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Research Article

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The Rat Hepatocyte Plasma Membrane Organic Anion Binding Protein Is Immunologically Related to the Mitochondrial F₁ Adenosine Triphosphatase β -Subunit

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Abstract

A 55-kD organic anion binding protein (OABP) was identified previously in liver cell plasma membrane sinusoidal subfractions. Although this protein was localized to the surface of hepatocytes by immunofluorescence, immunoblot analysis revealed reactivity toward both plasma membrane and mitochondrial fractions. To clarify these findings, an immunoreactive clone from a rat liver cDNA expression library was isolated, the 1,500-base pair cDNA insert was sequenced, and the corresponding β -galactosidase fusion protein was expressed and purified. The resulting sequence corresponded to that of the rat mitochondrial F₁-adenosine triphosphatase (F₁-ATPase) β -subunit. This protein and OABP are of similar size and are mutually immunologically cross-reactive. That the antigen was present on the cell surface as well as in mitochondria was suggested from studies of immunoprecipitation after cell-surface iodination, and light- and electron-microscopic immunocytochemistry. Photoaffinity labeling of bovine F₁-ATPase with high-specific-activity [³⁵S]sulfobromophthalein revealed binding only to the β -subunit. Hepatocyte uptake of bilirubin and sulfobromophthalein requires cellular ATP and mitochondria also transport these organic anions, which at high doses inhibit respiration. The presence of an organic anion binding site on the F₁-ATPase β -subunit suggests that it may play a role in these processes. (*J. Clin. Invest.* 1990. 86:220–227.)
Key words: bilirubin • liver • photolabeling • sulfobromophthalein • transport

Introduction

The organic anions bilirubin and sulfobromophthalein (BSP)¹ circulate tightly bound to albumin (1, 2). The avidity of albumin for these compounds is such that, at equimolar concentrations, < 0.1% of ligand remains unbound at equilibrium (3). Despite the fact that these organic anions are protein bound,

they are rapidly extracted from their protein carrier by the liver (2). Previous studies revealed that hepatocyte uptake of bilirubin and BSP had carrier-mediated kinetics (1, 4). In particular, recent studies performed in short-term cultured rat hepatocytes revealed that this uptake process was saturable, temperature sensitive, inhibited after depletion of cellular ATP, and dependent upon the presence of Cl⁻ in medium (5, 6).

We identified a 55-kD protein in sinusoidal surface-derived subfractions of liver cell plasma membrane as a putative organic anion transporter that bound photoactivated [³⁵S]BSP (7). Subsequently, we purified this organic anion binding protein (OABP) from deoxycholate-solubilized rat liver cell plasma membrane by using BSP-Sepharose affinity chromatography (7). Through the use of monospecific antibody, OABP was localized to the surface of hepatocytes by immunofluorescence (8). However, immunoblot analysis revealed reactivity towards both plasma membrane and mitochondrial fractions (8). To clarify these findings, we have screened a rat liver cDNA expression library with this antibody. DNA inserts of isolated clones were sequenced and expressed as β -galactosidase fusion proteins. The cDNA sequence was identical to the sequence of the 3' 1,500 base pairs of the β -subunit of rat mitochondrial F₁-adenosine triphosphatase (F₁-ATPase). These proteins are of similar size and are mutually immunologically cross-reactive. We present data suggesting that this antigen resides on both the plasma membrane and the inner mitochondrial membrane of the hepatocyte.

Methods

Rat liver cDNA library screening. A rat liver λ gt11 cDNA expression library (Clontech Laboratories, Inc., Palo Alto, CA) was screened for OABP expression by the method of Young and Davis (9). In brief, goat anti-rat OABP antiserum, diluted 1:100 in 50 mM Tris, pH 7.8, 15 mM NaCl containing 3% (wt/vol) bovine serum albumin (BSA) was used to identify OABP-expressing recombinants. Nitrocellulose filters were washed and blocked with 3% (wt/vol) BSA and 0.3% (wt/vol) dry milk in Tris-buffered saline at 4°C. Positive plaques were visualized using rabbit anti-goat IgG horseradish peroxidase conjugate (Sigma Chemical Co., St. Louis, MO) (1:400) followed by incubation with 4-chloro-1-naphthol peroxidase substrate solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) according to the manufacturer's directions. Positive plaques were recovered and purified by rescreening at lower plaque density.

Analysis of clones. Positive plaques were liquid amplified and phage DNA was purified. Insert size was determined after *Eco*RI digestion and agarose gel electrophoresis. Recombinant DNA was digested with *Eco*RI, and the cDNA insert was gel purified and subcloned into the *Eco*RI site of pGEM2 plasmid (Promega Biotech, Madison, WI) for restriction analyses and sequencing.

Restriction endonuclease digestions were performed with *Bam* HI, *Bgl* II, *Eco*RI, *Eco*RV, *Hind* III, *Kpn* I, *Pst* I, *Pvu* II, and *Sph* I

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1. Abbreviations used in this paper: BSP, sulfobromophthalein; OABP, organic anion binding protein.

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(Boehringer Mannheim Diagnostics, Inc., Houston, TX) according to the manufacturer's instructions. For sequence determination, restriction fragments of the OABP cDNA were subcloned into pGEM 3Z. The DNA sequence was determined from double-stranded DNA using SP6 and T7 primers by the dideoxy chain termination procedure using [³²P]dATP or [³⁵S]dATP (Amersham Corp., Arlington Heights, IL) and a DNA sequencing kit (Promega Biotec).

Preparation of recombinant fusion protein in lysogenic bacteria. *Escherichia coli* strain Y1089, infected with the recombinant encoding the protein recognized by goat anti-rat OABP antiserum, was grown overnight in lysogenic bacteria media supplemented with 10 mM CaCl₂ at 32°C. Aliquots of the culture, sufficient to yield 1,000 colonies per dish, were streaked on 150-mm agar plates. Randomly selected colonies were incubated overnight at 32°C or 42°C. Colonies that grew at 32°C but not at 42°C were presumed to be lysogenic.

Lysogenic colonies were grown in Luria-Bertani media at 32°C until OD₂₆₀ was 0.6. Incubation was continued for 20 min at 42°C. Isopropyl β-D-thiogalactopyranoside was added to a final concentration of 4.2 μM and incubation was continued for 1 h at 37°C. Bacteria were harvested by centrifugation at 4,000 g for 5 min in a rotor (model GSA, DuPont-Sorvall, Newtown, CT). The pellet was resuspended in 20 mM sodium phosphate, pH 7.4, then frozen in liquid nitrogen, thawed, sonicated (model W140 Sonifier cell disrupter, Heat Systems-Ultrasonics, Inc., Plainview, NY), and refrozen until used.

To purify the recombinant protein, the thawed pellet was then washed with 2 ml of 1% Triton X-100 (Sigma Chemical Co.) in 0.02 M sodium phosphate buffer (pH 7.4) followed by 2 ml of 1% sodium deoxycholate (United States Biochemical Corp., Cleveland, OH) in 0.2 M sodium phosphate (pH 7.4). The pellets were resuspended in 10% glycerol, 5% mercaptoethanol, 2.3% SDS, 0.0625 M Tris (pH 6.8) (sample buffer), and subjected to 7.5% SDS-PAGE. The recombinant fusion protein band at 160 kD was excised and electroeluted from the gel (10).

Antibody studies. A female New Zealand rabbit (Hazleton Research Products, Inc., Denver, PA) was injected subcutaneously, at 1-mo intervals, with 6–40 μg of electroeluted fusion protein (8). To determine loss of antibody activity after absorption with OABP or fusion protein, 10 ml of 1:1000 dilution of rabbit anti-rat OABP serum or anti-fusion protein serum in 5% BSA, 50 mM Tris, 0.15M NaCl, pH 7.6, was absorbed at 4°C overnight with ~5–10 μg of purified OABP or fusion protein. Residual antibody activity was assessed by immunoblot analysis (8).

Northern blot analysis. Rat liver total, poly A(+), and poly A(–) mRNA was prepared, electrophoresed on 1% agarose, and transferred to Gene Screen (New England Nuclear, Boston, MA) (11). Northern blot analysis was performed after ³²P labeling of the 1,250-base pair cDNA insert by primer extension using [³²P]dCTP (Amersham Corp.) (11).

Culture of isolated rat hepatocytes. Rat hepatocytes were isolated and cultured in monolayer at densities of 1.8 × 10⁶/60-mm dish in modified Waymouth's 752/1 medium containing 5% fetal bovine serum (6, 12). The medium was changed 2 h after plating and cultures were used for studies 16 h after plating.

Surface iodination of short-term cultured rat hepatocytes. Cultured cells were washed twice with ice-cold 20 mM PBS, pH 7.4, and incubated in 1 ml of PBS containing 25 mM glucose, 100 μg of lactoperoxidase (8 U, Sigma Chemical Co.), 50 μg of glucose oxidase (10 U, Calbiochem-Behring Corp., San Diego, CA) and 500 μCi of ¹²⁵I-Na (13–17 mCi/μg I, Amersham Corp.) for 45 min at 4°C. Cells were then washed three times in 2 ml of ice-cold PBS, and a final wash was performed with 2 ml of ice-cold PBS containing KI (1 μg/ml).

Immunoprecipitation. Surface-labeled cells were lysed in 1 ml of buffer containing 0.1% rat serum albumin, 1% Nonidet P40, 1 mM EGTA, 10 mM Tris, pH 7.6, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 150 mM NaCl. Cell lysates were centrifuged for 2 min in an Eppendorf centrifuge (model 5414, Brinkmann Instruments Co., Westbury, NY) and the resulting supernatant was incubated for 30 min at 4°C with constant mixing with 50 μl of 20% Protein A-Sepha-

rose (Sigma Chemical Co.) suspended in PBS. Beads were removed by centrifugation, the supernatant was incubated with 10 μl of nonimmune rabbit serum for 30 min at 4°C, and 50 μl of 20% Protein A-Sepharose was added for 30 min at 4°C. After centrifugation for 2 min at 4°C, the supernatant was incubated with anti-OABP or anti-fusion protein serum (10 μl) for 3 h at 4°C. Antigen-antibody complexes were recovered by adsorption to 50 μl of 20% Protein A-Sepharose for 30 min at 4°C followed by centrifugation for 2 min. Protein A-Sepharose beads were then washed five times with 1 ml of 10 mM Tris, pH 7.2, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS (13) and once with 0.05 M Tris-saline, pH 7.4. The antigen-antibody complexes were removed from the Protein A-Sepharose beads and denatured by boiling in SDS-PAGE sample buffer containing 5% β-mercaptoethanol for 3 min. The protein A-Sepharose beads were removed by centrifugation and the supernatant was subjected to 10% SDS-PAGE. Fixed gels were dried and subjected to radioautography (13).

Photoaffinity labeling of F₁-ATPase with [³⁵S]BSP. [³⁵S]BSP at a specific activity of 3,400 mCi/mmol was prepared by sulfonation of phenoltetrabromophthalein with H₂³⁵SO₄ (14). Purified bovine F₁-ATPase was a gift from Dr. Harvey Penefsky, SUNY Health Science Center, Syracuse, NY). Approximately 23 μg of protein was photoaffinity-labeled with 22 pmol of [³⁵S]BSP in 100 μl of PBS, pH 7.4, as previously described (7). An aliquot of this mixture was subjected to SDS-PAGE and fluorography.

Preparation of liver tissue for immunocytochemistry. Male Sprague-Dawley rats (250–300 g) (Marland Farms, Hewitt, NJ) were anesthetized by ether, and the livers were fixed either by perfusion or immersion at 4°C with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, 7.5% sucrose for a total of 4 h (15). After an overnight rinse in cold 0.1 M phosphate buffer, pH 7.4, 7.5% sucrose, nonfrozen sections ~30 μm thick were prepared with an Oxford Vibratome (Ted Pella, Tustin, CA), using a slow speed and high amplitude setting, and placed in cold 7.5% sucrose. Sections were exposed to rabbit anti-OABP serum at 4°C for 20–40 h, with intermittent, mild agitation. Single sections (~2 mm²) were placed in tissue culture U-shaped multiwell plastic trays (Linbro, Flow Laboratories, Inc., McLean, VA) that contained 0.2 ml of antiserum diluted 1:100 with PBS containing 1% BSA. After several rinses in cold PBS, the sections were treated with Protein A-horseradish peroxidase (0.1 ml/ml) (E. Y. Laboratories, San Mateo, CA) for 1 h at room temperature in the dark. After several rinses in PBS and in 7.5% sucrose, the sections were incubated in 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) at pH 7.6 (15) at room temperature for 10 min in the dark to reveal peroxidase activity, and examined by light microscopy. Some sections were processed for electron microscopy as previously described (15).

Immunogold localization studies. Immunogold-labeling was performed on LR-White (London Resin, Ltd., Hampshire, England) ultrathin sections. Fixation was done by vascular perfusion with 2% paraformaldehyde plus 0.1% glutaraldehyde in PBS (pH 7.4) for 5 min, followed by immersing dissected cubes of liver in PBS at 4°C for 12 h. The tissue was then rinsed in PBS at 4°C overnight, dehydrated in a graded series of ethanol concentrations, and immersed in 100% LR-White; the samples were encapsulated in resin and polymerized by irradiation using a black light source (UV light, 282 nm) at 4°C. Suitable consistency of the blocks was obtained after 3–4 d of polymerization. Ultrathin sections were cut by means of an Ultratome (Reichert Scientific Instruments, Buffalo, NY), mounted on Formavar/Carbon-coated Cu grids, and further processed for immunolabeling. Labeling with the primary antibody was achieved by using a 10-μl droplet of polyclonal anti-OABP (1:50). Affinity-purified goat anti-rabbit IgG coupled to colloidal gold (5–6 nm, Janssen Pharmaceutica, Beerse, Belgium) was used as a second marker.

Results

Identification of OABP-reactive clones from a λ gt11 rat liver expression library. Approximately 2 × 10⁶ plaques produced

from a λ gt11 cDNA rat liver library were screened with goat anti-rat OABP antibody, and six positive plaques were identified. These plaques were purified by three additional cycles of plating and screening with the same antibody. DNA was purified from these phage, digested with *EcoRI*, and subjected to agarose gel electrophoresis. The DNA patterns from the six recombinant DNAs were identical, consisting of two bands of $\sim 1,250$ and 300 base pairs, indicating the presence of an internal *EcoRI* site. The two *EcoRI* insert DNA fragments from one clone were subcloned into pGEM2.

Immunologic characterization of the recombinant protein in *E. coli*. Lysogenic *E. coli* were induced with isopropyl β -D-thiogalactopyranoside to produce the β -galactosidase-OABP fusion protein. This recombinant protein was virtually insoluble in detergent at 4°C, and was purified by preparative 7.5% SDS-PAGE after extraction of the lysate sequentially with 1% Triton X-100 and 1% deoxycholate (Fig. 1, left). Immunoblot analysis of the *E. coli* lysate revealed the presence of an ~ 160 -kD band reacting with both antibody to β -galactosidase and antibody to OABP (Fig. 1, right). Nontransformed *E. coli* lacked this immunoreactivity (Fig. 1, right).

Immunoblot analysis using antibody to the purified recom-

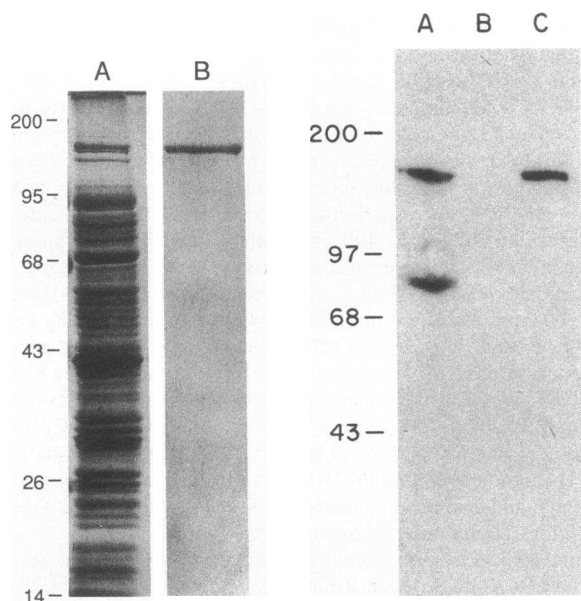


Figure 1. (Left) Expression of recombinant protein in *E. coli*. *E. coli* strain Y1089 was transfected with λ gt11 phage coding for immunoreactive OABP, and expression of the protein as a hybrid with bacterial β -galactosidase was induced as described in Methods. This recombinant protein was virtually insoluble in detergent at 4°C, and was purified by preparative 7.5% SDS-PAGE after extraction of the bacterial lysate sequentially with 1% Triton X-100 and 1% deoxycholate. Lane A, Coomassie-stained SDS-PAGE of the bacterial lysate. Lane B, Coomassie-stained SDS-PAGE of the purified fusion protein. This preparation was used to immunize rabbits. (Right) Immunoblot analysis of the recombinant protein. *E. coli* lysate was subjected to 10% SDS-PAGE, transferred to nitrocellulose, and probed with either antibody to *E. coli* β -galactosidase (lane A) or anti-OABP (lane C). Lane B represents a lysate from nontransformed *E. coli* probed with anti-OABP. These results indicate a single protein with immunoreactivity to β -galactosidase and OABP. The lower band in lane A presumably represents synthesis of an incomplete β -galactosidase protein. Molecular mass markers at left in both panels are in kilodaltons.

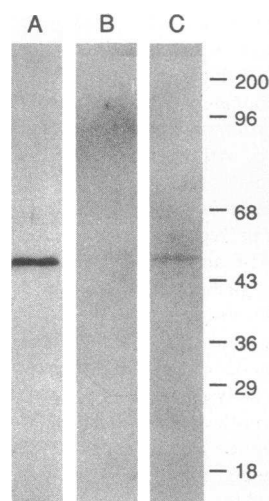


Figure 2. Immunologic identity of OABP and the recombinant protein. Three immunoblots performed against purified OABP are shown. Lane A, reaction of anti-fusion protein antibody with purified rat OABP. Lanes B and C, antibody reactivity was markedly reduced after preabsorption with OABP (lane B) or fusion protein (lane C). Similar results were obtained with the original anti-OABP antibody. Molecular mass markers at right are in kilodaltons.

binant fusion protein revealed a similarly sized 55-kD band with purified OABP (Fig. 2) and rat liver homogenate (data not shown). This immunoreactivity was markedly reduced after preabsorption of the antibody with either OABP or the fusion protein (Fig. 2). Similar results were obtained with the original anti-OABP antibody (data not shown).

Nucleotide sequence and deduced amino acid sequence of OABP cDNA. A partial restriction map of the 1500-base pair λ gt11 cDNA insert is shown in Fig. 3. The five DNA fragments labeled a-e were subcloned into either pGEM-2 (b, e), pGEM-3Z (c, d), or pGEM-3 (a). In addition the two *EcoRI* restriction fragments were subcloned into pGEM-2. These subclones were sequenced using the strategy shown in Fig. 3. Computer analysis of the DNA sequence revealed identity with bases 63-1558 of the published sequence of the β -subunit of rat mitochondrial F_1 -ATPase (16).

Immunological identity of OABP and the β -subunit of mitochondrial F_1 -ATPase. Rabbit antibody raised against the β -subunit of yeast F_1 -ATPase provided by Dr. Michael Douglas (University of Texas, Dallas) was used for immunoblot analy-

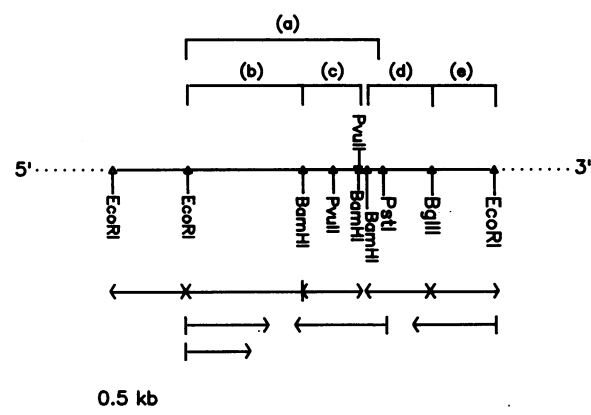


Figure 3. Restriction map and sequencing strategy of OABP cDNA. The five fragments labeled a-e were subcloned and inserted into either pGEM-2 (b, e), pGEM-3Z (c, d), or pGEM-3 (a). In addition, the two *EcoRI* restriction fragments were subcloned into pGEM-2. These subclones were sequenced and used to deduce the nucleotide sequence of the original cDNA using the strategy indicated by the arrows. The direction of the arrowheads indicates the direction in which the fragments were sequenced.

sis of rat liver OABP, bovine heart F₁-ATPase, and rat liver homogenate. A single immunoreactive protein band was detected (Fig. 4). Identical results were obtained when anti-rat liver OABP antibody was used (data not shown).

Northern blot analysis. As seen in Fig. 5, a single mRNA of ~ 1.7 kb hybridized with the 1,250-base pair [³²P]cDNA.

Immunoprecipitation with surface iodinated rat hepatocytes. The cell surface proteins of overnight cultures of rat hepatocytes were selectively radiolabeled with ¹²⁵I using soluble lactoperoxidase/glucose oxidase at 4°C for 45 min. A major band on SDS-PAGE migrating at 55 kD (Fig. 6) was immunoprecipitated with antibody against the recombinant protein. This band was not seen after immunoprecipitation with nonimmune serum or with antibody that had been preabsorbed with recombinant protein (Fig. 6).

Immunocytochemistry studies. To directly visualize antigenic localization, sections of rat liver were examined using immunocytochemistry. Bound antibody was detected by immunoperoxidase staining. As seen in Fig. 7, by light microscopy, anti-OABP antibody reacted with the sinusoidal and basolateral cell surface but not with the canalicular membrane. Diffuse granular cytoplasmic staining, compatible with a mitochondrial subcellular localization, was also seen. Immunoreactivity was not seen with nonimmune serum or with anti-OABP that had been preabsorbed with the recombinant protein. By electron microscopy, reaction product was clearly seen on sinusoidal and basolateral but not canalicular surfaces of hepatocytes (Fig. 8). Visualization of immunoreactivity in mitochondria was difficult. However, using immunogold methodology, antigen was clearly detected within mitochondria (Fig. 9).

Photoaffinity labeling of F₁-ATPase with [³⁵S]BSP. Bovine cardiac F₁-ATPase was photoaffinity-labeled with high-specific-activity [³⁵S]BSP. The labeled material was then subjected to 15% SDS-PAGE and fluorography. These studies revealed that [³⁵S]BSP bound only to the β-subunit (Fig. 10).

Discussion

In previous studies, we found high-affinity binding of [³⁵S]BSP to a sinusoidal surface-derived rat liver cell plasma membrane subfraction (7). Binding was eliminated by preincubation of membrane with trypsin. Photoaffinity labeling of this membrane subfraction with [³⁵S]BSP revealed a predominant 55-kD protein, and this was purified to high degree by BSP-Sepharose affinity chromatography (7). Antibody raised to this OABP reacted with the surface of short-term cultured rat hepatocytes as determined by immunofluorescence (8).

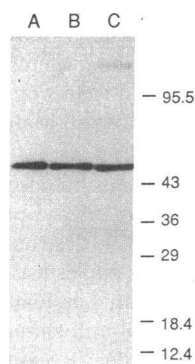


Figure 4. Immunologic cross-reactivity of OABP and the mitochondrial F₁-ATPase β-subunit. Antibody to the β-subunit of yeast F₁-ATPase was provided by Dr. Michael Douglas. It was used to probe by immunoblot OABP (lane A), bovine F₁-ATPase (provided by Dr. Harvey Panefsky, lane B), and rat liver homogenate (lane C). A single band is seen in all three lanes, indicating cross-reactivity between the β-subunit of mitochondrial F₁-ATPase and OABP.

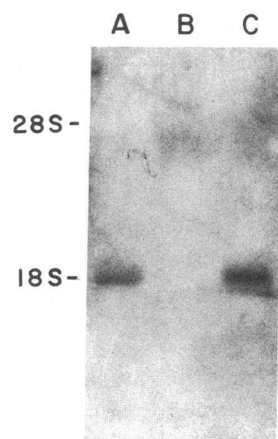


Figure 5. Northern blot analysis of OABP cDNA. Rat liver total (lane A), poly A(-) (lane B), and poly A(+) (lane C) mRNA was electrophoresed on 1% agarose and transferred to Gene Screen. Northern blot analysis was performed after ³²P labeling of the 1,250-base pair cDNA insert by primer extension using [³²P]dCTP. Hybridization was overnight in 50% formamide, 5× SSC, and 2× Denhardt's solution. The blot was washed twice at 65°C with 0.1% SDS in 0.1× SSC. A single, ~ 1.7-kb poly A(+) mRNA hybridized with this probe.

Two other candidate organic anion transport proteins have been identified (17, 18). Their relationship to OABP is not clear. Antibody to the protein isolated by Stremmel and Berk (17) partially inhibited BSP uptake by isolated hepatocytes. However, large amounts of antibody were necessary, and uptake was determined only at high concentrations of BSP in the absence of albumin. It is questionable whether a physiologic, or a higher capacity, lower affinity organic anion transporter was studied (5, 6). Sottocasa et al. (18) reconstituted BSP uptake in liposomes with their candidate transporter. The relationship of this protein to the other organic anion binding proteins and to physiologic organic anion transport is yet to be established. Ultimate determination of function of these proteins may await transfection of transport-deficient cells with specific full-length cDNAs.

In the present study, the antibody to purified OABP was used to clone a cDNA from a rat liver expression library. This cDNA coded for ~ 80% of the apparent molecular weight of mature OABP and was expressed in *E. coli* as a fusion protein with β-galactosidase. Antibody raised to the fusion protein or antibody to native OABP reacted with both proteins as indicated by loss of antibody activity after absorption against either protein. This immunologic cross-reactivity indicates a high degree of homology between the affinity purified OABP and the fusion protein encoded by the cDNA. Computer analysis of the cDNA sequence revealed identity with the published cDNA sequence for the β-subunit of rat mitochondrial F₁-ATPase (16). This F₁-ATPase subunit and OABP were of the same molecular mass as estimated by SDS-PAGE and were immunologically cross-reactive (Fig. 4).

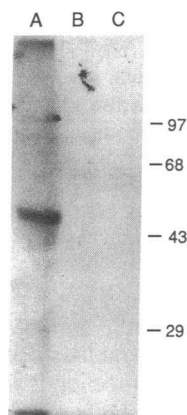


Figure 6. Immunoprecipitation of ¹²⁵I surface-labeled rat hepatocytes. Surface proteins of overnight cultures of rat hepatocytes were radiolabeled with ¹²⁵I using soluble lactoperoxidase/glucose oxidase at 4°C for 45 min. The cell lysate was subjected to immunoprecipitation, and SDS gel electrophoresis was performed. Immunoprecipitation with anti-fusion protein antibody revealed a major 55-kD band. No band was seen after immunoprecipitation with antibody that was preabsorbed with fusion protein (lane B) or with preimmune serum (lane C). Molecular mass markers at right are in kilodaltons.

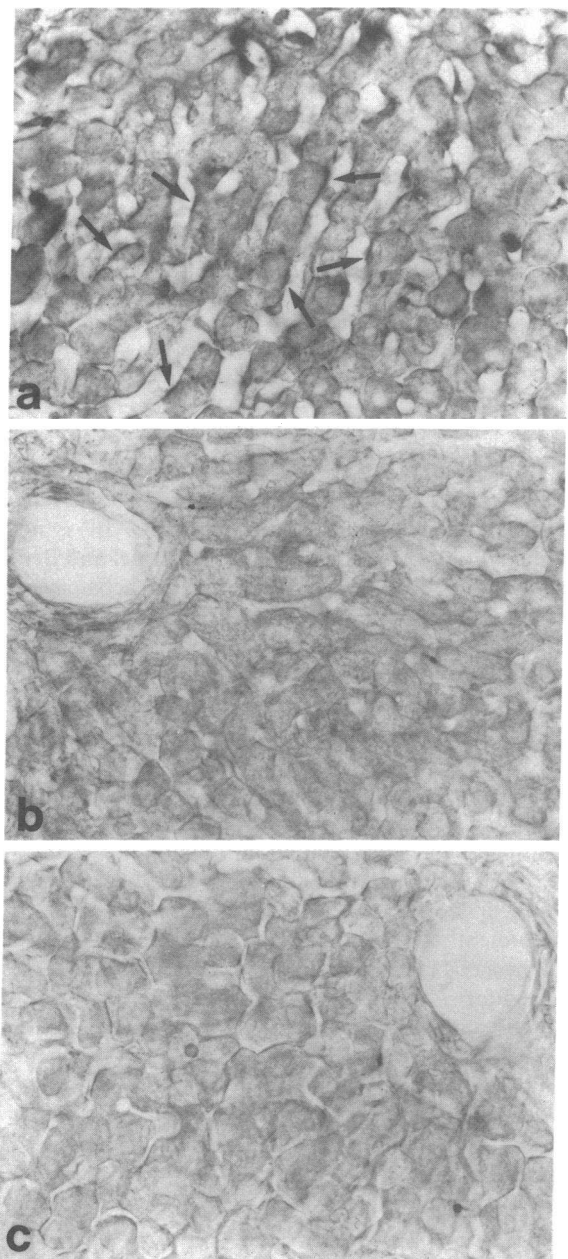


Figure 7. Hepatocyte surface localization of OABP after absorption of antibody with recombinant protein. (a) A section of aldehyde-fixed rat liver was exposed to anti-OABP antisera diluted 1:100, treated with Protein A-peroxidase, and incubated in diaminobenzidine at pH 7.6 to reveal sites of peroxidase activity. OABP antigen was localized only in hepatocytes and their basolateral plasma membrane domains. The apical plasma membrane domains (bile canaliculi) did not demonstrate the antigen. Punctate intracellular reaction product, consistent with mitochondrial localization, was also seen. Arrows indicate the portion of the OABP-positive membrane facing the sinusoids. $\times 400$. (b) Section exposed to anti-OABP antisera absorbed against fusion protein and processed as in a. Essentially little or no OABP antigen localization is evident in hepatocyte basolateral plasma membrane. $\times 400$. (c) Section exposed to nonimmune serum and processed as in a. This control section shows no staining at the hepatocyte basolateral surfaces. $\times 400$.

Although the presence of OABP in sinusoidal rat liver cell subfractions could be due to a small amount of mitochondrial contamination, several lines of evidence suggest both a liver

cell surface and inner mitochondrial localization of this antigen. By ELISA, OABP was ~ 14 -fold enriched in sinusoidal subfractions in which mitochondrial enzyme markers were deenriched (8). Surface iodination of cultured hepatocytes at 4°C and immunoprecipitation using anti-OABP (Fig. 6) resulted in specific recovery of a 55-kD protein (Fig. 6). These results suggesting hepatocyte surface localization of OABP were confirmed by immunocytochemistry at both the light- and electron-microscopic level. In rat liver, surface localization of OABP is seen only in hepatocytes. The widespread tissue homogenate distribution of the antigen reported previously (8), probably derives from its presence in mitochondria.

The β -subunit of F_1 -ATPase, an inner mitochondrial membrane protein, has not been previously described on the plasma membrane. The β -subunit precursor is coded from chromosomal DNA which is thought to be translated on free polysomes (19). The signal for mitochondrial localization of this protein resides at the NH_2 terminus (20). After binding, the precursor protein is translocated into mitochondria by an ATP-dependent process during which the NH_2 -terminal ~ 60 amino acids are cleaved, producing the mature form of the protein (20–22). These events differ from those characterizing the usual synthetic pathway for plasma membrane proteins, involving signal recognition protein-dependent synthesis on membrane-bound polysomes (23–25). However, since the liver cell mitochondrial membrane area is ~ 25 times that of the plasma membrane (26), it is possible that a small yet significant percent of mRNA coding for the β -subunit of F_1 -ATPase is translated via the signal recognition protein-dependent translocation mechanism. Alternatively, there may be heterogeneity in mRNA coding for the signal sequence of this protein at the 5' translated region. Such heterogeneity could arise from a mechanism such as tissue-specific alternate splicing of RNA, or alternative start sites. These possibilities will be the subject of future studies. Although Northern blot analysis revealed a single band upon hybridization of rat liver mRNA with our cDNA, microheterogeneity within this band must be considered. Both the cDNA obtained by us and the cDNA obtained by Garboczi et al. (16) lack the NH_2 -terminal 200–250 nucleotides as judged by comparison with the sequence of the human F_1 -ATPase β -subunit (27).

The presence of identical or closely related proteins in mitochondria and plasma membrane has been suggested in other systems. Berk et al. (28) reported that a rat hepatic plasma membrane, fatty acid binding protein and mitochondrial glutamate-oxaloacetate transaminase located in the mitochondrial matrix, share a number of properties. They found that the sequence of the 24 NH_2 -terminal amino acids of the fatty acid binding protein are identical to the published sequence for mitochondrial glutamate-oxaloacetate transaminase. Monospecific polyclonal antisera raised against each of the proteins cross-reacted with the other protein. Each of the antisera were reported to exhibit "cytoplasmic" (presumably mitochondrial) and plasma membrane immunofluorescence staining.

Cell surface localization of mitochondrial antigens has also been observed in primary biliary cirrhosis, a liver disease of unknown etiology characterized by slowly progressive destruction of biliary epithelium and signs of increasing cholestasis, resulting eventually in profound jaundice and death. Circulating antibodies that react with mitochondria (anti-mitochondrial antibodies) are characteristic of this disorder. Recent

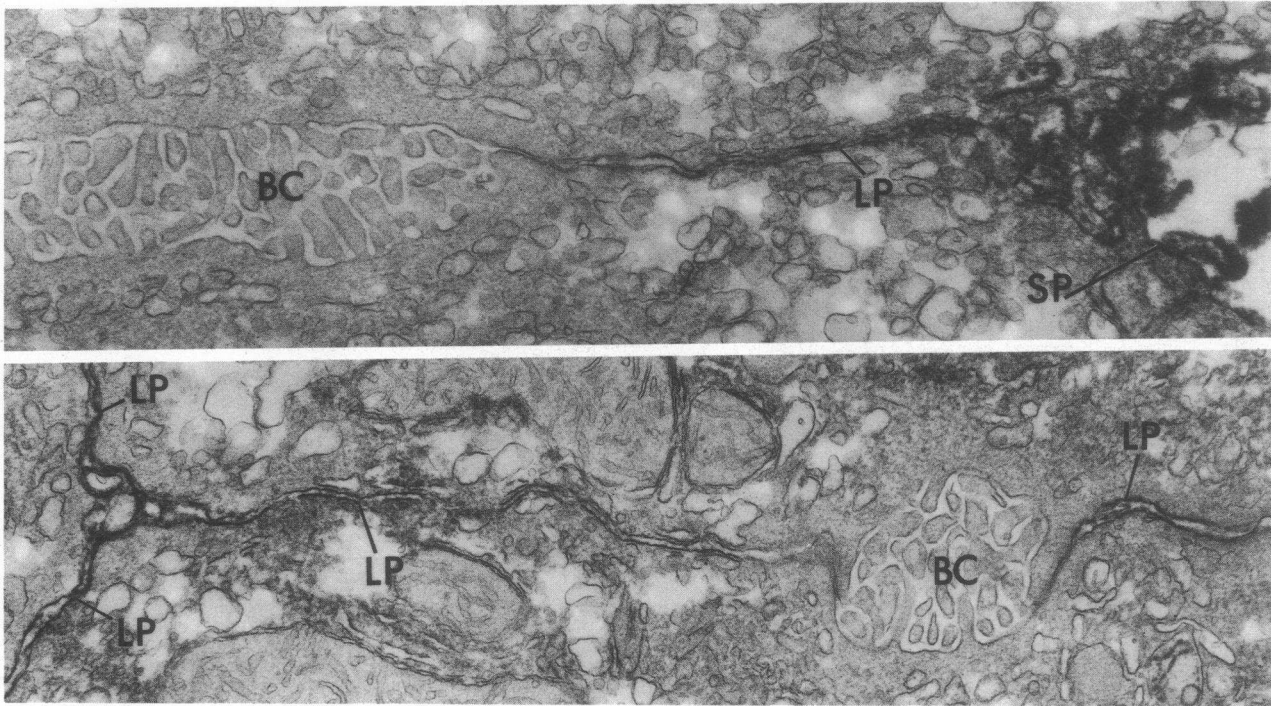


Figure 8. Electron-microscopic immunocytochemical localization of OABP in nonfrozen sections of aldehyde-fixed rat liver. (Top) Reaction product is present at both the sinusoidal (SP) and lateral (LP) hepatocyte plasma membrane but is absent from the bile canaliculus (BC). $\times 44,000$. (Bottom) Reaction product is present only in the lateral plasma membrane (LP). No reaction product is detected in the bile canaliculus (BC). $\times 42,000$.

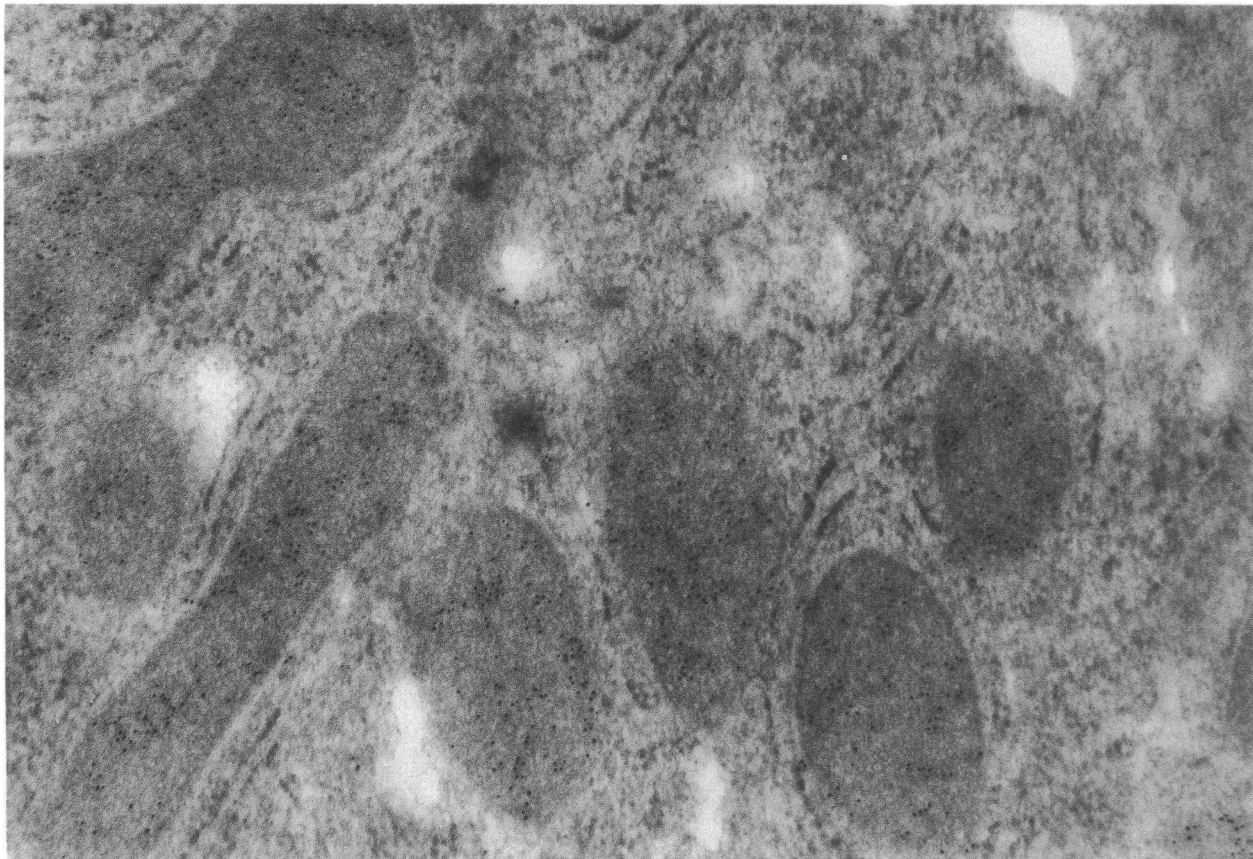


Figure 9. Immunogold localization of OABP in rat liver mitochondria. Ultramicrotome sections of LR-White ultrathin sections of 2% paraformaldehyde/0.1% glutaraldehyde-fixed rat liver were prepared as described in Methods and localization of anti-OABP was identified after incubation in goat anti-rabbit IgG coupled to colloidal gold (5–6 nm). Abundant gold particles are seen within mitochondria ($\times 100,000$).

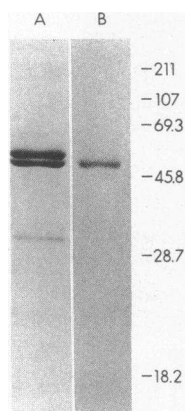


Figure 10. [³⁵S]BSP photoaffinity labeling of bovine F₁-ATPase. Approximately 23 μg of protein was photoaffinity-labeled with 22 pmol of [³⁵S]BSP (3,400 mCi/mmol) as described in Methods. SDS-PAGE on a 15% polyacrylamide gel was performed and stained with Coomassie blue (lane A). Bands corresponding to the α-(upper), β-(middle), and γ-subunits (lower) are seen. Fluorography of this gel was then performed and revealed localization of radioactivity only in the β-subunit (lane B).

studies suggest that the primary biliary cirrhosis specific antigen(s) are in plasma membrane as well as mitochondrial subcellular fractions (29). These studies also demonstrated by immunofluorescence reactivity of these sera with a hepatoma cell surface antigen(s); immunofluorescence was eliminated by preabsorption of antisera with bovine heart mitochondria (29).

The functional significance of these findings is not as yet known. Previous studies that we performed in cultured rat hepatocytes revealed that uptake of bilirubin and BSP required cellular ATP and the presence of Cl⁻ (6). Other studies revealed that mitochondria transport organic anions such as BSP (30, 31), and in high doses, BSP and bilirubin inhibit mitochondrial respiration (32). The [³⁵S]BSP photoaffinity-labeling studies, which were performed in an approximately threefold molar excess of native bovine F₁-ATPase using the molecular mass of 371,135 daltons as determined by Walker et al. (33), indicate that only the β-subunit bound BSP (Fig. 10). The presence of an organic anion binding site on the F₁-ATPase β-subunit suggests that it may play a role in these processes.

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