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

NAZZARENO BALLATORI, JAMES F. REBBEO, GREGORY C. CONNOLLY, DAVID J. SEWARD, BENJAMIN E. LENTH, JOHN H. HENSON, PAZHANI SUNDARAM, AND JAMES L. BOYER

Department of Environmental Medicine, University of Rochester School Medicine, Rochester, New York 14642; Williams College, Williamstown, Massachusetts 01267; Wesleyan University, Middletown, Connecticut 06459; Department of Biology, Dickinson College, Carlisle, Pennsylvania 17013; Liver 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 membrane protein 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 mamalian 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) for taurocholate of 40 ± 7 μM and a Km for ATP of 0.6 ± 0.1 mM, and was competitively inhibited by scymln 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 canalicus 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). The molecular mechanism by which bile salts are transported across the canalicular membrane into bile has remained elusive until recently. 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, but the molecular identity was unknown. Gerloff and co-workers 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. Moreover, Bsep/Spgp is selectively localized to the canalicular membrane of hepatocytes, indicating an important role in biliary secretion. Gerloff and co-workers 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, 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. A yeast bile salt transporter, Bat1p, 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 provided by Dr. Gert Fricker, Heidelberg, Germany. [3H]taurocholate was synthesized as described by Karlaganis et al. (10). N-7-[4-nitrobenzo-2-oxa-1,3-diazol]-7b-amino-3a,12a-dihydroxy-5b-cholan-24-oyl-2-aminoethanesulfonate taurocholate (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-dinitrophospho-glutathione [DNP-SG; (3)] were synthesized as described. All other chemicals were obtained from either J. T. Baker (Phillipsburg, NJ) 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 minced with scissors in 100 ml of the same elasmobranch Ringer solution (pH 7.4), and chilled on ice. Livers were homogenized further with 10 strokes of a loose-fitting pestle. The homogenates were transferred to a 40-ml Dounce homogenizer and homogenized in a glass Dounce (Millipore) for 2 ha t6 0V . Membranes were isolated from male Sprague-Dawley rats, as previously described (12).

**Uptake studies.** 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 membranes 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 phosphate, 10 mM MgCl₂, 100 μg/ml creatine phosphokinase, and either 5 mM Na₃ATP or 10 mM NaCl). Diluted vesicles were washed and incubated at 20°C for 15 min before starting the transport reaction. Transport was started by adding 20 μl of diluted plasma membranes 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 a 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 radiolabeled drug uptake was quantitated by liquid scintillation measure-
ments. Controls for nonspecific binding of substrate to filters and vesicles were performed by measuring retention of radiolabeled substrate 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 an 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 $K_i = [I]/(K_m/K_{m0} - 1)$, where [I] is inhibitor concentration and $K_{m0}$ and $K_m$ are $K_m$ 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 membranes and selectively binds to the canalicular 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 membranes that displayed an apparent molecular mass of ~210 kDa (Fig. 1). This predicted molecular mass is somewhat larger than that seen in rat liver canalicular 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 canaluli (Fig. 2, A and B). The bile canalicular 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 the inclusion of ATP in the medium (Fig. 3). In this mixed population of skate liver membrane vesicles (sinusoidal and canalicular), 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.

The ATP-dependent component of uptake was characterized further and was found to be saturable both in terms of taurocholate (Fig. 4A) and ATP (Fig. 4B). The apparent K_m for taurocholate was 40 ± 7 µM, and the V_max was 2.0 ± 0.1 nmol·mg^−1·30 min^−1; the apparent K_m for ATP was 0.6 ± 0.1 mM.

Scyminol sulfate, the major bile salt of the skate, is a competitive inhibitor of ATP-dependent [3H]taurocholate transport. Scyminol sulfate was an excellent inhibitor of taurocholate uptake (Fig. 5A). The inhibition was competitive in nature, with a K_i of 23 µM (Fig. 5B),

| 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 |
|-----------------|-----------------|-----------------|
| Compound        | Taurocholate    | Percent         |
|                 | Uptake, pmol·mg | protein·30 min^−1|       |
| MgATP (5 mM)    | 267 ± 12        | 100 ± 4         |
| MgAMP-PNP (5 mM)| 17 ± 5          | 7               |
| Gramicidin D (0.01 mM) | 230 ± 6        | 87              |
| Verapamil (0.01 mM) | 208 ± 1      | 78              |
| Vincristine (0.01 mM) | 141 ± 5      | 53              |
| Scyminol sulfate (0.1 mM) | 106 ± 3    | 38              |
| Cholic acid (0.1 mM) | 173 ± 7       | 65              |
| Taurocholate (0.1 mM) | 140 ± 7      | 52              |
| NBD-TC (0.1 mM) | 117 ± 9        | 44              |
| Glycocholate (0.1 mM) | 166 ± 6     | 62              |
| Ursodeoxycholate (0.1 mM) | 199 ± 9    | 75              |
| Taurodeoxycholate (0.1 mM) | 104 ± 6   | 39              |
| Glycodeoxycholate (0.1 mM) | 105 ± 2   | 39              |
| Lithocholate (0.1 mM) | 41 ± 4        | 15              |
| Taurolithocholate (0.1 mM) | 226 ± 9   | 85              |
| Glycolithocholate (0.1 mM) | 224 ± 10  | 84              |

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 taurocholate uptake. MgAMP-PNP was substituted for MgATP to establish the transport requirement for ATP hydrolysis. AMP-PNP, adenylylimidodiphosphate; BSP, sulfobromophthalein; DNP-SG, (2,4-dinitrophenyl)glutathione; LTC_4, leukotriene C_4; PAH, p-aminohippuric acid; GSH, glutathione; GSSG, glutathionedisulfide; NBD-TC, nitrobenzoxadiazole-taurocholate.
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-aminohippurate (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 inhi-

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**Fig. 4.** Concentration and ATP dependence of initial rates of taurocholate uptake in skate liver plasma membrane vesicles. Vesicles were incubated in a buffer containing an ATP-regenerating system and either varying concentrations of [3H]taurocholate with 5 mM ATP or 10 mM NaCl at 20°C for 0 and 30 min (A) or 10 µM [3H]taurocholate with increasing concentrations of ATP or NaCl (B). Difference between taurocholate uptake with ATP and with NaCl was plotted for each concentration of taurocholate, and data were fit according to the Michaelis-Menten equation. Each data point represents mean ± SE of at least 3 experiments, each performed in duplicate. Data were analyzed by a Woolf-Augustinsson-Hofstee plot.

**Fig. 5.** Scymnol sulfate (SS) competitively inhibits ATP-dependent taurocholate uptake in skate liver plasma membrane vesicles. A: ATP-dependent taurocholate uptake was measured in vesicles incubated in a buffer containing an ATP-regenerating system in the presence of no added scymnol sulfate or with 32, 56, or 100 µM scymnol sulfate. Each data point represents mean ± SE of at least 3 experiments, each performed in duplicate. B: data were analyzed by a Woolf-Augustinsson-Hofstee plot. v/s, Velocity (pmol·mg⁻¹·30 min⁻¹) per substrate concentration (µM).
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 (3α, 7α, 12α, 24α, 26, 27-hexahydroxy-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 (Km of 40 µM) is within the range reported for rat liver canalicular membranes (Km 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. Scymnol sulfate and its bile alcohol precursor chriol (3α, 7α, 12α-trihydroxy-5β-cholestan-3β) both stimulate bile flow in the isolated perfused skate liver and undergo a highly efficient enterohepatic circulation when injected into the duodenum of free-swimming skates (7). The functional similarity in ATP-dependent taurocholate transport between rat and skate liver membrane vesicles indicates that an evolutionarily conserved protein may be mediating this excretory step.

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.

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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).

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