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Isolation and partial characterization of a fatty acid binding protein in rat liver plasma membranes

(hepatocyte/hepatic uptake)

WOLFGANG STREMMEL*, GEORG STROHMEYER*, FRANZ BORCHARD*, SHAUL KOCHWA†, AND PAUL D. BERK†‡

*Medizinische Klinik, Poliklinik D und Pathologisches Institut der Universität Düsseldorf, Moorenstrasse 5, 4000 Düsseldorf, Federal Republic of Germany; and †The Polly Annenberg Levee Hematology Center and the Hepatic Research Group, Department of Medicine, Mount Sinai School of Medicine, New York, NY 10029

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ABSTRACT **When [^{14}C]oleate–bovine serum albumin complexes were incubated *in vitro* with rat liver plasma membranes (LPM), specific, saturable binding of oleate to the membranes was observed. Maximal heat-sensitive (i.e., specific) binding was 3.2 nmol/mg of membrane protein. Oleate-agarose affinity chromatography of Triton X-100-solubilized LPM was used to isolate a single 40-kDa protein with high affinity for oleate. On gel filtration, the protein comigrated with various fatty acids but not with [^{14}C]bilirubin, [^{35}S]sulfobromophthalein, [^{14}C]taurocholate, [^{14}C]phosphatidylcholine, or [^{14}C]cholesteryloleate. A rabbit antibody to this membrane fatty acid-binding protein gave a single precipitin line with the antigen but no reactivity with concentrated cytosolic proteins, LPM bilirubin/sulfobromophthalein-binding protein, or rat albumin or other rat plasma proteins. The antibody selectively inhibited heat-sensitive binding of [^{14}C]oleate to LPM. Immunofluorescence studies localized the antigen in liver-cell plasma membranes as well as in other major sites of fatty acid transport. These data are compatible with the hypothesis that this protein may act as a receptor in a hepatocellular uptake mechanism for fatty acids.**

Although the hepatic uptake of fatty acids is often described as a passive, diffusional process (1), recent kinetic studies have suggested that at least a portion of their cellular uptake may be carrier mediated (2–6). However, others have questioned carrier-mediated uptake of fatty acids and asserted that the apparently saturable uptake component observed in previous studies predominantly reflected fatty acid metabolism (7, 8). Previously, we presented evidence suggestive of a component of saturable and reversible binding of fatty acids to high-affinity membrane binding sites (9, 10). In the present study, we have further examined the binding of fatty acids to rat liver plasma membranes (LPM) and sought to isolate and characterize a putative membrane receptor protein responsible for the binding.

MATERIALS AND METHODS

Materials. [1-^{14}C]Oleic acid, [1-^{14}C]arachidonic acid, [1-^{14}C]palmitic acid, [1-^{14}C]linoleic acid, [24-^{14}C]taurocholic acid, L-α-1-palmitoyl-2-[1-^{14}C]oleoyl-phosphatidylcholine, cholesteryl[1-^{14}C]oleate, and Aquasol were from New England Nuclear. [^{35}S]sulfobromophthalein (disodium phenoltetrabromophthalein di[^{35}S]sulfonate; [^{35}S]BSP) and [1′,2,3′,4,5,6′,7′,8-^{14}C]bilirubin were from Amersham. The unconjugated [^{14}C]bilirubin was repurified as previously described (11). Unlabeled oleate, taurocholate, BSP, bilirubin, bovine serum albumin (fraction V), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, and Triton X-100 were from Sigma; Sephadex G-100 and G-150, Sepharose 4B, and AH-Sepharose 4B, from Pharmacia; Bio Beads SM-2, from Bio-Rad; rat serum albumin and rabbit antiserum against rat albumin, from Cappel Laboratories (Cochranville, PA); goat anti-rat fibronectin (IgG fraction), from Calbiochem–Behring; and anti-rat ligandin, by courtesy of A. W. Wolkoff (Department of Medicine and Liver Center, Albert Einstein College of Medicine, New York, NY).

Isolation and Characterization of LPM. LPM enriched in sinusoidal components were prepared from livers of male Sprague–Dawley rats by differential centrifugation (12, 13) and characterized by electron microscopy (14) and by analysis of enzymatic markers (13). Membrane protein content was determined by the method of Lowry *et al.* (15).

Binding of [^{14}C]Oleate. To avoid exceeding the solubility of oleate in the aqueous phase of incubation mixtures (10), membrane [^{14}C]oleate binding was determined by incubation of LPM aliquots with 60 μM [^{14}C]oleate bound to various measured quantities of defatted (16) bovine serum albumin. Nonspecific binding was evaluated in parallel incubations of LPM aliquots denatured by prior incubation for 4 hr at 70°C, avoiding agglutination of membrane particles. All incubations were carried out in 1 ml of P_i/NaCl (0.15 M NaCl/0.02 M sodium phosphate, pH 7.6) for 30 min at 37°C, after preliminary studies had established that binding was complete within 30 min. Binding was assessed by a centrifugation assay (17, 18).

Binding of Other Ligands. To examine the binding to LPM of various organic anions under specified conditions, as a basis for antibody inhibition studies (see below), aliquots containing 5 mg of membrane protein were incubated at 37°C for 30 min in 1 ml of P_i/NaCl with various concentrations of either [^{14}C]taurocholate, or ^{35}S-BSP bound to defatted bovine serum albumin (1:2 molar ratio), or [^{14}C]oleate bound to defatted bovine serum albumin (3:1 molar ratio). This system has been described in detail (18). In antibody inhibition studies, resuspended aliquots of LPM were incubated for 30 min at 37°C with 0.2 mg of the IgG-fraction of either anti-fatty acid binding membrane protein antiserum or anti-fibronectin (control) antiserum before ligands were added.

Preparation of Affinity Columns. Oleate-agarose was prepared by incubating 40 ml of swollen, washed AH-Sepharose 4B with 60 ml of 0.1 M sodium oleate, pH 10.0, and 2 g of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide for 3 days at 37°C with gentle rotation (19). BSP was coupled to Sepharose 4B with epichlorhydrin (20) as described (21).

Solubilization and Affinity Chromatography of Membrane Proteins. LPM proteins were solubilized with 1% (vol/vol) Triton X-100 (21). After centrifugation at 100,000 × *g* for 60 min, residual detergent was removed with Bio-Beads SM-2 (21). Aliquots containing 500 mg of the solubilized proteins

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Abbreviations: LPM, liver plasma membrane(s); FABP, fatty acid binding protein; BSP, sulfobromophthalein.

‡To whom reprint requests should be addressed.

were then loaded onto oleate-agarose and BSP-agarose columns and washed with P_i/NaCl at 20 ml/hr until no further protein (A_{280}) appeared in the washes. Bound protein was then eluted from the BSP-agarose column with 50 μM BSP (21) and from the oleate column with 8 M urea. The eluates were concentrated by ultrafiltration (Diaflo PM-10 membranes, Amicon), and the proteins were characterized by NaDodSO$_4$/PAGE (22) and analytical isoelectric focusing in polyacrylamide gels (2117 Multiphor, LKB). The protein bands were routinely stained with Coomassie blue; representative gels were also stained with Sudan black or periodic acid/Schiff reagent (23). Contamination of the isolated BSP- and oleate-binding proteins was examined by double-immunodiffusion in agar (24) with rabbit antisera against rat albumin, rat hepatocyte BSP-binding protein (18), and rat ligandin.

Further Characterization of the Oleate-Binding Protein. Aliquots (0.3 mg) of the purified oleate-binding protein were mixed with tracer amounts of [^{14}C]oleate and subjected to gel filtration through a Sephadex G-150 column, which was calibrated with purified standard proteins spanning a range of 20–158 kDa. Other 0.3-mg aliquots of the purified oleate-binding protein were equilibrated with tracer amounts of various ^{14}C-labeled fatty acids, other organic anions, and hydrophobic membrane constitutents and then subjected to gel filtration through a column of Sephadex G-100 equilibrated with 0.1 M NaCl/1 mM NaHCO$_3$, pH 7.6. Elution was carried out with the same solution at 14 ml/hr. Eluates, collected in 1-ml fractions, were monitored for protein (A_{280}) and for ^{14}C (liquid scintillation spectrometry) (18).

Preparation of Antibody to Oleate-Binding Protein. Outbred New Zealand White rabbits were immunized with the purified oleate-binding protein as described (18). Presence and purity of the antibody was examined by radial double-immunodiffusion on agar plates (24) against the purified LPM oleate-binding protein, Triton X-100-solubilized LPM proteins, rat serum albumin, concentrated rat liver cytosolic proteins, rat LPM BSP-binding protein (18), and whole rat serum. IgG fractions of the antisera were prepared by standard techniques (25).

Indirect Immunofluorescence Studies. Frozen 5-μm sections of various rat tissues were air-dried and then incubated for 30 min at room temperature with 1:50, 1:100, and 1:200 dilutions of rabbit antibody against the LPM oleate-binding protein. Sections were then washed with P_i/NaCl for 30 min, incubated with fluorescein isothiocyante-conjugated swine anti-rabbit antiserum diluted 1:20 or 1:50, thoroughly washed with P_i/NaCl for 60–90 min, and finally mounted with buffered glycerol. Controls consisted of incubations with antiserum preabsorbed with the LPM oleate-binding protein in equal dilution, incubations without immune serum, and incubations with pre-immune serum. Within a few hours after mounting, sections were examined with a fluorescence microscope (Zeiss), employing filter combination BP485/FT510/LP520.

RESULTS

Characterization of LPM Fractions. The LPM fractions were similar to those used previously (18) and showed homogeneous membrane structures in numerous randomly selected electron micrographs. Very few tight junctions, typical of canalicular membranes, were observed. 5′-Nucleotidase was enriched 25-fold, and glucagon-stimulated adenylate cyclase, 12-fold compared to the initial homogenate. Contamination with microsomes or mitochondria was negligible, as assessed by enzymatic markers. Albumin or ligandin were not detected by radial immunodiffusion against appropriate antibodies.

Binding of [^{14}C]Oleate to LPM. As the oleate/albumin molar ratio was increased from 0.25 to 5 in incubation mixtures containing a total oleate concentration of 60 μM, the initial free oleate concentration increased hyperbolically from 0.018 to 6.2 μM (10). [These concentrations represent maximal estimates, as they were calculated from the equilibria between oleate and albumin (10), ignoring the unknown binding parameters of the incubated LPM.] Over the same range of oleate/albumin ratios, total [^{14}C]oleate bound to native LPM increased nonlinearly (Fig. 1*A*). When the total-binding curve was corrected for heat-insensitive (i.e., nonspecific) binding, an apparent saturation curve was observed (Fig. 1*B*). In the system employed, maximal heat-sensitive oleate binding (16.04 ± 1.06 nmol/5 mg of membrane protein) occurred at an oleate/albumin ratio of 3, at which point 60% of the incubated oleate was still not membrane bound. Specific binding was not further increased by increasing the oleate/albumin ratio to 4 or 5, despite a progressive increase in the ratio of residual non-membrane-bound oleate to albumin and a further increase of more than 2-fold in the calculated free oleate concentration remaining in the incubation mixture.

Binding of Other Organic Anions to LPM. Incubation of LPM with increasing concentrations of [^{14}C]taurocholate, [^{35}S]BSP (as a 1:2 complex with bovine serum albumin) and [^{14}C]oleate (as a 3:1 complex with bovine serum albumin) resulted in saturable binding of each of these representative ligands, as previously described (18). In the presence of excess unlabeled ligand, nonspecific binding of [^{35}S]BSP and [^{14}C]taurocholate to LPM was less than 2% and 6%, respectively, of total binding and was ignored in subsequent antibody inhibition studies with these two ligands. Other than

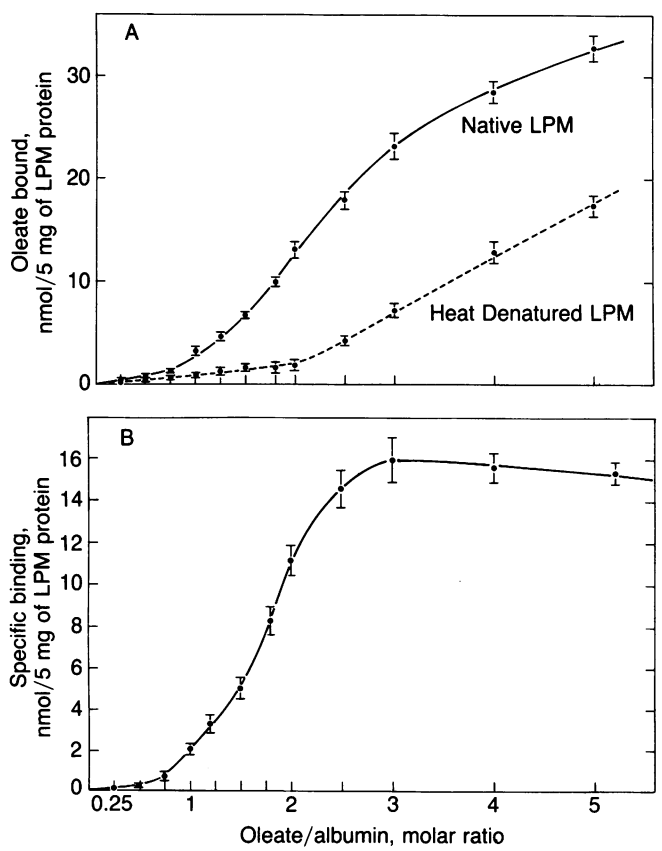

FIG. 1. *(A)* Binding of [^{14}C]oleate to native and heat-denatured LPM (mean ± SD, n = 5). Each incubation contained 5 mg of LPM protein and a total oleate concentration of 60 μM. The bovine serum albumin concentration was varied so that the oleate/albumin ratio ranged from 0.25–5. *(B)* Specific binding of [^{14}C]oleate to LPM, calculated (from the data in *A*) as total binding to native membranes minus binding to heat-denatured LPM.

establishing conditions for maximal binding of each test substance as a reference point for inhibition studies (see below), no attempt was made to further characterize ligand binding in this system.

Affinity Chromatography Studies. In contrast to the multiplicity of bands found for the initial solution of solubilized membrane proteins, $NaDodSO_4$/PAGE revealed only a single band at ≈40 kDa in the concentrated eluate from the oleate-agarose column (Fig. 2). As previously reported (18), a somewhat larger protein, of ≈55 kDa, was recovered from the BSP-agarose column.

Further Characterization of the Oleate-Binding Membrane Protein. On cylindrical gels, the oleate-binding protein recovered from oleate-agarose did not stain with either Sudan black or periodic acid/Schiff reagent. Analytical isoelectric focusing of the protein revealed only a single, sharp band with a pI of 9.0. The protein showed no immunologic reactivity with rabbit antibodies against rat serum albumin, ligandin, or the hepatocyte membrane BSP-binding protein. On Sephadex G-150 gel filtration, the oleate binding protein comigrated with [^{14}C]oleate as a discrete, sharp peak. By comparison with other marker proteins, its molecular mass in this nonreducing, nondetergent medium was again 40 kDa. When the purified protein was cochromatographed on Sephadex G-100 with various organic anions and other hydrophobic membrane constituents, binding to the protein was only exhibited by the various ^{14}C-labeled fatty acids tested (Fig. 3). Although oleate comigrated with the protein, the oleate ester of cholesterol and a phosphatidylcholine containing oleate in the 2-position did not. BSP, bilirubin, and taurocholate also showed no affinity for the oleate-binding membrane protein. These data suggest that the protein serves as a general fatty acid-binding protein and that its affinity spectrum is not limited solely to oleate. It is, therefore, referred to below as the LPM fatty acid binding protein (LPM-FABP).

Characterization of Rabbit Antibodies Against the LPM-FABP. Whole antisera from two rabbits repeatedly injected with the LPM-FABP produced a single precipitin line between the purified protein and the mixture of solubilized LPM proteins, but showed no reaction with concentrated rat liver cytosolic proteins [including cytosolic fatty acid-binding protein (Z protein)], rat liver LPM BSP-binding protein, rat albumin, and rat serum.

Antibody Inhibition of Organic Anion-Binding to Rat LPM. In studies employing various concentrations of a 3:1 complex of [^{14}C]oleate–albumin with constant amounts of membrane and antibody, the antibody against the LPM-FABP significantly inhibited binding of [^{14}C]oleate to native LPM whereas nonspecific oleate binding to heat-denatured LPM was unaffected (Fig. 4). The anti-LPM-FABP inhibited heat-sensitive (specific) binding by a maximum of 41% compared to control in this system. In incubations containing the same amounts of antibody (0.2 mg of IgG fraction) and [^{14}C]oleate but various amounts of LPM, a linear relationship was observed between the logarithm of the LPM concentration and the percent inhibition of specific [^{14}C]oleate binding, as determined by comparison with control incubations containing

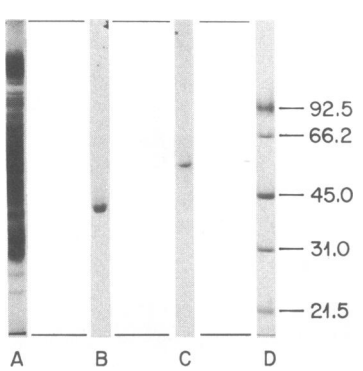

FIG. 2. Affinity chromatography of detergent-solubilized proteins from rat LPM. Shown are Coomassie blue-stained cylindrical gels after $NaDodSO_4$/PAGE of total solubilized membrane proteins (gel A), 40-kDa protein eluted from oleate-agarose with 8 M urea (gel B), 55-kDa protein eluted from BSP-agarose with BSP (gel C), and standards (gel D; molecular masses in kDa, to right). Tops of gels and tracking dye fronts are aligned with the horizontal lines between the gels.

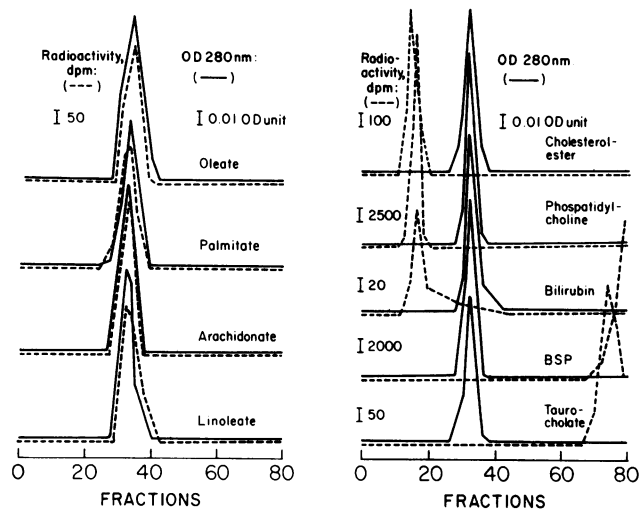

FIG. 3. Chromatography on Sephadex G-100 of 0.3-mg aliquots of purified LPM fatty acid (oleate)-binding protein with tracer amounts of [^{35}S]BSP, [^{14}C]bilirubin, or [^{14}C]taurocholate; or ultrasonically dispersed [^{14}C]phosphatidylcholine, cholesteryl [^{14}C]-oleate, [^{14}C]oleate, [^{14}C]arachidonate, [^{14}C]palmitate, or [^{14}C]linoleate. Only ^{14}C-labeled fatty acids were eluted with the protein peak. Ligands not soluble in aqueous solution (bilirubin, cholesteryl oleate, and phosphatidylcholine) appeared in the void volume (up to fraction 21), whereas BSP and taurocholate appeared in the effluent near the total volume of the column (starting at fraction 70) and not associated with protein.

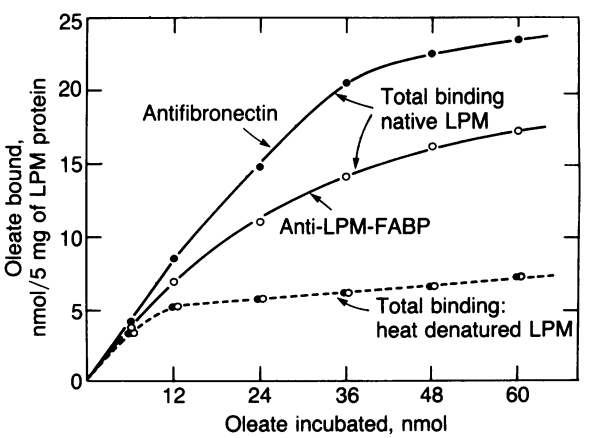

FIG. 4. Effect of the IgG fraction of a rabbit antibody to the LPM fatty acid binding protein (anti-LPM-FABP, ○) and a control IgG fraction (anti-fibronectin, ●) on total [^{14}C]oleate binding to native and heat-denatured LPM. Aliquots (5 mg) of native or denatured LPM were incubated in a volume of 1 ml for 30 min at 37°C with 0.2 mg of either IgG fraction and then incubated for an additional 30 min with 6–60 μM [^{14}C]oleate in the form of a 3:1 oleate–albumin complex. Data shown are mean values from 3–5 incubations (SD ≤ ±5% in all cases). Under the conditions employed, the anti-LPM-FABP inhibits heat-sensitive (specific) [^{14}C]oleate binding by ≤41%.

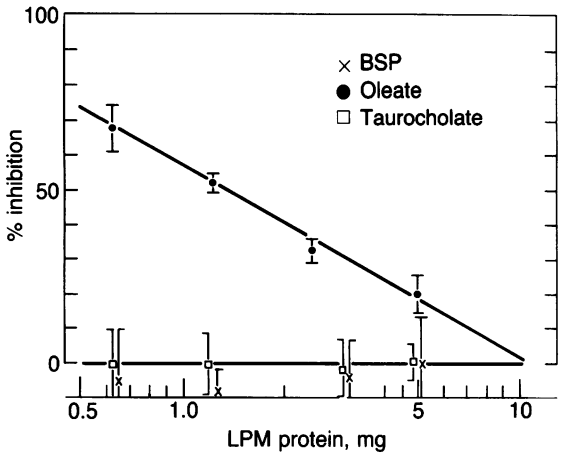

FIG. 5. Inhibition of the binding of [^{14}C]oleate to LPM by rabbit anti-LPM-FABP. In paired studies, various quantities of LPM (0.625–5 mg of protein) resuspended in 0.4 ml of P_i/NaCl were incubated for 30 min at 37°C with 0.2 mg (0.1 ml) of the IgG fraction of either anti-LPM-FABP or anti-rat fibronectin. After addition of [^{14}C]oleate–albumin (3:1), [^{35}S]BSP–albumin (1:2), or [^{14}C]taurocholate, the reaction mixture was incubated in a total volume of 1 ml for 30 min at 37°C. In each instance, sufficient ligand was added to saturate LPM under the conditions employed and, in the case of oleate, replicate incubations were conducted with heat-denatured LPM to permit determination of specific binding. Binding of ligand to LPM was determined in the presence of each antibody; results are expressed as percent inhibition by anti-LPM-FABP compared to binding observed in the presence of anti-fibronectin. Mean values ± SD (n = 3) are given for inhibition of specific (oleate) or total (BSP, taurocholate) binding. Nonspecific binding of BSP and taurocholate was very low (see text) and was ignored. Antibody had no effect on nonspecific binding of [^{14}C]oleate to rat LPM.

antibody against fibronectin. In this system, binding of [^{14}C]taurocholate and [^{35}S]BSP was not affected (Fig. 5).

Indirect Immunofluorescence Studies. In frozen sections of rat liver, there was fluorescence along the plasma membranes of the hepatocytes in sections incubated with rabbit anti-LPM-FABP (Fig. 6 *Top*), but not in control sections (Fig. 6 *Bottom*). All surfaces of the hepatocyte appeared to show similar staining. Except for nonspecific autofluorescence due to lipofuscin, principally in centrilobular hepatocytes, there was no cytoplasmic staining of liver parenchymal cells. Ito cells also failed to show specific staining. Within the portal parenchyma, faint but specific staining was observed in the surface membranes of the epithelial cells of small bile ducts.

The jejunal mucosa showed marked fluorescence, which did not appear in the control sections, in the apical parts of the brush-border villus and crypt cells. Similar staining was observed in the ileum, but no evidence of specific immunofluorescence was observed in esophageal or colonic mucosa. In studies of other tissues, specific immunofluorescence was observed in the intercalated discs of cardiac muscle.

DISCUSSION

Three major classes of organic anions—bile acids, non-bile acid cholephils such as BSP and bilirubin, and fatty acids—are efficiently extracted from blood by the liver (26–31) despite tight binding to albumin in the circulation (32–35). Studies of the uptake kinetics of conjugated bile acids (29, 36) and non-bile acid cholephils, such as BSP and bilirubin (26–28, 30), and of their respective binding to LPM (13, 18, 37) suggest that separate but analogous carrier-mediated mechanisms facilitate the hepatocellular uptake of both of these classes of ligands. Candidate carrier proteins have been isolated for both the BSP/bilirubin (18, 38, 39) and bile acid systems (40, 41), and reconstitution of bile acid transport by

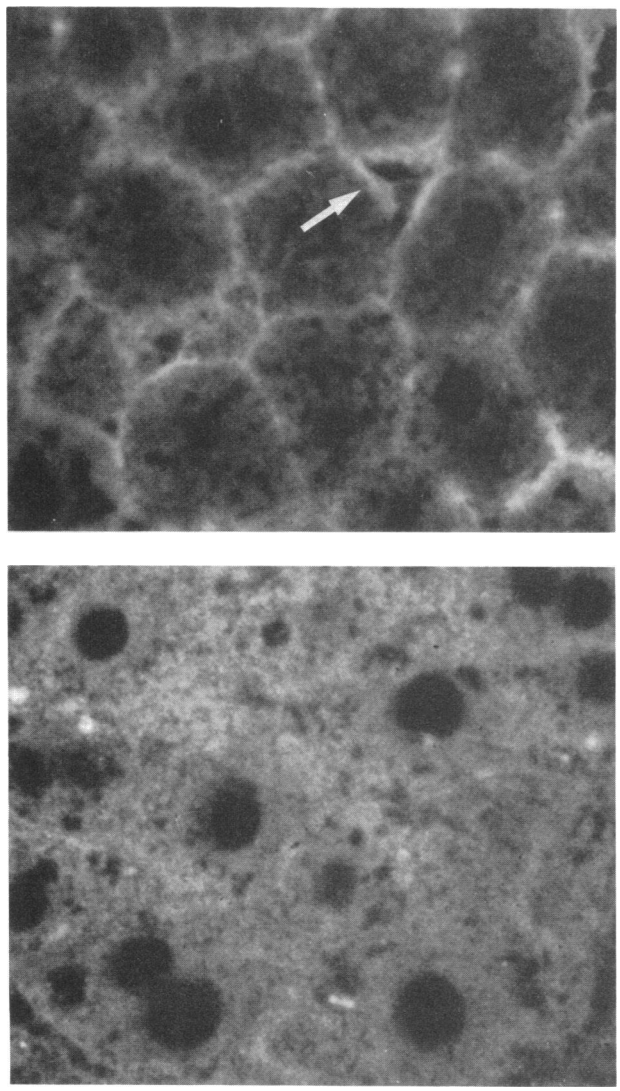

FIG. 6. *(Top)* Indirect immunofluorescence staining of the plasma membranes of hepatocytes in normal rat liver sections after incubation with rabbit antibody to rat LPM-FABP. *(Bottom)* Inhibition of staining by preabsorption of the primary antibody with purified LPM-FABP.

incorporation into liposomes of a bile acid-binding membrane protein has recently been described (42).

The hepatocellular uptake mechanism for fatty acids, the other major class of albumin-bound organic anions, is highly controversial but at least some studies suggest that these, too, are sequestered by a saturable, membrane-associated mechanism (6, 10). Previous observations from our laboratory suggested saturable binding of [^{14}C]oleate to high-affinity binding sites on LPM exhibiting a binding constant (K_a) of 2×10^8 M^{-1} (10). Binding was shown to be reduced by 50% following incubation of the membranes with trypsin. To establish specificity of oleate binding and to determine nonspecific association to plasma membranes, ligand interaction with native and heat-denatured LPM was further investigated in the present studies. The choice of the system employed, involving manipulation of the oleate/albumin molar ratio to influence the concentration of free ligand, and the use of heat-denatured LPM rather than excess unlabeled ligand to estimate nonspecific binding was dictated by the low solubility of fatty acids in aqueous media. The apparently saturable heat-sensitive (specific) binding (Fig. 1), coupled with the previously observed trypsin inhibition (10), suggested that high-affinity specific binding is due to an intrinsic membrane protein, a suggestion pursued by application of an

affinity chromatography technique previously successfully employed to demonstrate a BSP/bilirubin-binding protein in similar membrane preparations (18).

The affinity chromatography data have established that LPM do, in fact, contain a single protein with a high binding affinity for fatty acids but not for phospholipids, cholesterol esters, bile acids, BSP, or bilirubin. This protein (LPM-FABP) consists of a 40-kDa polypeptide chain with a pI of 9.0, lacking either lipid or carbohydrate components. Based on its molecular weight and the maximal specific binding capacity of LPM for oleate, this protein would appear to constitute no more than 12% by weight of the intrinsic membrane protein of the hepatocyte. The protein does not share immunologic determinants with either ligandin or the cytosolic fatty acid-binding protein (Z protein) previously described in liver (43) and small intestine (44). It is also distinguished from the latter by physicochemical properties, since the Z protein has a molecular mass of 12 kDa and a pI of 5.7.

Thus, we have demonstrated the presence in LPM of a single, basic protein with a high affinity for fatty acids but not for other hydrophobic membrane constitutents or for other classes of albumin-bound organic anions. Its precise physiologic function remains to be established but its pI of 9.0 is compatible with the hypothesis that, at physiologic pH, electrostatic interactions between a positively charged membrane protein and a negatively charged fatty acid anion may contribute to the binding process. Moreover, immunofluorescence detection of its presence in liver, small intestine, and myocardium, which are major sites of fatty acid transport, support the likelihood of a significant biologic function. Further support for this postulate derives from the specific inhibition of fatty acid binding to liver-derived plasma membranes by antibodies to this protein, as reported above.

The concept that insoluble fatty acids may be passed sequentially from albumin to the membrane binding protein described herein and thence to the cytosolic fatty acid-binding protein previously described is appealing, but remains only a working hypothesis. The existence and characteristics of this protein are compatible with the general concept that separate but analogous class-specific membrane transport systems mediate the hepatocellular uptake of the three major classes of albumin-bound organic anions.

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