Bile salt excretion in skate liver is mediated by a functional analog of Bsep/Spgp, the bile salt export pump

NAZZARENO BALLATORI,1 JAMES F. REBBEO,1 GREGORY C. CONNOLLY,1 DAVID J. SEWARD,2 BENJAMIN E. LENTH,3 JOHN H. HENSON,4 PAZHANI SUNDARAM,5 AND JAMES L. BOYER5

1Department of Environmental Medicine, University of Rochester School of Medicine, Rochester, New York 14642; 2Williams College, Williamstown, Massachusetts 01267; 3Wesleyan University, Middletown, Connecticut 06459; 4Department of Biology, Dickinson College, Carlisle, Pennsylvania 17013; 5Liver Center, Department of Medicine, Yale University School of Medicine, New Haven, Connecticut 06520; and Mount Desert Island Biological Laboratory, Salsbury Cove, Maine 04672

Bile salt excretion in skate liver is mediated by a functional analog of Bsep/Spgp, the bile salt export pump. Am. J. Physiol. Gastrointest. Liver Physiol. 278: G57–G63, 2000.—Biliary secretion of bile salts in mammals is mediated in part by the liver-specific ATP-dependent canalicular bile salt transporter Bsep/Spgp, a member of the ATP-binding cassette superfamily. We examined whether a similar transport activity exists in the liver of the evolutionarily primitive marine fish Raja erinacea, the little skate, which synthesizes mainly sulfated bile alcohols rather than bile salts. Western blot analysis of skate liver plasma membranes using antiserum raised against rat liver Bsep/Spgp demonstrated a dominant protein band with an apparent molecular mass of 210 kDa, a size larger than that in rat liver canalicular membranes, ~160 kDa. Immunofluorescent localization with anti-Bsep/Spgp in isolated, polarized skate hepatocyte clusters revealed positive staining of the bile canaliculi, consistent with its selective apical localization in mammalian liver. Functional characterization of putative ATP-dependent canalicular bile salt transport activity was assessed in skate liver plasma membrane vesicles, with [3H]taurocholate as the substrate. [3H]taurocholate uptake into the vesicles was mediated by ATP-dependent and -independent mechanisms. The ATP-dependent component was saturable, with a Michaelis-Menten constant (Km) of 4.0 ± 7 µM and a Vmax for ATP of 0.6 ± 0.1 mM, and was competitively inhibited by scymnol sulfate (inhibition constant of 23 µM), the major bile salt in skate bile. ATP-dependent uptake of taurocholate into vesicles was inhibited by known substrates and inhibitors of Bsep/Spgp, including other bile salts and bile salt derivatives, but not by inhibitors of the multidrug resistance protein-1 or the canalicular multidrug resistance-associated protein, indicating a distinct transport mechanism. These findings provide functional and structural evidence for a Bsep/Spgp-like protein in the canalicular membrane of the skate liver. This transporter is expressed early in vertebrate evolution and transports both bile salts and bile alcohols.

HEPATIC BILE SECRETION IS initiated at the level of the bile canaliculus by continuous vectorial secretion of bile salts and other solutes across this apical membrane domain. In mammals, bile salts are the major solutes secreted into bile, whereas sulfated bile alcohols predominate in many species of fish, including sharks, skates, and rays [elasmobranchs (10, 27)].

The molecular mechanism by which bile salts are transported across the canalicular membrane into bile has remained elusive until recently (8). Earlier studies had demonstrated that canalicular transport of bile salts is mediated by an ATP-dependent system that appears relatively selective for bile salts and structurally related compounds (1, 14, 16, 25, 28), but the molecular identity was unknown. Gerloff and co-workers (8) recently demonstrated that the “sister of P-glycoprotein,” Spgp, functions as a canalicular bile salt export pump in mammalian liver and is now designated as Bsep/Spgp. Bsep/Spgp is a member of the ATP-binding cassette (ABC) superfamily of membrane transporters and is expressed predominantly in the liver (5). Moreover, Bsep/Spgp is selectively localized to the canalicular membrane of hepatocytes, indicating an important role in biliary secretion (8). Gerloff and co-workers (8) expressed Bsep/Spgp in Xenopus laevis oocytes and SF9 cells and demonstrated that it transports bile salts with high affinity in an ATP-dependent manner. The substrate specificity of Bsep/Spgp is relatively narrow compared with the broad specificity of some ABC transporters and appears restricted to primary bile salts, in agreement with studies in rat liver canalicular membrane vesicles. However, overexpression of Bsep/Spgp also confers resistance to Taxol (6), suggesting perhaps a broader specificity. A defect in the human BSEP/ SPGP gene has recently been suggested to be the basis for one form of progressive familial intrahepatic cholestasis (26). A yeast bile salt transporter, Bat1p (18), has also been described that displays similar features to Bsep/Spgp: Bat1p is an ABC protein with a comparable substrate specificity, although its sequence homology with Bsep/Spgp is relatively low (23% identity).

The present study examined the mechanism for canalicular bile salt transport in an evolutionarily
primitive marine vertebrate, the little skate. As noted above, skates secrete mainly bile alcohols rather than bile acids; thus it is of interest to examine whether a similar canalicular transport mechanism is present in this marine organism. Our results demonstrate the presence of an ATP-dependent process for transport of bile salts and sulfated bile alcohols and indicate that this transport activity is mediated by an analog of rat liver Bsep/Spgp.

**EXPERIMENTAL PROCEDURES**

Animals. Skate hepatocytes and liver plasma membranes were isolated from male skates (Raja erinacea; 0.7–1.2 kg body wt), which were obtained by trawl from waters in the Gulf of Maine and transported to the Mount Desert Island Biological Laboratory by boat. At the laboratory, skates were maintained in large tanks filled with aerated flowing seawater for up to 4 days before use. Rat liver canalicular membranes were isolated from male Sprague-Dawley rats (220–260 g) obtained from Charles River Laboratories (Kingston, NY). Rats were fed ad libitum until time of experimentation.

Materials. A polyclonal antibody to Bsep/Spgp was provided by Dr. Bruno Stieger, Zurich, Switzerland. The antibody was raised in rabbits against the COOH-terminal 13 amino acids of rat liver Bsep/Spgp (8). [3H]taurocholic acid was obtained from DuPont NEN (Boston, MA). Scyymnol sulfate was provided by Dr. Gert Fricker, Heidelberg, Germany. It was purified from skate bile as described by Karlaginis et al. (10). N-7-(4-nitrobenzo-2-oxa-1,3-diazol)-7b-amino-3a,12a-dihydroxy-5β-cholan-24-yl-3a,7a-dimethoxy-8-oxo-4-endo-(nor)-5β-cholanic acid (NBD-TC), a fluorescent bile salt derivative, was provided by Dr. David S. Miller, National Institute of Environmental Health Sciences. NBD-TC (19, 20) and S-dinitrophenyl glutathione [DNP-SG; (3)] were synthesized as described. All other chemicals were obtained from either J. T. Baker (Phillipsburg, N.J.) or Sigma (St. Louis, MO).

Isolation of skate and rat liver plasma membrane vesicles. A mixed preparation of skate liver canalicular and sinusoidal plasma membranes was isolated by a modification of the method of Song et al. (24), as previously characterized in our laboratory (21). Briefly, three skate livers (45–60 g) were removed from skates anesthetized with pentobarbital sodium (5 mg/kg) administered via caudal vein, perfused with 40 ml phosphate-buffered saline solution (pH 7.4), and chilled on ice. Livers were minced with scissors in 100 ml of the elastomeric medium Tris-mineral oil mixture (210 mM sucrose, 10 mM HEPES-Tris, pH 7.5, 20 mM KCl) and homogenized in a glass Dounce homogenizer (loose-fitting pestle, type A) with 20 strokes. Portions of the homogenate were transferred to a 40-ml Dounce homogenizer and homogenized further with 10 strokes of a loose-fitting pestle. The homogenates were diluted to 690 ml with the branch Ringer buffer and homogenized in a glass Dounce homogenizer (loose-fitting pestle, type A) with 10 strokes. Portions of the homogenate were then centrifuged for 60 min at 70,000 g. The supernatant was collected and centrifuged in a Sorvall GSA rotor at 4,000 g for 10 min. The supernatant was collected and centrifuged in a Sorvall SS-34 rotor for 20 min at 10,000 g. The soft outer white pellets were collected, leaving darker adherent pigment material, and combined in a precooled 40-ml Dounce and homogenized with 10 strokes of a type B pestle. Homogenates (3.5 ml) were layered over a discontinuous sucrose gradient made of 15 ml of 45%, 8 ml of 32%, and 8 ml of 16% sucrose. Gradients were then centrifuged for 60 min at 70,000 g in a Beckman SW-28 rotor. The banded material at the 16%/32% interface was collected and pooled, diluted with 150 ml of transport buffer containing 10 mM HEPES-Tris, pH 7.4, 20 mM KCl, 0.1 mM CaCl₂, and 250 mM sucrose, and centrifuged in Sorvall SS-34 rotor for 10 min at 20,000 g. Supernatant was removed, pelleted plasma membranes were resuspended with transport buffer and passed through a 25-gauge needle 20 times, and aliquots were stored at −80°C. Rat liver canalicular membranes were isolated from male Sprague-Dawley rats, as previously described (12).

Vesicle transport assays. Uptake of [3H]taurocholate was measured by rapid filtration on Millipore 0.45-µm filter under vacuum, essentially as described previously (2). Skate liver plasma membrane vesicles were thawed by immersion in a 20°C water bath and diluted in transport buffer (10 mM HEPES-Tris, pH 7.5, 250 mM sucrose, 20 mM KCl, with an ATP-regenerating system consisting of 10 mM phosphocreatine, 10 mM MgCl₂, 100 mg/ml creatine phosphokinase, and either 5 mM Na₂ATP or 10 mM NaCl). Diluted vesicles were passed repeatedly through a 26-gauge needle (10 times) and incubated at 20°C for 15 min before starting the transport reaction. Transport was started by adding 20 µl of diluted plasma membrane vesicles to 80 µl of incubation transport buffer (with substrate) at 4°C or 20°C for timed intervals. Transport was quenched by adding 1 ml of ice-cold stop buffer (300 mM sucrose, 10 mM HEPES-Tris, pH 7.5, and 20 mM KCl), and vesicles were collected by applying 1 ml of quenched reaction solution to prewetted filter under vacuum and washing filter with an additional 4 ml of ice-cold stop buffer. Filters were collected and dissolved in 5 ml of Opti-Fluor (Packard Instrument, Meriden, CT), and radioactivity was quantitated by liquid scintillation measurement.
ments. Controls for nonspecific binding of substrate to filters and vesicles were performed by measuring retention of radiolabeled substrate on filters in the absence of vesicles or on vesicles incubated in transport buffer at 4°C for each time point.

Electrophoresis and immunoblotting. Skate liver plasma membranes and rat liver canalicular membranes were added to an equal volume of sample loading buffer (50 mM Tris·HCl, pH 6.8, 2% SDS, 0.1 mM dithiothreitol, and 10% glycerol) and subjected to SDS-PAGE on 7.5% gels. The separated polypeptides were electrotransferred to Immobilon P membranes (Millipore) for 2 h at 60 V. The membranes were blocked overnight with 10% nonfat milk and then incubated for 1 h at room temperature with the rabbit polyclonal antibody (1:10,000) raised against the rat Bsep/Spgp (8). The blot was washed and incubated with anti-rabbit IgG-horseradish peroxidase conjugate (1:3,000; Sigma) followed by detection of the immunoreactive bands by enhanced chemiluminescence according to the manufacturer’s instructions (Amersham).

Isolation of skate hepatocyte clusters. Skate hepatocytes were isolated as previously described (23). Immediately after isolation, cells were resuspended in elasmobranch Ringer solution (containing in mM: 270 NaCl, 4 KCl, 2.5 CaCl₂, 3 MgCl₂, 0.5 Na₂SO₄, 1 KH₂PO₄, 8 NaHCO₃, 350 urea, 5 D-glucose, and 5 HEPES-Tris, pH 7.5) and centrifuged at 250 g. The washed hepatocytes were resuspended at a concentration of 30–50 mg wet wt/ml (~3–5 × 10⁶ cells/ml) in elasmobranch Ringer and preincubated at 15°C in 50-ml polypropylene tubes for 20–30 min before the start of each experiment.

Immunofluorescent localization. Isolated skate hepatocytes were allowed to settle onto glass coverslips coated with 1 mg/ml poly-L-lysine and then fixed for 30 min in −20°C methanol. The cells were rehydrated in PBS, blocked with 1% BSA and 2% goat serum in PBS, incubated with a 1:50 dilution of a rabbit antiserum against rat liver Bsep/Spgp (8), and incubated with a rhodamine-conjugated goat anti-rabbit IgG secondary antibody. For double labeling of actin and Bsep/Spgp, cells were labeled with anti-Bsep/Spgp and a mouse monoclonal anti-actin antibody (clone C-4 from ICN), followed by rhodamine-conjugated goat anti-rabbit IgG (for Bsep/Spgp) and fluorescein-conjugated sheep anti-mouse IgG (for actin) secondary antibodies. Labeled cells were observed using a ×40 (1.0 numerical aperture) objective lens on a Olympus Fluoview confocal laser scanning microscope.

Statistical analysis. Kinetic data from experiments measuring uptake of radiolabeled substrate in membrane vesicles were fit to the Michaelis-Menten equation by nonlinear least-squares regression analysis using SigmaPlot 4.16. Maximal velocity (Vmax) and Michaelis-Menten constant (Km) values with standard errors were derived from these curves, and inhibition constant (Ki) values were calculated using the equation Ki = [I]/(Km(1 + [I]/Ki)), where [I] is inhibitor concentration and Km and Ki are constants in the presence and absence of the inhibitor, respectively. Comparison of data measuring initial rates of uptake of [³H]taurocholate in the presence and absence of inhibitors were performed by unpaired Student's t-test and correlated to P < 0.05.
RESULTS

Rat liver Bsep/Spgp antibody recognizes a ∼210-kDa protein in skate liver membrane vesicles and selectively binds to the canicular membrane of skate hepatocytes. The presence of a Bsep/Spgp analog in skate liver was examined using a polyclonal antibody to the rat liver protein. Western blot analysis indicated a predominant band in skate liver membrane vesicles that displayed an apparent molecular mass of ∼210 kDa (Fig. 1). This predicted molecular mass is somewhat larger than that seen in rat liver canicular membranes, ∼160 kDa (Fig. 1). No signal was detected in skate brain or gallbladder samples (data not shown).

Immunolocalization of Bsep/Spgp in isolated, polarized clusters of skate hepatocytes demonstrated clear labeling of the apical membranes of the hepatocytes, the bile canaliculi (Fig. 2, A and B). The bile canicular staining pattern was corroborated by double labeling of the hepatocytes for Bsep/Spgp and actin (Fig. 2, C and D); the latter is known to localize near the apical membrane. The staining for Bsep/Spgp codistributes with actin in the pericanalicular regions.

ATP-dependent uptake of [3H]taurocholate in skate liver plasma membrane vesicles. Taurocholate uptake into skate liver plasma membrane vesicles was markedly stimulated by the inclusion of ATP in the medium (Fig. 3). Uptake was not stimulated by a nonhydrolyzable analog of ATP (adenylylimidodiphosphate) and was inhibited by vanadate (Table 1), indicating that ATP hydrolysis is required. Gramicidin D had no effect (Table 1), demonstrating that the ATP effect is not secondary to an induced electrical or proton gradient.

In this mixed population of skate liver membrane vesicles (sinusoidal and canicular), the ATP-sensitive component represented ∼75% of the total taurocholate uptake (Fig. 3). Uptake was roughly a linear function of time for the first 30–45 min and reached steady-state values after 2 h of incubation. On the basis of the taurocholate uptake values illustrated in Fig. 3 and an intravesicular water space of ∼1 µl/mg of vesicle protein (21), the concentration of taurocholate inside the vesicles was calculated to be significantly higher than the concentration in the medium [i.e., 120 µM = (120 pmol/mg)/(1 µl/mg)], vs. an extravesicular concentration of only 1 µM. Although there are a number of assumptions in this calculation, the results are consistent with ATP-dependent pumping against a concentration gradient.

Table 1. Effects of substrates and inhibitors of ABC transporters on ATP-dependent uptake of 10 µM [3H]taurocholate in skate liver plasma membrane vesicles

<table>
<thead>
<tr>
<th>Compound</th>
<th>Taurocholate Uptake, pmol/mg protein -1 ·30 min -1</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgATP (5 mM)</td>
<td>267 ± 12</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>MgAMP-PNP (5 mM)</td>
<td>17 ± 5</td>
<td>7</td>
</tr>
<tr>
<td>Gramicidin D (0.01 mM)</td>
<td>230 ± 6</td>
<td>87</td>
</tr>
<tr>
<td>Vanadate (0.1 mM)</td>
<td>120 ± 7</td>
<td>45</td>
</tr>
<tr>
<td>DIDS (0.5 mM)</td>
<td>25 ± 8</td>
<td>9</td>
</tr>
<tr>
<td>BSP (0.1 mM)</td>
<td>131 ± 15</td>
<td>49</td>
</tr>
<tr>
<td>GSH (10 mM)</td>
<td>254 ± 13</td>
<td>95</td>
</tr>
<tr>
<td>DNP-SG (0.1 mM)</td>
<td>264 ± 5</td>
<td>99</td>
</tr>
<tr>
<td>GSSG (0.1 mM)</td>
<td>250 ± 8</td>
<td>94</td>
</tr>
<tr>
<td>LTC4 (0.0007 mM)</td>
<td>261 ± 6</td>
<td>98</td>
</tr>
<tr>
<td>PAH (0.1 mM)</td>
<td>238 ± 8</td>
<td>89</td>
</tr>
<tr>
<td>Verapamil (0.01 mM)</td>
<td>208 ± 1</td>
<td>78</td>
</tr>
<tr>
<td>Vinristine (0.01 mM)</td>
<td>141 ± 5</td>
<td>53</td>
</tr>
<tr>
<td>Scymnol sulfate (0.1 mM)</td>
<td>108 ± 3</td>
<td>38</td>
</tr>
<tr>
<td>Cholic acid (0.1 mM)</td>
<td>172 ± 7</td>
<td>65</td>
</tr>
<tr>
<td>Taurocholate (0.1 mM)</td>
<td>140 ± 7</td>
<td>52</td>
</tr>
<tr>
<td>NBD-TC (0.1 mM)</td>
<td>117 ± 9</td>
<td>44</td>
</tr>
<tr>
<td>Glycholate (0.1 mM)</td>
<td>166 ± 7</td>
<td>62</td>
</tr>
<tr>
<td>Ursodeoxycholate (0.1 mM)</td>
<td>199 ± 9</td>
<td>75</td>
</tr>
<tr>
<td>Taurodeoxycholate (0.1 mM)</td>
<td>104 ± 6</td>
<td>39</td>
</tr>
<tr>
<td>Glycocholate (0.1 mM)</td>
<td>105 ± 2</td>
<td>39</td>
</tr>
<tr>
<td>Lithocholate (0.1 mM)</td>
<td>41 ± 4</td>
<td>15</td>
</tr>
<tr>
<td>Taurolithocholate (0.1 mM)</td>
<td>226 ± 9</td>
<td>85</td>
</tr>
<tr>
<td>Glycolithocholate (0.1 mM)</td>
<td>224 ± 10</td>
<td>84</td>
</tr>
</tbody>
</table>

Values are means ± SE of triplicate measurements. Plasma membrane vesicles (100 µg) isolated from the little skate Raja erinacea liver were incubated for 30 min at 20°C in the presence and absence of 5 mM MgATP in a buffer containing an ATP-regenerating system. The difference was the initial rate of ATP-dependent [3H]taurocholate uptake. MgATP-PNP was substituted for MgATP to establish the transport requirement for ATP hydrolysis. AMP-PNP, adenylylimidodiphosphate; BSP, sulfobromophthalein; DNP-SG, (2,4-dinitrophenyl)glutathione; LTC4, leukotriene C4; PAH, p-aminobenzoic acid; GSH, glutathione; GSSG, glutathione disulfide; NBD-TC, nitrobenzoxadiazole-taurocholate.

Scymnol sulfate, the major bile salt of the skate, is a competitive inhibitor of ATP-dependent [3H]taurocholate transport. Scymnol sulfate was an excellent inhibitor of taurocholate uptake (Fig. 5A). The inhibition was competitive in nature, with a K, of 23 µM (Fig. 5B),...
suggesting that this endogenous bile alcohol is a high-affinity substrate of the ATP-dependent bile salt transport system. ATP-dependent [3H]taurocholate uptake was also inhibited by other primary bile acids and bile acid derivatives (Table 1). Cholate and glycocholate, 0.1 mM, decreased uptake to ~65% of control, whereas NBD-TC, taurodeoxycholate, and glycodeoxycholate decreased uptake to ~40% of control. Interestingly, lithocholate was a powerful cIs inhibitor, whereas taurolithocholate and glycolithocholate had only small effects (Table 1).

In contrast to the bile salts, ATP-dependent [3H]taurocholate uptake was not as sensitive to inhibitors of canalicular multidrug resistance-associated protein (Mrp2) or multidrug resistance-associated protein 1 (Mdr1) (Table 1). The glutathione S-conjugates DNP-SG (0.1 mM), leukotriene C4 (0.7 µM), and glutathione disulfide (0.1 mM) had no effect. Glutathione (10 mM) and p-aminophenylamine (0.1 mM) also had no effect. Verapamil (0.01 mM) and vincristine (0.1 mM), two P-glycoprotein substrates, decreased uptake to 78% and 53% of control, respectively. The organic anions sulfobromophthalein (BSP; 0.1 mM) and DIDS (0.5 mM) were effective inhibitors, supporting previous studies in rat liver canalicular membrane vesicles that reported inhibi-
bition of ATP-dependent taurocholate transport by BSP (16, 25) and DIDS (25, 28).

**DISCUSSION**

Marine elasmobranchs selectively remove $[^3H]$taurocholate from plasma and efficiently excrete this bile salt into bile, generating bile-to-plasma concentration ratios of nearly 1,000 to 1 (4). Studies in isolated skate hepatocytes indicate that hepatic taurocholate uptake mechanisms are sodium independent, saturable, and inhibited by structurally related compounds, indicating the presence of carrier-mediated uptake systems (7, 13, 22). Unconjugated bile salts inhibit this transport process more effectively than conjugated bile salts, in contrast to findings in mammalian liver cells (11, 15, 17). Injection of skate liver mRNA into Xenopus laevis oocytes also results in the expression of sodium-independent taurocholate uptake (9). Thus there is ample evidence that the livers of lower vertebrates have evolved specific transport proteins for mediating bile salt uptake and excretion, although the molecular basis of these transporters remains to be determined.

Unlike mammalian livers, the major bile salt in elasmobranch bile is scymnol sulfate, a sulfated bile alcohol that is the major bile salt in skate bile, is a substrate for the ATP-dependent system. Competitive inhibition of ATP-dependent bile salt transport by scymnol sulfate in isolated plasma membrane vesicles (Fig. 5) strongly suggests that these substrates share the same transport mechanism for excretion into bile. Scymnol sulfate and its bile alcohol precursor chtriol (3α,7α,12β-trihydroxy-5β-cholestan-26(27)-sulfate) (10, 27). The concentration of scymnol sulfate in skate gallbladder bile is ~25 mM, compared with only 0.1 mM for cholic acid (10). Uncharged bile alcohols inhibit bile salt uptake in skate hepatocytes by noncompetitive mechanisms, whereas taurocholate and scymnol sulfate are competitive inhibitors of uptake (7), suggesting that these anions share a similar transport protein for entry into the hepatocytes.

The present studies extend these observations on the hepatic transport of bile salts and scymnol sulfate to the canalicular excretory step. Our results demonstrate that $[^3H]$taurocholate transport by a mixed population of skate liver plasma membrane vesicles is mediated by both ATP-dependent and -independent mechanisms. The ATP-independent component was not characterized in the present study but is probably mediated by proteins responsible for sodium-independent sinusoidal bile salt uptake in skate liver (7, 13, 22). The ATP-dependent component was examined further, and its properties were found to be similar to those measured in rat liver canalicular membrane vesicles (1, 14, 16, 25, 28). Taurocholate uptake into skate liver membrane vesicles was not enhanced by a nonhydrolyzable ATP analog and was inhibited by vanadate (Table 1), indicating that ATP hydrolysis is required for transport. The apparent affinity for taurocholate ($K_m$ of 40 µM) is within the range reported for rat liver canalicular membranes ($K_m$ of 2–47 µM), and the substrate specificity and inhibitor profile appear to be comparable.

Our results also indicate that scymnol sulfate, a sulfated bile alcohol that is the major bile salt in skate bile, is a substrate for the ATP-dependent system. Competitive inhibition of ATP-dependent bile salt transport by scymnol sulfate in isolated plasma membrane vesicles (Fig. 5) strongly suggests that these substrates share the same transport mechanism for excretion into bile.

Additional studies were therefore carried out to test whether skate liver has a protein that is similar to the bile salt exporting protein of mammalian liver, namely Bsep/Spgp. Western blot analysis using antibodies to rat liver Bsep/Spgp indicated a predominant band of ~210 kDa (Fig. 1), which is somewhat larger than that seen in rat liver canalicular membranes, ~160 kDa. Immunolocalization of Bsep/Spgp in skate hepatocytes demonstrates selective labeling of the bile canaliculi (Fig. 2, A and B), supporting previous findings in mammalian liver (8).

Together, these studies indicate that a Bsep/Spgp-like protein with specificity for bile alcohol and bile salt excretion has evolved early in vertebrate evolution. The structural basis for this transport system and its similarity to or differences from mammalian Bsep/Spgp await its molecular cloning.

We thank Bruno Steiger and Peter J. Meier for kindly providing the Bsep/Spgp antibody, Gert Fricker for the scymnol sulfate, and David S. Miller for the NBD-TC.

This work was supported in part by National Institutes of Health Grants DK-48823, DK-25636, ES-06484, and DK-34989, by Technology Training Program Grant ES-07026, and by Grant ES-03828 to the Center for Membrane Toxicity Studies at the Mount Desert Island Biological Laboratory.

Address for reprint requests and other correspondence: N. Ballatori, Dept. of Environmental Medicine, Box EHSC, Univ. of Rochester School of Medicine, Rochester, NY 14642 (E-mail: ballatorin@envmed.rochester.edu).

Received 11 March 1999; accepted in final form 9 September 1999.

**REFERENCES**


