Immunological Studies of an Organic Anion-binding Protein Isolated from Rat Liver Cell Plasma Membrane

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Abstract

The mechanism of organic anion uptake by hepatocytes has kinetics that suggest facilitated diffusion, and carrier-mediated membrane transport has been postulated. In previous studies, we purified a 55,000-mol wt organic anion-binding protein (OABP) by affinity chromatography on sulfobromophthalein (BSP)-Sepharose of deoxycholate solubilized liver cell plasma membrane preparations. Using specific goat and rabbit antibodies to OABP, we have now investigated the distribution of this protein in liver fractions and other tissues by an enzyme-linked immunosorbent assay and by the immunoblot (Western blot) procedure. These studies indicated that OABP is present in significant amounts in all tissues examined except for blood. Although OABP has not as yet been isolated from each of these tissues and characterized, OABP in heart retained the ability to bind organic anions, and was purified by affinity chromatography on BSP-sepharose. In liver, OABP was membrane bound and remained so after extraction with 0.9 M NaCl, which suggests that it is an intrinsic membrane protein. OABP did not have a ubiquitous subcellular distribution within the hepatocyte. Preparation of subfractions of liver cell plasma membrane revealed that OABP is present in the sinusoidal and absent from the canalicular membrane. Immunofluorescence studies performed in short-term cultured hepatocytes suggest that OABP is associated with the surface of these cells and does not have a significant intracellular distribution.

Introduction

Bilirubin, sulfobromophthalein (BSP),[1] and other organic anions circulate bound to albumin (1, 2). Within the liver, the ligand-albumin complex enters the space of Disse where the ligand interacts with the hepatocyte plasma membrane and enters the cell free of albumin (3, 4). The mechanism for organic anion uptake by hepatocytes has kinetics suggesting facilitated diffusion (5, 6), and a carrier-mediated membrane transport mechanism has been postulated. The nature of the putative organic anion carrier or carriers is not known. In previous studies (7), we purified a 55,000-mol wt organic anion-binding protein (OABP) by affinity chromatography on BSP-Sepharose of deoxycholate solubilized liver cell plasma membrane preparations. We have prepared specific goat and rabbit antibodies to OABP. Using these antibodies, we investigated the tissue distribution of OABP by the immunoblot (Western blot) procedure, and quantitated OABP in tissues and liver fractions by an enzyme-linked immunosorbent assay (ELISA).

Methods

Preparation of OABP. Male Sprague-Dawley rats (200–250 g) were obtained from Marland Farms (Peekskill, NY) and fed ad lib. with derived subfraction of liver cell plasma membrane (LPM) was prepared by discontinuous sucrose gradient centrifugation as previously described (7, 8). The LPM subfraction (1–2 ml) containing 2–3 μg protein/ml was mixed with sodium deoxycholate (0.375% final volume) and allowed to stand on ice for 30 min. After centrifugation for 20 min at 10,000 *g*, the supernate was charged onto a 25-ml GSH-BSP agarose affinity column (7, 9). The column was washed with 190 ml of 20 mM phosphate-buffered saline (PBS) at pH 7.4, and eluted with 0.01 M Tris, pH 8.8 (4°C). Protein content in the eluate was determined by the Biorad dye binding assay (10). Analysis of the final preparation by SDS-polyacrylamide gel electrophoresis (PAGE) (11) and isoelectric focusing in 6 M urea (12) revealed a single band when stained for protein (7).

Preparation of antibody to OABP. Goat and rabbit antibodies were prepared by injecting animals with 0.5 ml of Pertussis vaccine (Eli, Lilly, & Co., Indianapolis, IN), followed 2 h later by multiple subcutaneous injections with 140 μg of OABP in 2 ml homogenized OABP with 3 ml of complete Freunds adjuvant (Sigma Chemical Co., St. Louis, MO). Booster injection of 50 μg OABP in incomplete Freunds adjuvant were given every 4–6 wk. The presence of antibody was assessed by ELISA (see below), and antiserum used in these studies had detectable titers of >1:4000.

Preparation of rat organ homogenates. Rats were anesthetized with ether, and organs removed and immediately placed in ice-cold 20 mM PBS, pH 7.6, containing 400 U/ml aprotinin (Sigma Chemical Co.), 5 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma Chemical Co.). Intestinal mucosal cells were obtained by scraping opened intestine with a glass slide. Organs were homogenized on ice in 8 vol of this buffer using a Polytron type P710/35 (Brinkman Instruments Inc., Westbury, NY) at setting 4 for three 10-s periods. These homogenates were used as antibody absorbent before performing the quantitative ELISA.

In several additional studies, liver membranes were washed in 0.9 M NaCl to remove loosely associated proteins. Liver was homogenized in 8 vol of ice-cold 1 mM $NaHCO_3$, 0.5 mM $CaCl_2$, pH 7.6, by 15 strokes in a loose Dounce homogenizer. After filtration through two layers of cheesecloth, homogenates were centrifuged at 350 *g* for 10 min. The resulting supernatant was centrifuged at 105,000 *g* for 3 h and the pellet resuspended to a total volume four times the original with 2–3 strokes of a loose Dounce homogenizer in 0.9 M NaCl, 20

A preliminary report of this work was presented at the 34th meeting of the American Association for the Study of Liver Diseases, Chicago, IL, and appeared in abstract form in *Hepatology.* 1983. 3:875.

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Received for publication 9 October 1984 and in revised form 12 February 1985.

1. *Abbreviations used in this paper:* BSP, sulfobromophthalein; ELISA, enzyme-linked immunosorbent assay; LPM, liver cell plasma membrane; OABP, organic anion-binding protein; PAGE, polyacrylamide gel electrophoresis.

J. Clin. Invest.

0021-9738/85/08/0454/06 $1.00
Volume 76, August 1985, 454–459

mM Na phosphate, pH 7.4. The suspension remained on ice for 10 min before centrifugation at 105,000 *g* for 60 min. These steps were repeated a second time and the final pellet was resuspended in 20 mM PBS, pH 7.4.

Preparation of liver cell plasma membrane subfractions. Sinusoidal and canalicular-derived plasma membrane subfractions were prepared from rat liver by the rate-zonal centrifugation method of Wisher and Evans (7, 8).

Enzyme-linked immunosorbent assay of OABP. The procedure for the ELISA was modified from that previously described (13). A 0.1-ml aliquot of OABP (1 μg/ml) in 85 mM sodium carbonate, pH 9.6, was added to each well of a 96-well U microtiter plate (Flow Laboratories, Inc., McLean, VA), and incubated at 37°C for 1 h. To each well was added 0.2 ml of 5% BSA (35% solution, Sigma Chemical Co.) in 50 mM PBS, pH 7.2, containing 0.9 mM $MgCl_2$ (buffer A), and incubation was continued at 37°C for an additional hour. Each well was washed six times with buffer A containing 0.5% Tween 20 (Sigma Chemical Co.) and then aspirated to dryness. Goat antiserum diluted 1:800 (vol/vol) in 0.1 ml of buffer A was added to each well (0.1 ml). Some of these dilutions of sera had been preincubated with known amounts of OABP or membranes (prepared as above) overnight at 4°C. The plate was again incubated at 37°C for 1 h, washed six times with buffer A containing 0.5% Tween 20, and aspirated to dryness. Rabbit anti-goat IgG conjugated to horseradish peroxidase (Sigma Chemical Co.), diluted 1:400 (vol/vol) in buffer A was added (0.1 ml) to each well, and the plate was incubated at 37°C for 1 h followed by washing six times in buffer A containing 0.5% Tween 20.

To quantitate IgG-associated horseradish peroxidase remaining in each well, 0.1 ml of a solution containing 0.04% *o*-phenylenediamine (Sigma Chemical Co.) and 0.01% H_2O_2 in 24 mM citrate, 51 mM phosphate, pH 5.0, was added and the plate was incubated at 37°C. At 1 h, 0.1 ml of 1 N NaOH was added to each well, and reaction product was determined in an Artek (Artek Corp., Farmingdale, NY) V-beam computerized ELISA plate reader at 405 nm. In all studies, reaction product resulting from a parallel incubation containing nonimmune serum was subtracted from that resulting with immune serum.

Immunoblot detection of OABP. OABP was detected after SDS-PAGE by the method of Towbin et al. (14). Protein samples boiled for 90 s in 4% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.0625 M Tris, pH 6.8, were resolved on 10% SDS-PAGE. Proteins were transferred to 0.45-μm pore size nitrocellulose paper (Schleicher & Schuell, Inc., Keene, NH) in a Bio-Rad transblot cell (Bio-Rad Laboratories, Richmond, CA) at 300 mA for 16 h. After transfer, the nitrocellulose sheet was soaked for 1 h in 300 ml of 0.35% BSA in 0.15 M NaCl containing 50 mM Tris, pH 7.6 (buffer B), followed by 1 h in 10 ml of goat or rabbit anti-rat OABP or nonimmune serum (1:100 dilution in 3.5% BSA in buffer B). The sheet was next washed for two 30-min periods in 200 ml of 1% Tween-20 in buffer B. All procedures were performed at room temperature.

To detect protein-bound IgG on the nitrocellulose, we used rabbit IgG against goat IgG (Cappel Laboratories Inc., Cochranville, PA) or protein A (used in studies of rabbit anti-rat OABP) (Sigma Chemical Co.), which had been radioiodinated by a chloramine-T procedure (15). The nitrocellulose sheet was incubated for 1 h in 10^6 cpm of ^{125}I-protein in 10 ml of 3.5% BSA in buffer B. The sheet was then washed for 30 min in 700 ml of 1% Tween 20 in buffer B followed by a second 30-min wash in 30 ml of buffer B. The sheet was blotted dry with paper towels and exposed to Kodak XAR-5 X-ray film (Eastman Kodak Co., Rochester, NY) for 2–48 h at −70°C.

Immunofluorescence studies in short-term cultured hepatocytes. Glass coverslips were sterilized in 95% ethanol, flame dryed, and placed individually in 35-mm sterile Lux contur plastic culture dishes (Lux Scientific Inc., Newbury Park, CA). To each dish was added 1 ml of type III calf skin collagen (Sigma Chemical Co.) at 1 mg/ml in 0.5 N acetic acid. The dishes were left uncovered in a laminar flow hood and allowed to dry overnight under ultraviolet light.

Rat hepatocytes were isolated after perfusion with collagenase (Worthington, type I) and suspended in medium consisting of Waymouth's 752/1 (Gibco Laboratories, Grand Island, NY), 25 mM Hepes, pH 7.2, 5% heat inactivated fetal bovine serum (Gibco Laboratories), 1.7 mM additional $CaCl_2$, 5 μg/ml bovine insulin (Sigma Chemical Co.), 100 U/ml penicillin (Gibco Laboratories), and 0.1 mg/ml streptomycin (Gibco Laboratories). Approximately 1×10^6 cells in 1 ml were put into each dish and cultured in 5% CO_2 atmosphere at 37°C. Approximately 2 h into the culture, medium was changed and cells cultured for a total of 16–18 h (16).

After culture, dishes were washed twice with 2 ml of PBS, pH 7.4. Coverslips were carefully removed with a scalpel and placed in clean 30-mm culture dishes. They were then washed twice with 2 ml PBS, pH 7.4, and fixed in acetone at −70°C for 2 min. Coverslips were washed twice with PBS, pH 7.4, at room temperature. 0.2 ml of goat anti-rat OABP or nonimmune goat serum (1:100 dilution in PBS, pH 7.4, containing 0.5% BSA) was placed on each coverslip and incubated for 30 min at room temperature. Coverslips were then washed twice with 2 ml of PBS, pH 7.4. Subsequently, 0.2 ml of rhodamine conjugated rabbit anti-goat IgG (Cappel Laboratories), 1:100 dilution in PBS, pH 7.4, containing 0.5% bovine serum albumin was placed on each coverslip and incubated for 30 min at room temperature. Coverslips were washed twice in PBS, pH 7.4, and mounted on glass slides in Galvatol.

Results

A standard curve for the ELISA of OABP is seen in Fig. 1 and reveals a log linear region from ~2–30 ng of protein. Duplicate determinations were within 5% of each other. The reduction in ELISA reaction product after absorption of the goat anti-OABP with organ homogenates indicated the presence of significant amounts of OABP in all tissues tested (Table I). In all cases, the quantity of homogenate protein was such that the resulting optical density was within the log linear region of the simultaneously performed standard curve. Although homogenates were prepared in the presence of protease inhibitors, it is possible that reduced antibody activity resulted from protease digestion rather than specific absorption of antibody by membrane-bound antigen. For this reason, we directly examined whether these organs contained OABP by the Western blot procedure.

Organ homogenates were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose. As seen in Fig. 2, incubation with rabbit anti-OABP followed by ^{125}I-protein A revealed a single 55,000-mol wt band in all organs. There was no reactivity seen on Western blot analysis of rat red cells

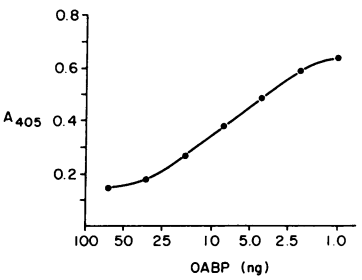

Figure 1. Standard curve for ELISA of OABP. Microtiter plates were coated with OABP (1 μg/ml). Goat anti-OABP (1:800 dilution) was preincubated with known amounts of OABP (abscissa) overnight at 4°C before addition to each well. After a 1-h incubation at 37°C, plates were washed, and rabbit anti-goat IgG conjugated to horseradish peroxidase was added to each well and incubated for an additional hour at 37°C. After washing, horseradish peroxidase that remained in each well was quantitated as the absorption at 405 nm (ordinate) of the reaction product of *o*-phenylenediamine. In all studies, reaction product resulting from a parallel incubation with nonimmune serum was subtracted from that obtained with anti-OABP.

*Table I. Content of OABP in Tissue Homogenates as Determined by ELISA**

Tissue	OABP (% Total protein)
Brain	0.19
Heart	0.90
Lung	0.20
Stomach	0.49
Duodenum	0.24
Jejunum	0.35
Ileum	0.32
Large intestine	0.56
Spleen	0.23
Kidney	0.51
Skeletal muscle	0.48
Liver	0.18

* Anti-OABP (0.36 ml of a 1:800 dilution) was absorbed overnight at 4°C with 4–15 μg of organ homogenate prepared in protease inhibitors (400 U/ml aprotinin, 5 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride). Absorbed antibody was used in an ELISA performed in microtiter plates coated with OABP (1 μg/ml). Results were compared with that obtained with antibody preabsorbed against known amounts of OABP as in Fig. 1.

or rat serum (Fig. 3), or when nonimmune serum was used. Similar results were obtained with the goat antibody.

Heart contained large amounts of immunoreactive OABP. To determine whether the heart protein possessed organic anion-binding activity, BSP-affinity chromatography of deoxycholate solubilized heart homogenate was performed. The eluted protein, subjected to SDS-PAGE followed by immunoblotting on nitrocellulose, migrated to the same position as did OABP in heart homogenate (Fig. 4).

To confirm the presence of OABP on the liver cell plasma membrane, we performed immunofluorescence studies on short-term cultured hepatocytes. As seen in Fig. 5, after anti-OABP incubation, there was surface reactivity that was not seen after incubation with nonimmune serum or absorption of serum against liver or heart homogenates.

Subfractions of rat liver cell plasma membrane were examined to determine whether there was a domain specificity

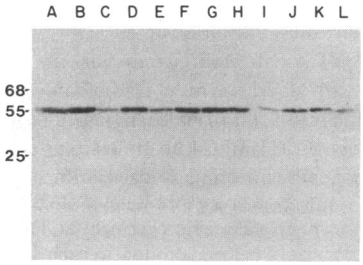

Figure 2. Radioautogram of immunoblot of rat organ homogenates with rabbit anti-rat OABP. Rat organ homogenates (≈40 ug) were subjected to 10% SDS-PAGE in 5% β-mercaptoethanol. Proteins were transferred to nitrocellulose paper which was soaked for 1 h in rabbit anti-rat OABP (1:100 dilution). After washing, the nitrocellulose was incubated with 10^6 cpm of ^{125}I-protein A, washed, and exposed to Kodak XAR-5 X-ray film (Eastman Kodak Co.) for 4 h at −70°C. A parallel experiment using nonimmune rabbit serum revealed no reactivity. The organs studied are in lanes as follows: (*A*) brain; (*B*) heart; (*C*) lung; (*D*) stomach; (*E*) duodenum; (*F*) jejunum; (*G*) ileum; (*H*) colon; (*I*) spleen; (*J*) kidney; (*K*) skeletal muscle; and (*L*) liver. Migration of molecular weight standards (e.g., 68 = 68,000) is indicated at the left.

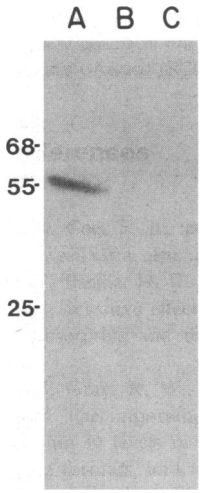

Figure 3. Radioautogram of Immunoblot of rat liver, plasma, and red cells with rabbit anti-rat OABP. Experimental details were as in Fig. 2. Liver homogenate is in lane *A;* plasma and red cells are in *B* and *C,* respectively. Migration of molecular weight standards (e.g., 68 = 68,000) is indicated at the left.

in the distribution of OABP. There was no detectable OABP in the canalicular fraction by ELISA assay and Western blot, despite a 120-fold enrichment in 5′ nucleotidase activity over homogenate levels. OABP in the sinusoidal fraction, previously shown to be enriched in insulin and glucagon receptors (7, 17), was enriched 13.5-fold as compared with homogenate by ELISA. Western blot analysis revealed a single 55,000-mol wt band in this fraction. After high speed centrifugation of liver homogenate and high salt wash of the pellet, >85% of starting OABP remained membrane bound.

Discussion

Although organic anions such as bilirubin and BSP circulate bound to albumin, they are rapidly and efficiently removed from the circulation by hepatocytes (3, 5). These compounds are extracted from albumin (3, 4, 18, 19) and enter the hepatocyte by a process having carrier-mediated kinetics (5, 6). The nature of this putative carrier is not known. Previous investigators suggested that hepatocyte cytosolic OABPs, of

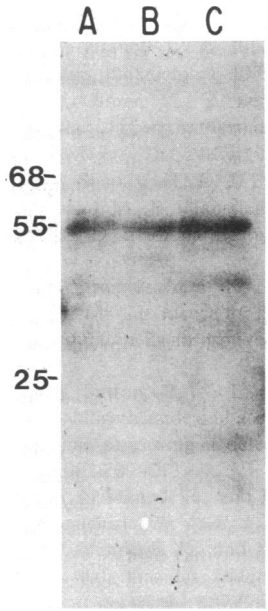

Figure 4. Radioautogram of immunoblot of OABP, rat heart homogenate, and BSP-sepharose purified heart protein with goat anti-rat OABP. OABP (≈1 μg) (lane *A*), heart homogenate (≈40 μg) (*B*), and BSP-sepharose purified heart protein (≈1 μg) (*C*) were subjected to 10% SDS-PAGE in 5% β-mercaptoethanol. Proteins were transferred to nitrocellulose paper that was soaked for 1 h in goat anti-rat OABP (1:100 dilution). After washing, the nitrocellulose was incubated in 10^6 cpm of ^{125}I-rabbit anti-goat IgG. The sheet was washed and exposed to Kodak XAR-5 X-ray film (Eastman Kodak Co.) for 12 h at −70°C. A parallel experiment using nonimmune goat serum revealed no reactivity. Migration of molecular weight standards (e.g., 68 = 68,000) is indicated at the left.

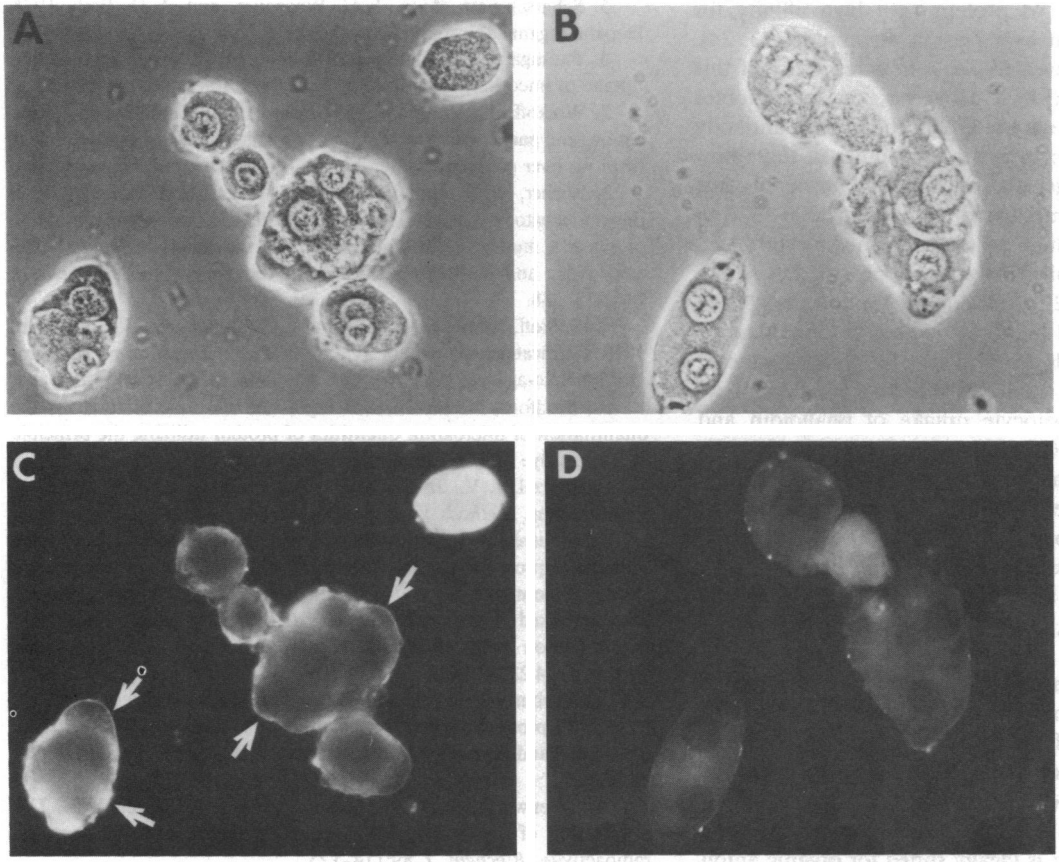

Figure 5. Immunofluorescence of goat anti-rat OABP in short term cultured hepatocytes. Rat hepatocytes were isolated after collagenase perfusion and cultured on collagen-coated coverslips for 24 h. After a 2-min fixation in acetone at −70°C, coverslips were incubated with goat anti-rat OABP (A-phase micrograph, C-fluorescence micrograph) or nonimmune goat serum (B-phase, D-fluorescence), followed by rhodamine conjugated rabbit anti-goat IgG. As indicated by the arrows in *C*, surface reactivity is seen in cells incubated with anti-OABP but not with nonimmune serum.

which ligandin is the most abundant, mediated uptake of organic anions (20). However, that influx of organic anions results from interaction with the cell surface was suggested from studies performed in isolated perfused rat liver in which altered ligandin concentration had no influence on influx of bilirubin (19). Ligandin content correlated inversely with efflux of bilirubin that had entered cells. Thus, ligandin influences only net uptake (the difference of influx and efflux) of bilirubin by reducing efflux of ligand from the cell.

To account for the rapid influx of organic anions into hepatocytes, an OABP on the liver cell plasma membrane was postulated (7). Photoaffinity activation of BSP in the presence of a sinusoidal liver plasma membrane fraction revealed specific binding to a 55,000-mol wt protein (7). This OABP was purified from deoxycholate solubilized membrane by BSP-Sepharose affinity chromatography (7, 9). Using specific antisera to OABP, we have now studied its tissue distribution.

The ELISA used in these studies takes advantage of the fact that OABP adsorbs readily onto plastic. Its advantage over a radioimmunoassay is that OABP loses antigenicity even with mild radioiodination procedures. Quantitation of OABP in tissues was performed after absorption of antisera against tissue homogenates that contained protease inhibitors. Loss of antibody activity could be due to factors other than interaction with antigen, such as retained protease activity or nonspecific absorption of antibody to membrane. For this reason, immunoblotting was used to confirm the presence of OABP in all tissues that were investigated by ELISA.

These studies indicate that OABP is present in significant amounts in all tissues examined except for blood. Although OABP has not as yet been isolated from each of these tissues and characterized, OABP in heart retains the ability to bind organic anions, and was purified by affinity chromatography on BSP-sepharose. In liver, the protein is membrane bound, and remains so after extraction with 0.9 M NaCl, which suggests that OABP is an integral membrane protein. OABP does not have a ubiquitous subcellular distribution within the hepatocyte. Preparation of subfractions of liver cell plasma membrane reveal that OABP is present in the sinusoidal and absent from the canalicular membranes. The immunofluorescence studies performed in short-term cultured hepatocytes suggest that OABP is associated with the surface of these cells and does not have a significant intracellular distribution, although this technique may not be sensitive enough to reveal small amounts of OABP in intracellular locations.

Several other groups have identified liver cell surface binding proteins for organic anions. Lunazzi and colleagues (21) have purified a protein from an acetone powder of rat liver. This protein, which has been named bilitranslocase, is comprised of 37,000- and 35,500-mol wt subunits (21), and has been reported as mediating BSP transport into liposomes in which it has been incorporated (22). Berk et al. reported

isolation of a 55,000-mol wt protein with high affinity for bilirubin and BSP from rat liver plasma membrane (23, 24). Preliminary studies (25) revealed weak cross-reactivity of this protein and OABP on ELISA. However, on immunoblot analysis, there was no reactivity of OABP to the Berk antibody (unpublished data). Further comparative studies of these proteins will be necessary to elucidate structural and immunological similarities. Liver cell membrane proteins of 54,000 mol wt that bind bile acids with high affinity and mediate bile acid transport have been described by von Dippe and Levy (26–28) and by Ziegler et al. (29). Although it has been suggested that the organic anion and bile acid binding proteins may be related (29), there is no direct evidence for this hypothesis. Recent observations have indicated that the 54,000-mol wt protein also mediates hepatocyte uptake of phalloidin and antaminide but not BSP (30, 31). Also suggesting that the 54,000-mol wt bile acid transport protein differs from OABP is the finding that it is absent from small intestine, as defined by photoaffinity methodology (32). The immunological distribution of this protein in tissues is not as yet available.

Although these studies were not designed to elucidate the possible function of OABP, the widespread distribution of this protein raises doubt as to whether it plays a unique role in hepatic organic anion transport. Failure of other organs to transport organic anions could be due to internal rather than cell surface localization of OABP; recent light microscopic immunocytochemical studies have revealed that this is not the case (33). However, specificity of the liver for organic anion transport may be due to factors in addition to requisite transport proteins. The liver is ideally suited for organic anion removal. Many of these compounds are avidly bound to albumin in the circulation, and only in the liver do sinusoidal fenestrations permit protein-bound ligand to interact directly with the plasma membrane (19, 34, 35). Thus, even if other organs have an identical transport system for organic anions, the liver may extract them preferentially. In liver dysfunction, other organs, such as kidney, may take up these molecules. Definition of the function of OABP requires additional investigation. The presence of OABP in short-term cultured hepatocytes suggests that this may be an ideal system in which to conduct such studies.

Acknowledgments

These studies were supported by National Institutes of Health grants AM-23026, AM-17702, AM-32419, and AM-32972.

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