The Adipocyte Fatty Acid-binding Protein Binds to Membranes by Electrostatic Interactions*

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The adipocyte fatty acid-binding protein (AFABP) is believed to transfer unesterified fatty acids (FA) to phospholipid membranes via a collisional mechanism that involves ionic interactions between lysine residues on the protein surface and phospholipid headgroups. This hypothesis is derived largely from kinetic analysis of FA transfer from AFABP to membranes. In this study, we examined directly the binding of AFABP to large unilamellar vesicles (LUV) of differing phospholipid compositions. AFABP bound LUV containing either cardiolipin or phosphatidic acid, and the amount of protein bound depended upon the mol % anionic phospholipid. The $K_a$ for CL or PA in LUV containing 25 mol % of these anionic phospholipids was approximately $2 \times 10^8$ M$^{-1}$. No detectable binding occurred when AFABP was mixed with zwitterionic membranes, nor when acetylated AFABP in which surface lysines had been chemically neutralized was mixed with anionic membranes. The binding of AFABP to acidic membranes depended upon the ionic strength of the incubation buffer: $\approx 200$ mM NaCl reduced protein-lipid complex formation in parallel with a decrease in the rate of FA transfer from AFABP to negatively charged membranes. It was further found that AFABP, but not acetylated AFABP, prevented cytochrome c, a well characterized peripheral membrane protein, from binding to membranes. These results directly demonstrate that AFABP binds to anionic phospholipid membranes and suggest that, although generally described as a cytosolic protein, AFABP may behave as a peripheral membrane protein to help target fatty acids to and/or from intracellular sites of utilization.

The adipocyte fatty acid-binding protein (AFABP)† belongs to the family of small (14–15 kDa) intracellular proteins that noncovalently bind hydrophobic ligands, principally unesterified fatty acids (FA). AFABP, also known as AP2 and adipocyte lipid-binding protein, is expressed only in differentiated adipocytes, where it constitutes 1–6% of the total cytosolic protein in the mature adipocyte, such that the majority of intracellular FA are most likely bound to AFABP (1). FABPs are generally believed to function in the solubilization, transport, and/or metabolism of FA in cells. Given their cell-specific expression, abundance, and high (nanomolar) affinity for FA (2), individual FABPs may perform distinct roles within various tissues; however, specific functions have not yet been established for any FABP.

To begin to understand the intracellular function of these proteins, our laboratory has investigated the mechanism of transfer of FA from FABPs to small unilamellar vesicles. Previous results have shown that AFABP, as well as heart and intestinal FABPs, transfers fluorescent anthroyloxy-labeled FA (AOFA) to model membranes via a collisional mechanism, in which it is proposed that the protein at least transiently must associate with membranes (3–5). The rate-limiting step for transfer, therefore, appears to involve an effective physical interaction between the protein and the membrane rather than the dissociation of FA from the binding site, the latter mechanism having been found for liver-type FABP (6). A number of observations suggest that electrostatic forces between AFABP and membranes modulate these physical interactions. 1) The phospholipid composition and surface charge of the membranes influence the rate of FA transfer (7). Anionic phospholipids incorporated into egg phosphatidylcholine (EPC) small unilamellar vesicles (SUV) increased the AOFA transfer rate relative to zwitterionic SUV, whereas transfer was slowed to positively charged SUV. 2) High salt concentrations diminished the rate of FA transfer to anionic membranes but had no effect on transfer to neutral vesicles (7). 3) Selective chemical neutralization of lysine residues located on the protein surface resulted in a greater than 35-fold decrease in the rate of fluorescent FA transfer and shifted the mechanism of fatty acid transfer from collisional to diffusional (8). 4) Fourier transform infrared spectroscopic studies demonstrated large decreases in lipid phase transition temperatures of anionic but not zwitterionic vesicles in the presence of AFABP, indicative of protein-membrane interactions (9). In addition, site-directed mutagenesis studies show that specific surface lysine residues are involved in collision-mediated fatty acid transfer to membranes (10). Thus, positively charged lysines on the surface of AFABP probably mediate ionic interactions with negatively charged phospholipid headgroups.

To investigate the interaction between AFABP and membranes, we have focused on the particularly dramatic enhancement of FA transfer (>20-fold) to membranes containing CL (7). CL is located in the mitochondrial membrane, and a substantial fraction of cellular CL has been found to be localized to the outer mitochondrial membrane (11). CL is known to interactselectively with peripheral and integral membrane mitochondrial proteins via electrostatic and hydrophobic interactions.

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§ The abbreviations used are: AFABP, adipocyte fatty acid-binding protein; FA, fatty acids(s); AOFA, anthroyloxy-labeled fatty acid; 2-AP, 2-(9-anthroyloxy)palmitic acid; CL, cardiolipin; EPC, egg phosphatidylcholine; EPE, egg phosphatidylethanolamine; FABP, fatty acid-binding protein; LUV, large unilamellar vesicle; SUV, small unilamellar vesicle; PA, phosphatidic acid; dansyl-PE, N-(5-dimethylaminonaphthalene-1-sulfonyl)-sn-glycero-3-phosphoethanolamine; NBD-PE, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine.
One of the best characterized examples is cytochrome c, a peripheral protein of the mitochondrial inner membrane. Cytochrome c binds avidly to acidic phospholipids, particularly to CL, which probably serves as the in vivo binding site for cytochrome c either solely or in a complex with cytochrome c oxidase (13). The binding is sensitive to ionic strength, surface charge density, and pH (13, 14) and thus affords a comparative model with which to characterize AFABP interactions with membranes. The results of this study indicate that, as predicted by kinetic analyses of AOFA transfer to membranes, AFABP binds to negatively charged membranes through electrostatic interactions.

**EXPERIMENTAL PROCEDURES**

**Materials**—The fluorescent probe 2-(9-anthroyloxy)palmitic acid (2-AP) was obtained from Molecular Probes (Eugene, OR). Phospholipids, CL (from bovine heart), monosyndecanoidipin and disylsyndecanoidipin (both from bovine heart), dansyl-PE, and NBD-PE came from Avanti Polar Lipids (Alabaster, AL) and were stored in chloroform at −20 °C. Nalidixic acid and isopropyl-1-thio-β-D-galactoside were purchased from Sigma and Fisher, respectively. Cytochrome c (Type VI, from horse heart) came from Sigma. All other reagents were of the highest grade available. The AFABP expression systems pMON-AFABP and pET-AFABP were generously provided by Dr. David Bernlohr (University of Minnesota) and Dr. Alan Kleinfeld (Salk Institute, San Diego, CA), respectively.

**AFABP Purification**—Murine AFABP was expressed in two Escherichia coli systems: 1) JM101 containing the pMON-AFABP plasmid, induced by nalidixic acid and 2) E. coli BL21(DE3) harboring pET-AFABP, induced by isopropyl-1-thio-β-D-galactoside (2, 15). As detailed previously, AFABP was purified using two sequential size exclusion chromatographic steps (Sephadex G-50; Amersham Pharmacia Bio-tech), followed by anionic exchange chromatography (DE52, Whatman) and delipidation using Lipidx-1000 (Sigma) (8). FABP purity was assessed by polyacrylamide gel electrophoresis. Protein concentrations were determined using the molar extinction coefficient for AFABP at 280 nm, 1.55 M−1 cm−1. FABP was assessed using the Lowry protocol (21). For assays examining the effect of salt concentration on binding, the pellet was resuspended in 25 mM HEPES, pH 7.2, and the protein in all samples was recovered by precipitation in chloroform-methanol-water (22), prior to quantitation using the Lowry assay. Calculations have been corrected for lipid loss in the supernatant, as determined by P, assay.

An apparent association constant, K (M−1), for AFABP binding to anionic phospholipid membranes was calculated using the following equation:

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K = \frac{[\text{AFABP}]_{\text{bound}} [\text{AFABP}]_{\text{free}}}{[\text{Lipid}]} \quad \text{(1)}
\]

where [Lipid] equals the concentration of the anionic lipid on the outer surface of the LUVs, estimated as one-half of its total concentration (18, 19). These estimations assume a 1:1 stoichiometry for AFABP:lipid. Because this is not certain, the values obtained must be considered comparative rather than absolute.

**Cytochrome c Binding to SUV**—The binding of cytochrome c to membranes was monitored as the Perrin-Förster resonance energy transfer between dansyl-labeled PE located in SUVs and the heme moiety of cytochrome c (13, 14, 23), as described previously by Corsico et al. (19). Cytochrome c concentration was determined by absorbance at 403 nm. The final incubation at 37 °C appeared especially important in optimizing the phospholipid recovery in EPC vesicles. The concentration of vesicles is expressed as the molar concentration of phospholipid; composition is expressed as the mol % of phospholipid. The phospholipid concentrations were determined by quantitation of inorganic phosphate (20) and for vesicles containing CL have been corrected for two phosphate groups/CL molecule. Vesicles were generally used immediately after determining the phospholipid concentration.

**Fluorescent FA Transfer Assay**—The rate of 2-AP transfer from recombinant murine AFABP to acceptor membrane vesicles containing 10% NBD-PE was determined using a resonance energy transfer assay, as described previously (3, 8). AFABP was incubated with 2-AP for 20 min to form the donor complex. The time-dependent decrease in 2-AP fluorescence upon mixing with acceptor SUV was assessed using an Applied Photophysics DX-17MV stopped flow spectrofluorometer (Surrey, UK), with the excitation wavelength set at 383 nm and emission monitored using a 408-nm cutoff filter. Unless otherwise specified, the final assay mixtures contained 10–15 μM AFABP, 1–1.5 μM 2-AP, and 100–150 mM SUV, such that the assay conditions maintained AFABP, 2-AP, and SUV concentrations of the molar ratio of 10:1:100, respectively. Transfer was monitored at ambient (24 °C) temperature. Controls to ensure that photobleaching was eliminated were performed prior to each experiment, as described previously (5). Data were analyzed using software provided with the instrument, and all curves were well described by a single exponential function.

**Sucrose-loaded Vesicle Binding Assay**—In the membrane-binding assay (19), AFABP was mixed with sucrose-loaded LUVs in the ratio of AFABP:LUV equaling 1:400 in a total volume of 100 μl of binding buffer, 25 mM HEPES, 100 mM KCl, pH 7.2. After 15 min of equilibration at room temperature, bound protein was separated by centrifugation for 1 h at 100,000 rpm at 20 °C in a Beckman Optima TL100 ultracentrifuge. The supernatant was immediately removed, and the pellet was solubilized in 100 μl of the binding buffer. Protein concentrations were determined in the supernatant ([AFABP]bound) using a modified Lowry protocol (21). For assays examining the effect of salt concentration on binding, the pellet was resuspended in 25 mM HEPES, pH 7.2, and the protein in all samples was recovered by precipitation in chloroform-methanol-water (22), prior to quantitation using the Lowry assay. Calculations have been corrected for lipid loss in the supernatant, as determined by P, assay.

**RESULTS**

**AFABP Interaction with Membranes**—As described above, previous studies have suggested that AFABP may directly interact with membranes and thereby modulate the rate of FA transfer (7–9). To investigate these interactions more directly, the equilibrium binding of AFABP to model membranes of differing lipid compositions was assessed using ultracentrifugation of sucrose-loaded LUV to separate bound and free protein (18, 19). AFABP that binds to LUV membranes is pelleted along with the LUV (which pellet because of the increase in density due to the sucrose), whereas unbound AFABP remains in the supernatant. As shown in Fig. 1, AFABP bound to LUV that were composed of 25 mol % of the anionic phospholipids, 1-palmitoyl-2-oleoyl phosphatidic acid (PA) and cardiolipin (CL) but not to zwitterionic egg PC vesicles containing

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**Table I**

| Lipid | K (M⁻¹) |
|-------|---------|
| EPC   | 65 ± 17 |
| EPE   | 111 ± 91|
| SM*   | 42 ± 34 |
| PA    | 1760 ± 490|
| CL    | 2400 ± 200|

*SM, sphingomyelin.

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**TABLE I**

**Apparent association constant Kₙ of AFABP with phospholipids in LUVs**

The apparent binding affinity of AFABP for phospholipids in LUV was determined using the sucrose-loaded vesicle binding assay and calculated using Equation 1 as described under “Experimental Procedures.” The lipid concentration equals the concentration of the anionic lipid on the outer surface of the LUVs, estimated as one-half of its total concentration. Vesicle composition is the same as detailed for Fig. 1. Data represent the means ± S.E. of 3–9 determinations.
energy transfer assay of Struck rather than fusion of vesicles was verified by the fluorescence 2-AP transfer to SUV (Fig. 3), indicating that electrostatic protein and membrane binding was also observed for the rate of 5–25 mol % CL. This dependence on surface charge density for bound to LUV was approximately linear for LUV containing 25% CL (EPC:CL:EPE, 65:25:10). Acetylated AFABP (Acet. AFABP) (5 μm) was incubated with 25% CL LUV. The protein-LUV mixture in 25 mM HEPES, 100 mM KCl, pH 7.2, was incubated for 15 min at room temperature and centrifuged, and the amount of protein in the pellet was quantitated as described under “Experimental Procedures.” Background, representing protein in buffer alone, has been subtracted from all values. Results are expressed as the percentage of the total protein in the initial mixture that binds the pelleted LUV and represent the means ± S.E. of 3–9 determinations. Inset, a representative binding curve for AFABP to CL-containing LUV. AFABP (5 μm) was mixed with increasing concentrations of LUV containing 25 mol % CL, and the percentage of AFABP bound was determined versus the concentration of CL, as described above.

egg phosphatidylethanolamine (EPE), EPC:EPE (90:10), and EPC:EPE (65:35), or 25 mol % sphingomyelin. Additionally, little to no binding was detected when acetylated AFABP was mixed with 25 mol % CL LUV (Fig. 1). The apparent Kd for CL in LUV was approximately $2.4 \times 10^3$ M$^{-1}$ and that for PA in LUV was $1.7 \times 10^3$ M$^{-1}$, whereas the apparent affinity for neutral or zwitterionic phospholipids was 100-fold lower (Table I). The values for Kd obtained in these studies are similar to those reported for binding of the peripheral proteins cytochrome c and protein kinase C to negatively charged membranes (25, 26).

AFABP also associated with anionic SUV and showed a similar preference for anionic phospholipids (CL > PA); however, the interaction induced aggregation of protein-membrane complexes that could be pelleted by low speed centrifugation ($14,000 \times g$) (data not shown). Aggregation of anionic SUV rather than fusion of vesicles was verified by the fluorescence energy transfer assay of Struck et al. (27). Although it is not surprising that AFABP would interact with SUV similarly as with LUV, it is not known why AFABP causes aggregation of the SUV. Thus, binding measurements and calculations of affinity were performed using the sucrose-loaded LUVs.

It was further found that the extent of protein and LUV interaction depended upon the mol % of anionic phospholipid in the LUV (Fig. 2). The increase in the percentage of AFABP that bound to LUV was approximately linear for LUV containing 5–25 mol % CL. This dependence on surface charge density for protein and membrane binding was also observed for the rate of 2-AP transfer to SUV (Fig. 3), indicating that electrostatic forces modulate the rate of FA transfer from AFABP to membranes. When the dependence of LUV binding on the mol % of CL in the vesicles is compared with the dependence of AOFA transfer rate on mol % CL, binding appears to plateau at 25 mol %, whereas the transfer rate continues to increase with increasing mol % CL (Figs. 2 and 3). This suggests that differences in membrane packing as well as headgroup conformation for the anionic phospholipids in SUV versus LUV may result in differential interactions of AFABP with these two vesicle types.

We have previously shown that the rate of AOFA transfer from AFABP to large vesicles is approximately an order of magnitude slower than transfer to highly curved small vesicles of identical composition and total phospholipid concentration (7). Despite this quantitative difference, however, the regulation of AOFA transfer by acceptor membrane concentration and the apparent collisional transfer mechanism were similar for SUV and LUV. Thus, although it appears that the fatty acid transfer mechanism is similar for both types of model membrane acceptors, quantitative differences, presumably secondary to membrane structural properties, are present.

In addition to electrostatic forces, hydrophobic forces have been proposed to be involved in the collisional mechanism of fatty acid transfer from AFABP to membranes (7, 8). We examined, therefore, the effects of CL acyl chain properties on AFABP-membrane interactions. The rate of transfer of 2-AP to
membranes composed of 25 mol % monolysocardiolipin or dilsyocardiolipin was decreased over 50% compared with 25 mol % CL (Table II). These analogs, formed by the hydrolysis of one and two sn-2 acyl chains, respectively, present on the glycerol side chains of heart CL, carry the same net negative charge on the headgroup as CL but differ in acyl chain packing (28). It was also found that compared with vesicles composed of 12.5 mol % tetrastearoyl-CL (C18:0 present at all acyl positions), the 2-AP transfer rate was three times faster than vesicles of equimolar heart CL consisting of approximately 90% C18:2 acyl chains at the sn-1 and sn-2 positions of the two glycerol backbones (data not shown). Thus, alterations in the acyl chain packing and/or composition of the membrane appear to significantly influence the ability of AFABP to associate with membranes, although these changes are of lesser magnitude than the effects of headgroup charge.

**Effects of NaCl on AFABP Interaction with Membranes**—High salt concentrations diminish the effective surface charge of acidic membranes; this charge shielding would consequently be expected to reduce the electrostatic interactions between positively charged residues on the surface of AFABP and negatively charged headgroups of membrane phospholipids. Therefore, we investigated the effect of NaCl concentration on the binding of AFABP to LUV containing 25 mol % CL (Fig. 4). Increasing the concentration of NaCl decreased the effective association of AFABP and membrane. Similarly, the rate of 2-AP transfer declined as NaCl concentrations were elevated (Fig. 5). Transfer data were obtained between 0.1 and 1.0 M NaCl and the concentration at which a 50% decrease in transfer rate was observed is approximately 0.4 M. For binding examined between 0.1 and 1.0 M NaCl, a concentration of approximately 0.5 M results in a 50% diminution of binding. These results agree with our previous observation that high salt concentrations slowed the rate of AOFAP transfer from AFABP to acidic membranes (7) and indicate, moreover, that the FA transfer rate is directly related to the extent of AFABP-membrane electrostatic interactions. The AOFAP transfer rate was slightly more sensitive to salt concentration than was AFABP binding to LUV (Fig. 5, inset), possibly owing, in part, to differential phospholipid packing of LUV compared with SUV.

To compare the results obtained for AFABP with another protein of similar size that is well known to interact with acidic membranes, we monitored cytochrome c interaction with membranes under identical conditions. The percentage of the total cytochrome c that bound CL membranes in low salt buffer (~0.5 M) was very similar to AFABP. Cytochrome c binding, however, appeared more sensitive to ionic strength, because 50 mM NaCl reduced binding by 60%, and higher concentrations essentially eliminated binding of the protein to LUV (Fig. 4), in agreement with the results of others (13, 14).

**Competition of AFABP with Cytochrome c for Binding to**

![Image](https://via.placeholder.com/150)

**FIG. 4. The effect of ionic strength on protein-membrane interaction.** AFABP or cytochrome c was mixed with 25 mol % CL LUV in 25 mM HEPES, 0–1000 mM NaCl, pH 7.2, for a final concentration of 5 μM protein and 2 mM LUV in 100 μl. After incubation for 15 min at room temperature, the mixture was centrifuged, and the amount of protein was determined in the pellet. Back grounds were subtracted from all values, as described in the legend to Fig. 1. Results are expressed as the percentages of the initial protein that binds and represent the means ± S.D. of two separate determinations.

**FIG. 5. The effect of ionic strength on 2-AP transfer from AFABP to SUV.** Transfer of 1 μM 2-AP from 10 μM AFABP to 100 μM SUV containing 25 mol % CL, 65 mol % EPC, and 10 mol % NBD-PE (EPC) or 90 mol % EPC and 10 mol % NBD-PE (EPC) was measured at 24 °C, as a function of increasing concentrations of NaCl, as described under “Experimental Procedures.” Results are the means ± S.D. of two separate experiments. Inset, the % AFABP bound to LUV (●) and the rate of transfer to SUV (○) are shown relative to the value determined in 0.1 M NaCl. All vesicles contained 25 mol % CL.

**Anionic Membranes**—The interaction of AFABP with membranes is sensitive both to surface charge density and ionic strength, similar to cytochrome c and other proteins that bind to membranes via electrostatic interactions. To investigate these properties further, we determined whether AFABP could compete with cytochrome c for binding to membranes containing CL or PA and 1 mol % of the fluorophore dansyl-PE. Cytochrome c caused concentration-dependent quenching of dansyl fluorescence, with 0.5 μM cytochrome c reducing the relative fluorescence intensity of SUV containing 25 mol % CL and 25 mol % PA by 55% and 70%, respectively. In contrast, c had little effect on EPC:EPE (90:10) SUV (Fig. 6A). Fig. 6B shows that preincubation of CL- or PA-containing vesicles with...
AFABP binding to anionic membranes by AFABP. A, cytochrome c was incubated with 12.5 μM EPC, 25 mol % PA, or 25 mol % CL vesicles containing 1% dansyl-PE. The quenching of dansyl fluorescence is expressed as the percentage of the relative fluorescence intensity of vesicles without cytochrome c. B, 12.5 μM SUV containing 1% dansyl-PE were incubated with increasing concentrations of AFABP (●, ■) or 2.5 μM acetylated AFABP (▲) for 5 min, and 0.5 μM cytochrome c was then added, as described under "Experimental Procedures." The compositions of SUV were: PA (●), (EPC:EPE:PA:dansyl-PE, 65:9:25:1), and CL (●, ▲)(EPC:EPE:CL:dansyl-PE, 65:9:25:1). Acetylated AFABP was incubated only with CL SUV. Results are expressed as the percentages of relative fluorescence intensity, where 100% represents the relative fluorescence intensity of SUV incubated in the presence of cytochrome c but without AFABP or acetylated AFABP. Results are the averages of five experiments ± S.E. for AFABP and the averages ± S.D. of two experiments for acetylated AFABP.

AFABP was effective in preventing subsequent cytochrome c binding. When AFABP (2.5 μM) was added to CL- and PA-containing SUVs prior to the addition of cytochrome c, the dansyl fluorescence relative to emission in the presence of cytochrome c alone was increased by approximately 52 and 33%, respectively (Fig. 6B). In other words, preincubation with AFABP prevented the subsequent quenching of dansyl fluorescence by cytochrome c. AFABP added after cytochrome c did not relieve quenching of dansyl fluorescence (data not shown), suggesting that AFABP was unable to displace cytochrome c once it bound to CL membranes. This result is similar to previous reports, in which excess liposomes were unable to dissociate cytochrome c from CL-containing membranes and high ionic strength only partially detached the protein from the membrane (14). Acetylated AFABP (2.5 μM) had little effect on cytochrome c-induced quenching of CL SUV, indicating the absence of effective interactions with the membrane when positively charged surface lysine residues were neutralized (Fig. 6B).

**DISCUSSION**

This study demonstrates that the major fatty acid-binding protein of the adipocyte behaves like a peripheral membrane protein. AFABP mixed with phospholipid membranes formed stable associations that were isolatable by ultracentrifugation. The degree of binding of the protein depended upon the phospholipid composition and charge density of the membrane, the ionic strength of the medium, and the net surface charge of the protein, indicating that electrostatic forces most likely govern the interaction. The binding affinity of AFABP for anionic membrane phospholipids was in the range of that reported for other peripheral proteins (25, 26). Additionally, AFABP, but not acetylated AFABP, prevented cytochrome c binding to anionic phospholipid membranes, and neither AFABP nor cytochrome c bound to zwitterionic phospholipid membranes. AFABP showed half-maximal interaction with anionic membranes at near physiological ionic strength, in contrast to cytochrome c, which was much more sensitive to the salt concentration and showed minimal interaction at 100 mM NaCl under our assay conditions. The findings are consistent with recent studies that used infrared spectroscopy to qualitatively demonstrate AFABP interactions with anionic vesicles (9) and corroborate previous studies of AOFABP transfer kinetics, in which we suggested that AFABP utilizes direct effective collisions with phospholipid membranes to transfer fatty acids. The present studies also support the importance of charged residues on the protein surface in mediating the association with membranes (3–5, 8, 29).

The previous AOFABP transfer studies suggested that in addition to electrostatic interactions, hydrophobic interactions might be involved in formation of the AFABP-membrane complex. Transfer from heart FABP and AFABP was fastest to less ordered fluid phase membranes containing unsaturated acyl chains compared with fully saturated acyl chains and was decreased to membranes containing cholesterol or sphingomyelin, both of which are known to increase the lipid order of fluid phase membranes (7). Here we found that 2-AP transfer to CL analogs with either three or two acyl chains was slower than transfer to four-chained CL, and this may be attributable to differences in acyl chain packing (28). Furthermore, it was found that transfer was slower to vesicles that contained 12.5 mol % of tetra-18:0 CL relative to vesicles with equimolar heart CL (essentially tetra-18:2 CL). The acyl chain differences may give rise to differential packing in the hydrocarbon region (30). Phase transition temperatures of the vesicles were not determined; therefore, it is possible that the tetraestearoyl-CL may form gel phase domains of relative immiscibility with the predominant fluid phase egg phosphatidylcholine (31). In a general sense these data demonstrate that alterations in acyl chain properties influence the degree of interaction of AFABP with membranes. Although these results support the idea that AFABP may penetrate further into the hydrocarbon core of the membrane rather than interact solely via surface electrostatic interactions, it is also possible that the changes observed here are due not to deeper protein penetration but rather to an alteration in the accessibility or motional freedom of the charged CL headgroup secondary to changes in the hydrocarbon region of the bilayer. In addition, changes in the orientation of the PC headgroup caused by addition of CL (32) may be involved in AFABP-membrane interactions.

The greater effect of NaCl on cytochrome c relative to AFABP interactions with membranes also suggests that AFABP-membrane interactions may include a hydrophobic component, because cytochrome c is well known to bind via electrostatic interactions. Nevertheless, work from several laboratories has shown that cytochrome c interactions with vesicles involves not solely electrostatic but also hydrophobic interactions (14, 33). Thus, the fact that AFABP binds to membranes under conditions of higher ionic strength than cytochrome c does not un-
equivocally support the presence of hydrophobic interactions for AFABP. Indeed, in other studies we found that addition of AFABP to anionic membranes caused a 5 °C shift in the gel to fluid phase transition temperature with little or no change in cooperativity of the transition, suggesting that AFABP does not penetrate into the hydrophobic core of the membrane but rather interacts primarily at the surface of the membrane (9).

Although the exact site(s) or region(s) of AFABP that form these contacts is not known, site-specific mutagenesis has shown that the helix-turn-helix domain of the FABPs is critical for collisional transfer of AOFA to acceptor membranes (24, 29). This domain forms part of the putative “portal” for fatty acid entry and exit from the protein. Deletion of the α-helical domain of intestinal FABP altered the regulation of AOFA transfer to acceptor membranes, making the normally collision-mediated process more characteristically diffusion-mediated and eliminating the sensitivity to membrane surface charge. In addition, the helix-less variant was 80% less efficient than native intestinal FABP in blocking cytochrome c binding to membranes (24). Mutagenesis of single lysine residues in the β-turn, α-I, and α-II domains of heart FABP all altered the absolute rate of ligand transfer, whereas mutations elsewhere on the protein surface had little effect (29). AFABP and heart FABP share greater than 75% sequence homology in these domains, compared with 64% overall homology (16), and the α-helical cap appears to function similarly in AFABP (10). The α-I domain of AFABP and other FABPs, moreover, forms an amphipathic helix, which is known to be involved in numerous lipid-protein interactions by orienting the protein at the polar/nonpolar interface of a membrane (34). Thus the α-helical domain may serve as either an initial or a major site of electrostatic interaction between AFABP and membranes.

FABPs have generally been considered to be cytosolic proteins. Myelin P2, however, which shares greater than 67% sequence identity and 90% sequence homology with AFABP (35), was characterized as a peripheral membrane protein by its extraction from membranes using low ionic strength (36) and has been immunolocalized as a peripheral membrane protein in actively myelinating rat Schwann cells (37). In addition, approximately 16% of the intestinal FABP was localized to the particulate fraction of enterocytes, even after extensive washing (38). Recently, we have used a monospecific polyclonal antibody to murine AFABP to examine its subcellular distribution, and preliminary results show that approximately 10% or more of AFABP is specifically localized to the lipid droplet fraction (the “fat cake”).

In conclusion, the present studies provide direct evidence that AFABP associates specifically with acidic phospholipids in membranes. As such, AFABP may function not simply as a soluble cytosolic protein but rather as a so-called “amphitropic” protein, interacting with anionic phospholipids and/or, anionic peptides in membranes or at the surface of lipid droplets. Such interactions may permit specific targeting of FA to or between intracellular sites to modulate the efficiency of FA metabolism within the adipocyte.

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