Biochemical and Biophysical Research Communications **255,** 34–39 (1999)
Article ID bbrc.1998.0123, available online at http://www.idealibrary.com on IDEAL®

Identification of Caveolin-1 as a Fatty Acid Binding Protein

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Received December 18, 1998

In an attempt to identify high affinity, fatty acid binding proteins present in 3T3-L1 adipocytes plasma membranes, we labeled proteins in purified plasma membranes with the photoreactive fatty acid analogue, 11-*m*-diazirinophenoxy[11-^{3}H]undecanoate. A single membrane protein of 22 kDa was covalently labeled after photolysis. This protein fractionated with caveolin-1 containing caveolae and was immunoprecipitated by an anti-caveolin-1 monoclonal antibody. Furthermore, 2D-PAGE analysis revealed that both the α and β isoforms of caveolin-1 could be labeled by the photoreactive fatty acid upon photolysis, indicating that both bind fatty acids. The saturable binding of the photoreactive fatty acid suggests caveolin-1 has a lipid binding site that may either operate during intracellular lipid traffic or regulate caveolin-1 function. © 1999 Academic Press

Fatty acids (FAs) are important substrates both for metabolic reactions involving energy storage (triglyceride synthesis in adipocytes), energy production (FA β-oxidation in hepatocytes and cardiomyocytes) and lipid synthesis, and for signal transduction (1–6). To fulfill these roles, exogenous FAs must first be taken up by cells and then delivered to appropriate internal sites. Transport of FAs across the plasma membrane, therefore, is a critical first step for both FA mediated signal transduction and metabolism.

3T3-L1 adipocytes have been used extensively as a model for studying FA uptake in mammalian cells (7–10). When exposed to the appropriate hormonal stimuli in culture, 3T3-L1 preadipocytes differentiate to adipocytes, acquiring an increased capacity for high affinity FA uptake (7, 8). This is accompanied by an increased expression of a number of genes involved in FA metabolism (4, 11), including three which have been implicated in FA transport (8–10, reviewed in 12 and 13): A 43 kDa peripheral membrane protein (called FABPPM, for plasma membrane FA binding protein) related to mitochondrial glutamate-oxaloacetate transaminase (8); CD36 (also called FAT for *FA T*ranslocase) (9), a transmembrane protein with broad ligand specificity (14); and FATP (*FA T*ransport *P*rotein), a 63 kDa multimembrane spanning protein identified by expression cloning (10). Expression of each of these has been shown to confer increased long chain FA uptake in transfected cells (10, 15, 16). Whether these proteins function individually or in concert with each other or other factors is not yet clear (12, 13).

One of the hallmarks of 3T3-L1 cell differentiation into adipocytes is the dramatic increase in the number of invaginated caveolae (17). Caveolae are a surface membrane domain that appears to have several functions, including the delivery of small molecules such as reduced folate to the cytoplasm (18), the delivery of macromolecules to the ER (19), the efflux of cholesterol (20) and the transcytosis of macromolecules across cells (21, 22). Caveolae also appear to contain multiple signaling molecules that participate in specific signaling activities (23, 24). Rapid-freeze, deep-etch electron microscopy has shown that fibroblast caveolae have a distinctive striated coat (25).

One of the components of the caveolae coat is a 22 kDa protein called caveolin-1 (25). The expression of caveolin-1 increases nearly ten fold during 3T3-L1 differentiation into adipocytes (26, 27). At the cell surface, caveolin-1 is found exclusively associated with caveolae (25). Caveolin-1 functionally interacts with multiple signaling molecules (28), suggesting it plays a role in modulating signal transduction from caveolae. However, it also may function in the bi-directional movement of cholesterol between caveolae and the ER (29, 30). Caveolin-1 is a component of vesicles derived

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Abbreviations used: BSA, bovine serum albumin; DAP, *m*-diazirinophenoxy; 2D, 2 dimensional; FA, fatty acid; GPI, glycosyl-phosphatidylinositol; IEF, isoelectric focusing; mAb, monoclonal antibody, PBS, phosphate-buffered saline; PM, plasma membrane; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

0006-291X/99 $30.00

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from the trans-Golgi network that contain glycosylphosphatidylinositol- (GPI) anchored membrane proteins, suggesting an involvement in the traffic of these vesicles to the plasma membrane (31, 32).

Using photoaffinity labeling with a photoreactive FA analogue, we have previously identified a unique, 22 kDa plasma membrane-associated FA-binding protein in 3T3-L1 adipocytes (7) and have shown that binding of the photoreactive FA to this protein in plasma membranes is saturable and exhibits a high affinity for the photoreactive FA (33). We now report that this protein localizes to plasma membrane caveolae and has been identified immunologically as caveolin-1. These results suggest either that caveolin-1 is involved in lipid traffic or lipid binding plays a role in the regulation of caveolin-1 function.

MATERIALS AND METHODS

Materials. 11-DAP[11-^{3}H]undecanoic acid was synthesized as described previously (34). Amplify fluorography reagent, ECL immunoblotting detection reagents and horseradish peroxidase- (HRP-) anti-mouse IgG were from Amersham. Anti-caveolin-1 monoclonal antibody (mAb 2234) was from Transduction Laboratories. Anti-adipocyte lipid binding protein (ALBP) was the generous gift of D. Bernlohr. All other reagents, were obtained as described (7).

Cell culture and subcellular fractionation. 3T3-L1 adipocytes were cultured, released from dishes and homogenized as described previously (7). All further steps were at 4°C. Homogenates were centrifuged at 10,000xg for 20 min. Supernatants were centrifuged at 100,000xg for 60 min to yield fraction A, a low density microsomal pellet (35). The 100,000xg supernatant was centrifuged at 454,000xg for 60 min, to yield fractions B (pellet) and C (supernatant). The 10,000xg pellet was suspended in homogenization buffer (1.0 mM Hepes, 1.0 mM EDTA, pH 7.4) containing 0.25 M sucrose, layered onto a discontinuous gradient (0.87, 1.1 and 1.5 M sucrose in homogenization buffer) and centrifuged for 90 min at 23,000xg in a swinging bucket rotor. Membrane fractions D, E, and F were recovered at the 0.25–0.87 M, 0.87–1.1 M, and 1.1–1.5 M sucrose interfaces, diluted with homogenization buffer, and centrifuged at 100,000xg for 60 min. Membrane pellets were suspended in homogenization buffer and stored at −80°C. Protein was determined by the method of Lowry et al. (36). Marker enzymes analyzed included 5′-nucleotidase (37) and alkaline phosphodiesterase (38) for plasma membranes, glucose 6-phosphatase for ER (39), α-mannosidase II for Golgi (38), and cytochrome C oxidase for mitochondria (40).

Caveolae were prepared by a modification of the method of Chang et al. (41). Plasma membranes (50 μl at 1 mg/ml, protein), treated for 30 min with 1.0% Triton X-100 in isolation buffer (50 mM Mops pH 7.1 containing 300 mM NaCl, 5.0 mM EDTA, 5.0 mM EGTA, 1.0 mM dithiothreitol, and 0.10 mM PMSF) at 4°C, were centrifuged for 90 min at 100,000xg on a discontinuous sucrose gradient (25 μl each of 5, 10, 20, 30, 40 and 80% sucrose in isolation buffer) in a Beckman TLS55 rotor. Fractions (25 μl) were recovered from the top of the gradient.

Photoaffinity labeling. Membrane samples (0.25 mg protein/ml in PBS) were incubated for 15 min at 37°C in the dark with the specified concentrations of 11-DAP-[11-^{3}H]undecanoate and BSA (see figure legends). Samples were subjected to photolysis and washed as described previously (7, 33, 34, 42).

Immunoprecipitation. All steps were at 4°C. Membranes (0.52 mg/ml protein) were solubilized for 4 h with 1.0% Triton X-100 and 60 mM octylglucoside (31, 32) in 25 mM Tris-HCl, pH 7.5, containing 5.0 mM EDTA, 250 mM NaCl, 20 μM 4-amidinophenyl methanesulfonyl fluoride, bestatin (40 μg/ml), 1.0 μM leupeptin and 1.0 μM pepstatin, and precleared with mouse IgG and Protein A-Sepharose. Immunoprecipitation was done in 25 mM Tris-HCl, pH 7.5, containing 5.0 mM EDTA, 250 mM NaCl, 1.0% Triton X-100 and BSA (30 mg/ml) after an overnight incubation with either anti-caveolin-1 monoclonal antibody (mAb 2234) or an irrelevant antibody followed by a 30 min incubation with Protein A-Sepharose. Pellets were boiled for 1.5 min in 40 μl of Laemmli sample buffer (7, 44) containing 100 mM dithiothreitol in place of 2-mercaptoethanol prior to SDS-polyacrylamide gel electrophoresis (PAGE).

Electrophoresis and immunoblotting. Samples for 2 dimensional (2D)-PAGE analysis were solubilized with SDS and NP-40 and isoelectric focusing (IEF) was performed in a BioRad Mini-IEF apparatus (500 V for 10 min, 750 V for 2 h) as described previously (42, 43). IEF gels were equilibrated for 5 min in Laemmli sample buffer (7, 44). SDS–PAGE (12.5% gels) and fluorography were performed as described previously (7, 44). For immunoblotting, proteins were transferred to PVDF membranes (45) that had been blocked overnight at 4°C in TBS (20 mM Tris-HCl pH 7.6, 137 mM NaCl) containing 5% dry milk and 0.5% Tween-20. Antibody reactions (1 h at room temperature, 1:2000 dilution unless otherwise indicated) and washes were in TBS containing 0.2% dry milk and 0.2% Tween-20. Bound antibodies were detected using the ECL kit (Amersham).

RESULTS AND DISCUSSION

Previously we reported that a 22-kDa protein in crude plasma membrane preparations from 3T3-L1 adipocytes is the principal protein labeled by a photoreactive FA, 11-DAP[11-^{3}H]undecanoate, added as a BSA complex (7), and that incorporation of the probe into the protein was saturable, exhibiting a high affinity (K_d of 216 nM) for the uncomplexed photoreactive FA (33).

We wanted to determine if this protein was associated with caveolae, which required that we first isolate plasma membranes prepared from 3T3-L1 adipocytes using a procedure that yielded purer membranes than used previously (7, 33). A variety of enzymatic markers were used to distinguish the density gradient fractions that contained plasma membrane from other membranes (Fig. 1a). These included 5′-nucleotidase and alkaline phosphodiesterase for plasma membranes, glucose 6-phosphatase for ER, α-mannosidase II for Golgi, and cytochrome C-oxidase for mitochondria. Immunoblotting for caveolin-1 and the cytosolic adipocyte lipid binding protein (ALBP) (46) was also used (Fig. 1b). Based on the distribution of these markers, fraction D contained the purest plasma membranes and is hereafter referred to as PM (plasma membranes).

The photoreactive FA bound the 22-kDa protein in these membranes (Fig. 2). PM samples were incubated for 15 min at 37 °C with 25 μM 11-DAP-[11-^{3}H]-undecanoate in the presence of 50 μM BSA. The presence of BSA in the incubation buffers maintains the concentration of uncomplexed FA in the nanomolar range (7, 33, 42, 47). Upon photolysis, the photoreactive probe was incorporated into two proteins (+ Photolysis). One was the BSA used in the labeling reaction

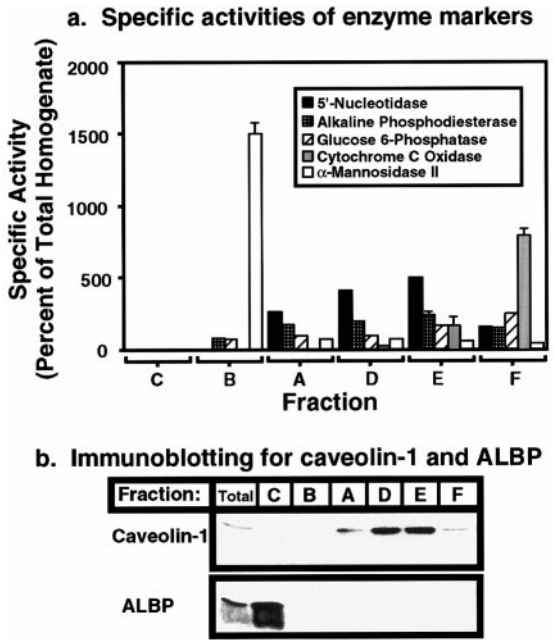

FIG. 1. Subcellular fractionation of 3T3-L1 adipocytes. 3T3-L1 adipocytes were homogenized and fractionated by a combination of differential and sucrose density gradient centrifugation and fractions A–F were collected as described. **a.** The specific activities (normalized for protein content) of 5′-nucleotidase (black), alkaline phosphodiesterase (stipled), glucose 6-phosphatase (hatched), cytochrome C-oxidase (grey) and α-mannosidase II (open) are shown for each fraction (A–F) expressed as the percentage of the specific activity in the total homogenate. Results are the means ± standard errors of three replicate assays and represent a typical profile. **b.** Equal amounts of protein (10 μg) from the total homogenate (lane marked "Total") and each fraction (A–F, as indicated) were also analyzed by SDS-PAGE, followed by immunoblotting with either anti-caveolin-1 mAb (upper panel; 1:16,000 dilution of HRP anti-mouse secondary Ab), or anti-ALBP polyclonal Ab (lower panel; 1:500 dilution of primary Ab, 1:20,000 dilution of HRP anti-rabbit secondary Ab).

(upper band). The mobility of this band was identical to unlabeled BSA and its intensity was reduced by washing labeled membranes in the presence of unlabeled BSA (data not shown). The other protein had an apparent molecular weight of 22 kDa. The probe was not incorporated into proteins in the absence of photolysis (− Photolysis).

We next isolated caveolae from 3T3-L1 adipocyte PM that had been labeled with the photoreactive FA (Fig. 3). Triton X-100 insoluble caveolae membrane was separated from Triton X-100 soluble plasma membrane on a discontinuous sucrose density gradient. Fractions from the gradient were separated by SDS-PAGE and immunoblotted with anti-caveolin-1 mAb. The highest concentration of reactive caveolin-1 was in fraction 7. The photoaffinity-labeled 22-kDa protein co-fractionated with the immunoreactive caveolin-1 (compare lanes marked fraction 7 in Fig. 3a and b). Labeled BSA (66-kDa band) and contaminating proteins of approximately 40 to 45-kDa were primarily in the Triton-

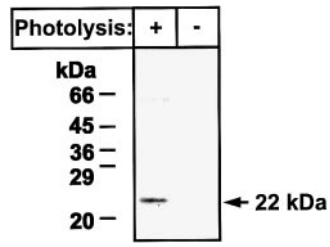

FIG. 2. A 22 kDa protein is labeled by 11-DAP-[11-^{3}H]-undecanoate upon photolysis of isolated plasma membranes from 3T3-L1 adipocytes. The plasma membrane (PM) fraction (fraction D, of Fig. 1) from 3T3-L1 adipocytes was incubated for 15 min in the dark at 37°C with 25 μM 11-DAP-[11-^{3}H]undecanoate in the presence of 50 μM BSA. The membranes were then either photolyzed (lane marked + Photolysis) or kept in the dark (lane marked − Photolysis) before pelleting at 100,000 rpm for 18 min at 4°C. Samples were washed with 1.0% BSA in PBS and 25 μg samples were analyzed by SDS-PAGE. Gels were treated for fluorography, dried and exposed to Kodak XAR-5 film. The molecular weight markers are indicated to the left of the gel.

soluble fractions (Fig. 3b, Fractions 1–4), which did not contain caveolin-1.

The similar apparent molecular weight and subcellular location of caveolin and the 22-kDa photoreactive FA-labeled protein suggested they might be the same protein. We used a standard immunoprecipitation assay to see if the photolabeled protein was caveolin-1 (Fig. 4). PMs were labeled with photoreactive FA, the membranes solubilized and proteins immunoprecipi-

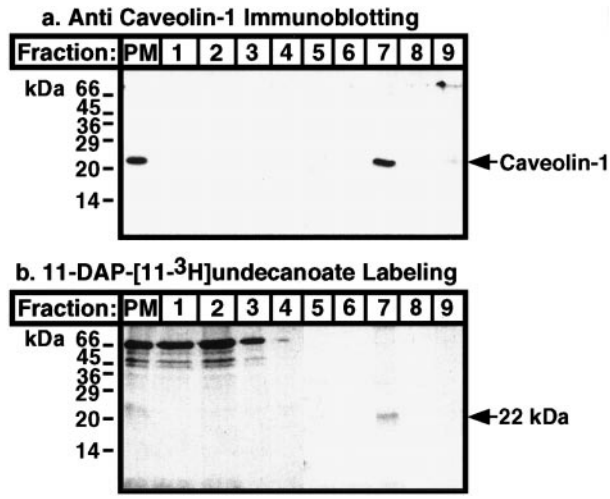

FIG. 3. The 11-DAP-[11-^{3}H]undecanoate-labeled protein co-fractionates with caveolin-1. PM were labeled by photolysis after incubation with 50 μM 11-DAP-[11-^{3}H]undecanoate in the presence of 50 μM BSA as described. Membranes (50 μg) were incubated in the presence of Triton X-100 at 4°C, and separated on a discontinuous sucrose gradient by centrifugation at 100,000xg for 90 min at 4°C. Fractions (25 μl) were recovered, separated by SDS-PAGE and either immunoblotted with anti-caveolin-1 mAb (**panel a**) or exposed to x-ray film for detection of 11-DAP-[11-^{3}H]undecanoate-labeled bands (**panel b**). PM = unfractionated plasma membrane. Fraction 1 is the top of the gradient and fraction 9 the pellet.

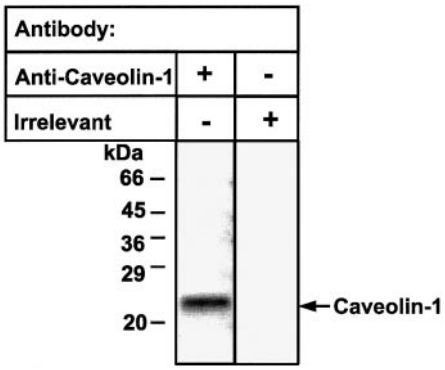

FIG. 4. The 22 kDa, 11-DAP-[11-^{3}H]undecanoate-labeled protein is immunoprecipitated by anti-caveolin-1 mAb. 3T3-L1 adipocyte plasma membranes, labeled with 10 μM 11-DAP-[11-^{3}H]undecanoate, were solubilized, precleared and allowed to react with either anti-caveolin-1 mAb 2234 or an irrelevant antibody as described. Immunoprecipitated proteins were solubilized, separated by SDS-PAGE, and fluorographed as described. Molecular weight standards are indicated on the left.

tated using either an anti-caveolin-1 mAb or an irrelevant IgG as a control. A single 22-kDa, photoreactive FA-labeled protein was in the precipitate only when anti-caveolin-1 mAb was used. Therefore, the single, photoaffinity-labeled 22-kDa protein in 3T3-L1 adipocyte PM is caveolin-1.

Caveolin-1 occurs as two variants, α and β, which differ in their N-terminus (48, 49). The monoclonal antibody (mAb 2234) used for immunoprecipitation in Fig. 4 is specific for α-caveolin-1 (49). To determine whether β-caveolin-1 also binds the photoreactive FA, PM were photo-labeled with the photoreactive probe and proteins were analyzed by 2 D-PAGE (Fig. 5). The 22 kDa photolabeled band resolved into two spots (Fig. 5a). Corresponding spots were detected with an anti-C-terminal caveolin-1 IgG that reacts with both α- and β-caveolin-1 (Fig. 5b). The more basic spot corresponded to α-caveolin-1 as determined by re-probing the blot with an anti-N-terminal caveolin-1 antibody (Fig. 5c). Therefore, both α- and β-caveolin-1 can bind the photoreactive FA, indicating that the unique N-terminus of α-caveolin-1 is not necessary for FA binding. Preliminary results indicate that both forms of caveolin-1 bind the photoreactive FA saturably and with comparable affinities (data not shown). While the photoreactive FA labels a single α-caveolin-1 spot (Fig. 5a, solid arrow), immunoreactive α-caveolin-1 contains multiple spots that trail toward the basic end of the gel (Fig. 5b and c, solid arrow). This suggests there are multiple isoforms of α-caveolin-1 in these cells but only one or a subset are able to bind the photoreactive FA. Caveolin-1 is a known substrate for both tyrosine and serine/threonine kinases (50–52), raising the possibility that the various isoforms correspond to multiple phosphorylated caveolins. If this is the case, then phosphorylation of α-caveolin-1 may regulate FA binding. Alternatively, FA binding to α-caveolin-1 may prevent phosphorylation (53, 54).

Caveolin-1 is palmitoylated (55) and the photoreactive FA can substitute for palmitate in the Cys-acylation of proteins (56). However, the binding of the photoreactive FA to caveolin-1 was dependent on irradiation (Fig. 2), indicating that labeling was via the photoreactive diazirinophenoxy moiety of the probe rather than a reaction of the carboxylate. In combination with the finding that labeling is saturable, this suggests the labeling is due to an interaction between the probe and a high affinity lipid-binding site in caveolin-1 (33, 57).

Previously caveolin-1 was shown to bind cholesterol in an overlay assay (58), and cholesterol appears to enhance the incorporation of caveolin-1 into lipid bilay-

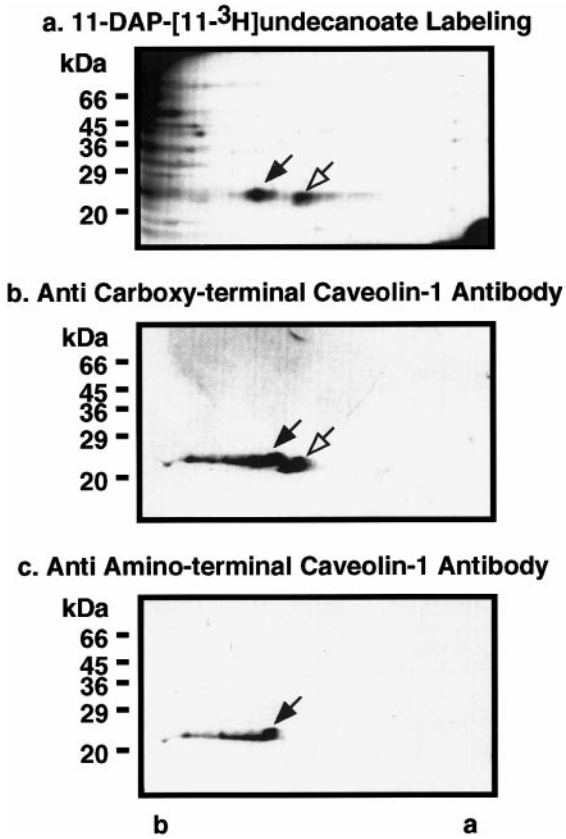

FIG. 5. Both α and β caveolin-1 are FA binding proteins. 3T3-L1 adipocyte plasma membranes, labeled with 10 μM 11-DAP-[11-^{3}H]undecanoate, were solubilized and 37.3 μg of protein was separated by IEF as described. Gels were either analyzed by fluorography to reveal 11-DAP-[11-^{3}H]undecanoate-labeled proteins (**panel a**) or immunoblotted using a rabbit-pAb specific for the carboxy-terminus of caveolin-1 (**panel b**). The blot was stripped and reprobed with a rabbit-pAb specific for the amino-terminus of caveolin-1 (**panel c**). The positions of α- and β-caveolin-1 and the corresponding photoaffinity labeled proteins are indicated by the solid and open arrows, respectively. The molecular weights standards are indicated on the left. The basic ("b") and acidic ("a") ends of the gel are indicated at the bottom.

ers (58, 59). Cholesterol is also associated with caveolin in a soluble complex with other proteins (30). We do not know if 11-DAP[11-^{3}H]undecanoate binds to the same site as cholesterol. Further work will also be needed to determine if FAs are bound to soluble caveolin-1 (30) or other isoforms of caveolin, such as caveolins-2 (60) and 3 (61).

The ability of caveolin-1 to bind FAs saturably and with high affinity suggests it could be involved either in lipid traffic, the organization of caveolae membrane or the regulation of caveolin-1 function. Caveolin-1 continuously moves from caveolae to the Golgi apparatus and back to the cell surface (62, 63). It may also shuttle cholesterol between the ER and caveolae (29). Therefore, caveolin-1 could transport FAs between membrane compartments. Since immunoprecipitation of GPI anchored membrane proteins such as the urokinase receptor co-precipitates caveolin-1 (64), the binding of FAs to caveolin-1 may be important for organizing this class of proteins in caveolae. Finally, the recent evidence that caveolin-1 is able to regulate the activity of multiple signaling molecules *in vitro* suggests the binding of FAs could modulate cell signaling events that are under the control of caveolin-1 (28).

ACKNOWLEDGMENTS

We thank J. Capone for use of his cell culture facility, D. Bernlohr for generously providing anti-ALBP antibody, K. Rothberg and L. Craig for help with immunoprecipitation and immunoblotting, and N. Fernandes and D. Zuccolo for technical assistance. This work was supported by a Medical Research Council of Canada Grant MA6488 to G.E.G. B.L.T. was the recipient of a Medical Research Council of Canada Studentship.

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