assay's reproducibility. However, a concentration of 0.1% Triton X-100 was necessary to assay the CD36 protein as a result of its high hydrophobicity. Reducing the Triton below this concentration led to precipitation of the protein-fatty acid complex (*data not shown*). Since this concentration is higher than that used by Vork et al. (1990), we examined the effect of the additional Triton on binding measurements. Duplicate blank samples containing 0.1% Triton X-100 and no protein were included for all FA concentrations used and were processed alongside those containing protein. The fraction of FA kept in solution by Triton, calculated from the blanks, amounted to about 30% of the FA added and it varied only slightly (± 2%) between assays. This fraction was subtracted from the binding values.

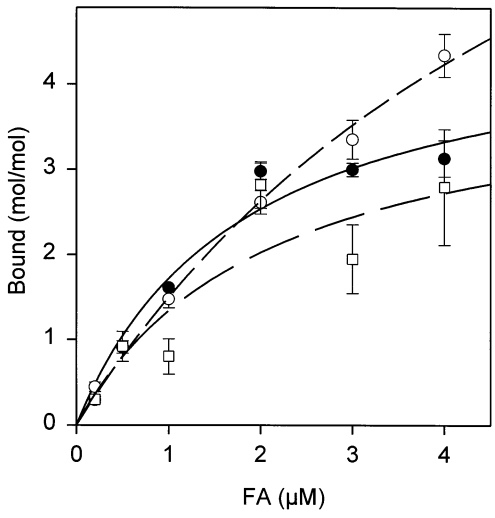

Fig. 2. Binding of oleic acid by bovine serum albumin as a function of FA concentration and with 0% (filled circles), 0.005% (open circles) or 0.1% (open squares) Triton X-100 in the assay buffer. Curves were generated by fitting the data (Sigma Plot) to the Michaelis-Menten equation, as described in the text. Data shown are representative of four experiments.

Using bovine serum albumin (BSA) we found that binding measurements obtained at fatty acid/protein molar ratios less than 1.0 were reproducible and were not significantly altered when Triton was increased from 0% to 0.005%, the concentration recommended by Vork et al. (1990), and to 0.1% the concentration necessary for our experiments (Fig. 2). However, as the ratio of fatty acid to protein was increased above 1:1, assays containing 0.1% Triton X-100 exhibited more variability. This reflected the fact that as FA concentration was increased, the fraction of FA partitioned in Triton micelles was very high relative to that bound to the protein as a result of a saturation of protein-FA binding sites.

All long-chain FA tested bound to the CD36 protein

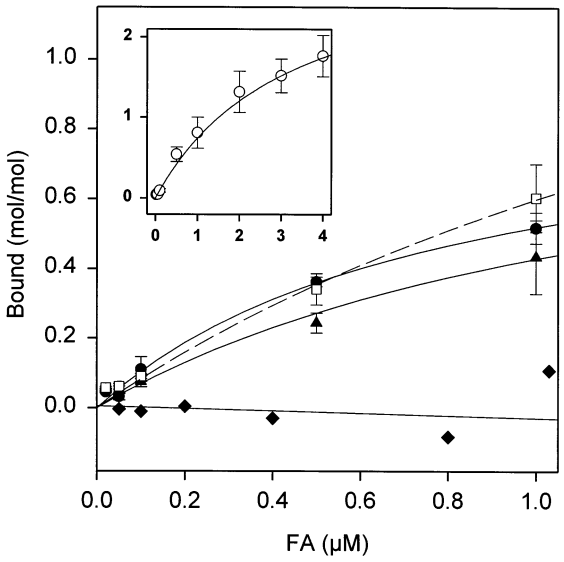

Fig. 3. Binding of fatty acids by CD36. CD36 was purified as described in the text and under legend to Fig. 1. The protein (0.5 μM) was assayed in TT buffer, and binding was determined as described in the text. Binding of arachidonic acid (filled triangles) and stearic acid (filled circles) is shown along with the combined data for all FA tested (open squares). Gelatin (filled diamonds), was assayed as a negative control, since it does not bind FA. The inset shows the binding by CD36 of oleic acid (open circles), over a wider range of FA concentrations. Data are representative of three to five experiments for each fatty acids.

at low ligand to protein ratios (0.2–2), similar to those used for albumin. Protein bound counts then averaged between 6–8% of those added. First, it was determined that the binding was very rapid. Binding values measured at 5, 10, 15, and 30 min were identical and indicated that equilibrium had been reached (*data not shown*). Second, the binding was a function of protein concentration. When the concentration of protein used was varied (0.1, 0.15 and 0.2 μM) while the added FA was kept constant, the protein-bound FA varied proportionally (0.14, 0.23 and 0.34) and the correlation coefficient exceeded 0.95.

Binding of Long-chain FA to CD36

Binding curves for adipose CD36 are shown in Fig. 3 with three representative FA, oleic acid (inset), a C18 monounsaturated (9) FA, and stearic and arachidonic acids, respectively, a C18 saturated and a C20 polyunsaturated (5,8,11,14) FA. Similar results were obtained for palmitic (C16 saturated) and linoleic (C18 unsaturated, 9,12) acids (*data not shown*). At similar concentrations of FA and protein, there were no significant differences in the binding values measured for the various FA and all data could be grouped and fitted to a single curve without significant increases in the standard deviation of the measurements (Fig. 3, broken line). As observed with albumin, binding measurements were more variable when the ratio of FA to protein was increased above 1:1 (Fig. 3, inset) again as a result of the fraction of FA partitioned in Triton becoming too high relative to that bound to CD36. This reflected saturation of protein binding sites which becomes apparent at FA concentrations exceeding 1 μM (inset). At free FA concentrations lower than 1μM, binding was almost linearly dependent on FA concentration and the data from each experiment could be fitted to a linear function with correlation coefficients that always exceeded 0.9. Based on the data, an approximate binding capacity of up to 3 moles of FA per mole of CD36 could be estimated (as opposed to about 5 for BSA, based on the data in Fig. 2). Determination of true dissociation constants was not possible since the concentration of the free (not micelle-partitioned) FA in the aqueous solution cannot be measured or calculated. If the total FA concentrations are factored in, the apparent dissociation constants obtained for CD36 and for BSA are similar and both about 1μM. Since the FA partitioned in Triton micelles is likely to be similar in both cases, the very similar binding curves would suggest that CD36 and BSA have similar affinities for long-chain FA.

The binding of FA by CD36 was reversible. The binding assay used in this study relies on the characteristic of Lipidex 1000 of only binding protein-free FA when kept at 0°C. In contrast, when the temperature of the protein-Lipidex mixture is increased to 37°C, almost all of the labeled fatty acid is bound by the Lipidex if the protein-ligand binding is reversible. Consistent with this, when mixtures of CD36/Lipidex were warmed to 37°C for 10 min, bound radioactivity was reduced by greater than 80%, as compared with samples processed without a warming period.

Additional experiments were carried out to establish reversibility and specificity of FA binding to CD36. The protein (0.2 μM) was equilibrated for 15 min with 0.6 μM labeled FA. Then an excess (60 μM) of unlabeled FA was added and the mixture was incubated for 5 more min before addition of the Lipidex resin and centrifugation. A large fraction (72% ± 2) of protein-bound radioactivity was displaced by the addition of the unlabeled FA.

Homology with FA-binding Proteins

Regions exhibiting similarities between sequences of CD36 and human muscle fatty acid-binding protein (M-FABP), for which crystal structure is available, were obtained using the sensitive alignment described by Patrick Argos (1987). Homologies are indicated by the diagonal lines on the diagram of Fig. 4, Panel *A*, and only regions exhibiting what is deemed as significant homology (requiring a minimum of 3 standard deviations

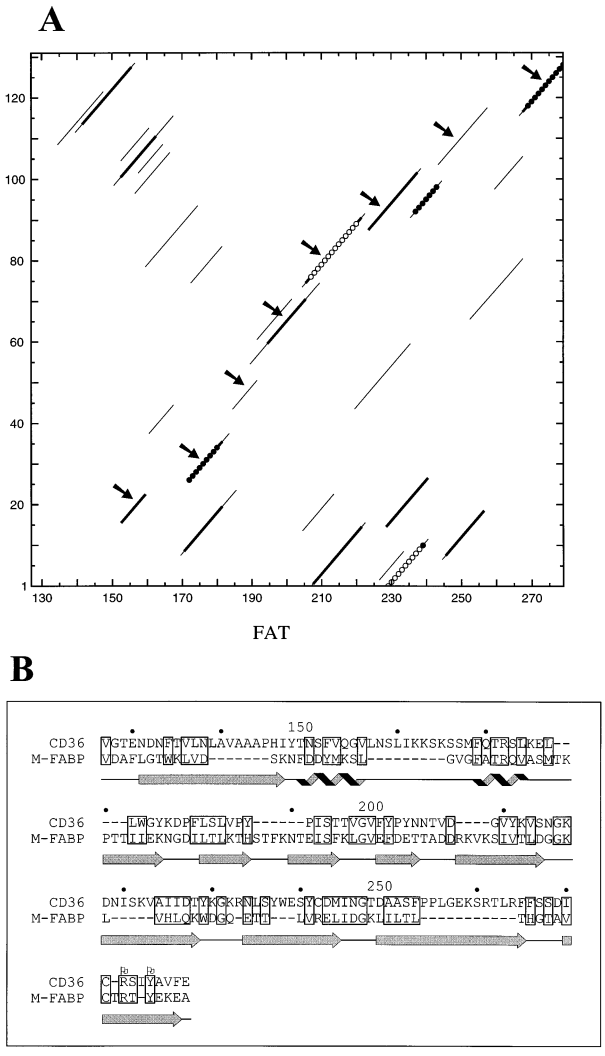

Fig. 4. Homology of CD36 with human muscle fatty acid-binding protein (M-FABP). Panel *A:* Search matrix based on the Dayhoff 5-parameter scoring method comparing CD36 and M-FABP. Multiple search window lengths of 7 to 15 residues were used in steps of 1. Symbols indicate the fractional standard deviation (σ) range of the search values (S) based on the following: Thin lines: $3.0\sigma \leqslant S < 3.28\sigma$; thick lines: $3.28\sigma \leqslant S < 3.57\sigma$; filled circles: $3.85\sigma \leqslant S < 4.13\sigma$. The segments used in the alignments of the two sequences are marked by arrows. Panel *B:* Alignment of a putative domain of rat CD36 (residues 127–279) with M-FABP. The alignment is based on the homology search matrix shown in Panel *A.* Boxed residues indicate identical or conserved ones according to the groupings KR, ST, PG, QNED and to hydrophobicity HYWFILVMCA. Residues Arg126 and Tyr128 of M-FABP which bind the fatty acid head group are marked with flags. The secondary structure of M-FABP is also shown below the sequence with alpha helices shown as zig-zag elements and beta strands as arrows.

above the mean; Feng, Johnson & Doolittle, 1985) are shown. The region comprising amino acids 127–279 clearly exhibited significant homology to M-FABP throughout 73% of its sequence.

The alignment based on the search diagram is shown in Panel *B* of Fig. 4. Strict sequence identity is low (14%) suggesting a distant relation between the two sequences. Structure predictions (Rost & Sander, 1993; 1994; Rost, Sander & Schneider, 1994) suggest that the secondary structure within this region may be similar to that of M-FABP. These predictions suggest that this region contains a single beta strand followed by two alpha helices in tandem and then by at least seven beta strands. The alpha-helical region of M-FABP aligns with a portion of a predicted alpha-helical region of CD36 (residues 139–154). The alignment also shows that the amino acids in M-FABP known to interact with the charged fatty acid head (Arg126 and Tyr128) are conserved in CD36 as Arg272 and Tyr275.

Discussion

Rat adipose CD36, was purified and tested directly for binding of native fatty acids. The purification procedure we describe combined phase partitioning in Triton X-114, to concentrate integral membrane proteins, followed by ion-exchange chromatography. Further purification using a wheat germ agglutinin column yielded very pure protein as determined by silver staining and Western blotting.

Following its purification, CD36 was kept solubilized with 0.1% Triton X-100 and assayed for binding under those conditions. The primary sequence of CD36 predicts a large heavily glycosylated extracellular domain, one or possibly two membrane spanning segments and very short intracellular segments (Abumrad et al., 1993; Pearce, Wu & Silverstein, 1994). Based on that, the protein is likely to incorporate into detergent micelles with its glycosylated segment protruding into the aqueous layer. Our data documented binding by CD36 of various long-chain FA. This binding occurred at low FA to CD36 ratios, similar to the case with BSA, and appeared to saturate as the ratio neared 3. In addition, although dissociation constants could not be calculated, the dependence of FA binding by CD36 on FA concentration was similar to that for BSA, a protein documented to have a very high affinity for FA. The results would suggest that CD36 has a high affinity for FA and can compete effectively for FA bound to albumin.

FA binding by CD36 was reversible and distinct from the covalent palmitoylation of the protein, that was shown to occur in rat adipocytes, presumably on an extracellular domain (Jochen & Hays, 1993). Reversibility of the binding was evidenced by dissociation of the FA and its binding to the Lipidex resin when the protein-FA-Lipidex mixture was warmed to 37°C, and also by unlabeled FA displacing labeled FA off the protein. A potential site for FA binding may exist in the extracellular segment of CD36 between residues 127–279, as predicted from homology searches with FA-binding pro-

teins. As mentioned under Results, this area had 73% homology with M-FABP. In addition, there is alignment of the alpha-helical region of M-FABP with sequence 139–154 in CD36 predicted to be alpha-helical. This sequence is highly hydrophobic and on that basis was hypothesized to dip in the plasma membrane (Greenwalt et al., 1992). It is possible that this alpha-helical segment might function as an entrance for the FA into the binding domain of CD36.

In summary, the data indicate the CD36 binds native long-chain FA at low concentrations and low ligand to protein ratios. Binding was similar to that determined for albumin under the same conditions and would suggest that CD36 can remove the FA off albumin at the low FA:albumin ratios present physiologically. Since these ratios are associated with limited partitioning of FA in the bilayer, it would seem efficient for cells with high FA metabolism to express CD36 in the membrane as a high affinity receptor to concentrate FA. Such a receptor system has been shown to exist for folate which, like FA, is present at nM concentrations as a result of its tight binding to plasma proteins (Kamen, Smith & Anderson, 1991). The distribution of CD36 that favors cells active in lipid uptake and metabolism is consistent with this interpretation (Abumrad et al., 1993).

It remains to be determined whether the FA, following binding to CD36, is delivered by the protein directly to the FA-acyl-CoA synthase on the inside of the membrane or whether it is transferred to another membrane protein that functions as a FA carrier. Most carriers, unlike CD36, have multiple membrane spanning domains. However, proteins with a membrane configuration similar to CD36 have been shown recently to function in the uptake of amino acids and of potassium (Takumi, Ohkubo & Nakanishi, 1988; Palacín, 1994). In addition, CD36 expression in fibroblasts increases uptake or long-chain FA at low molar ratios of FA to albumin, supporting induction of a high affinity FA uptake system that is added to the diffusion-like system present constitutively in the cell (Ibrahimi et al., 1996).

Other functions have been hypothesized for CD36 and binding of FA by the protein may be part of one or more of these functions. The protein was shown to be associated with Src kinases and may function in signal transduction (Huang et al., 1991; Schuepp et al., 1991) and FA binding may initiate the signaling process. CD36 has also been implicated in the uptake of oxidized low density lipoproteins, Ox-LDL (Endemann et al., 1993) and may recognize FA peroxidation products on apoproteins. More recently, CD36 was shown to bind anionic phospholipids like phosphatidylinositol and phosphatidylserine (Rigotti, Acton & Krieger, 1996). This, together with binding of long-chain FA, would suggest that hydrophobicity and a negative charge may be required for binding. To elucidate the role(s) of FA binding to CD36, configuration of the protein in the membrane, the FA-binding site, and whether binding induces a conformational change in the protein will have to be determined. In addition to adipose CD36, several other membrane proteins have been implicated as receptors or transporters for long-chain FA. These proteins were identified by binding to a FA-affinity column (Schwieterman et al., 1988), to a photoreactive FA derivative (Gerber, Mangroo & Trigatti, 1993) or by expression cloning using fluorescent FA (Schaffer & Lodish, 1994). These proteins coexist in adipocytes and it will be important to determine whether they interact functionally.

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