ATP-dependent Transport of Taurocholate across the Hepatocyte Canalicular Membrane Mediated by a 110-kDa Glycoprotein Binding ATP and Bile Salt

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Direct photoaffinity labeling of liver plasma membrane fractions enriched in sinusoidal and canalicular membranes using [35S]adenosine 5'-O-(thiotriphosphate) ([35S]ATP[S]) allows the identification of ATP-binding proteins in these domains. Comparative photoaffinity labeling with [35S]ATP[S] and with the photolabile bile salt derivative (7,7-azo-3,12a-dihydroxy-6β-[35S]-H]-cholano-24-oyl-2'-aminoethanesulfonate) in the ATP-dependent precipitation with a monoclonal antibody (Be 9.2) revealed the identity of the ATP-binding and bile salt-binding canalicular membrane glycoprotein with the apparent Mr of 110,000 (gp110). The isoelectric point of this glycoprotein was 3.7.

Transport of bile salt was studied in vesicles enriched in canalicular and sinusoidal liver membranes. Incubation of canalicular membrane vesicles with [3H]taurocholate in the presence of ATP resulted in an uptake of the bile salt into the vesicles which was sensitive to vanadate. ATP-dependent taurocholate transport was also observed in membrane vesicles from mutant rats deficient in the ATP-dependent transport of cysteiny1 leukotrienes and related amphiphilic anions. Substrates of the P-glycoprotein (gp170), as verapamil and doxorubicin, did not interfere with the ATP-dependent transport of taurocholate.

Reconstitution of purified gp110 into liposomes resulted in an ATP-dependent uptake of [3H]taurocholate. These results demonstrate that gp110 functions as a carrier for the ATP-dependent transport of bile salts from the hepatocyte into bile. This export carrier is distinct from hitherto characterized ATP-dependent transport systems.

Liver parenchymal cells represent polarized epithelial cells that exhibit efficient transport of a wide variety of endogenous and exogenous substances from blood into bile (2-5). Only part of these vectorially transported compounds originates from blood, while others are either metabolically transformed or synthesized within the hepatocytes. Biliary excretion is thus an important step in the detoxication of endogenous and exogenous compounds. The canalicular domain of the plasma membrane of hepatocytes exhibits highly specialized transport functions that can mediate active canalicular excretion of a variety of structurally unrelated molecules. Recently two ATP-dependent primary-active transport systems were kinetically characterized using canalicular membrane vesicles from rat liver: the export system for hydrophobic, mostly basic compounds (P-glycoprotein) (6, 7) and the transport system for cysteiny1 leukotrienes (8) and structurally related amphiphilic anions including glutathione S-conjugates (8-10). There is good evidence that P-glycoprotein (gp170),1 one of the multidrug resistance gene products (11, 12), functions as a "multidrug export carrier" in the hepatocyte canalicular membrane (6, 7). The molecular properties of the transport system for cysteiny1 leukotrienes and related amphiphilic anions remain to be elucidated. A powerful method for identification of unknown binding sites of interest is photoaffinity labeling using an appropriate ligand (13, 14). In most instances the ligand must be modified in order to obtain photolabile properties. For identification of nucleotide binding sites azido derivatives of the radioactively labeled nucleotides are useful (13, 14). Direct photoaffinity labeling using unmodified nucleotides was previously used to characterize binding sites of isolated enzymes (15-19). For identification of ATP-binding proteins in hepatocyte plasma membranes we used direct photoaffinity labeling with the ATP derivative [35S]ATP[S], which is rather resistant to hydrolysis of the γ-thio phosphate residue. This approach has the advantage of a more selective labeling of ATP-binding proteins than ADP- or AMP-binding proteins, which will be labeled using [α-32P]ATP because of high activities of nucleotide hydrolases like Ca2+/Mg2+-dependent ATPase and nucleotide pyrophosphatase in the plasma membrane (4, 7).

This study demonstrates that the method of direct photoaffinity labeling with [35S]ATP[S] allows the identification of different ATP-binding proteins in plasma membrane subfractions. Some of the ATP-binding proteins in the canalicular membrane may function as part of ATP-dependent transport systems. Direct evidence is presented for an ATP-dependent export carrier for taurocholate in the hepatocyte canalicular membrane, which differs from hitherto identified primary-

1 The abbreviations used are: gp170, P-glycoprotein (1); [35S] ATP[S], adenosine-5’-(γ-[35S]thiotriphosphate; [3H]ATC, (7,7-azo-3,12a-dihydroxy-6β-[3H]-cholano-24-oyl-2'-aminoethanesulfonate; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propane sulfonate; EGT, [ethylendehisoxoyetheneminitriilo]lustraceta; IEF, isoelectric focusing; mAab, monoclonal antibody; PAGE, polyacryl-
active transport systems in this membrane domain. These findings are of considerable importance for the understanding of the hepatobiliary transport phenomenon of bile salts, since canalicular secretion represents the rate-determining step in the vectorial transport of taurocholate.

**EXPERIMENTAL PROCEDURES**

**Materials**—ATP, ADP, AMP, GTP, UTP, CTP, creatinine phosphate, phosphomonoesterase, Triton X-100, creatine kinase, peptide N-glycosidase F, and the glycan detection kit were obtained from Boehringer Mannheim (Mannheim, Federal Republic of Germany). Octyl glucoside (octyl-β-D-glucopyranoside) and 3-10, and the low molecular weight electrophoresis calibration kit were purchased from Pharmacia LKB (Freiburg, F. R. G.). Leupeptin, benzamidine, Servalyte 3-5, Servalyte 5-7, and Servalyte 8-10 were from Serva (Heidelberg, F. R. G.). Biotinyl-L-Serum Albumin and the anolyte was 25 mM NaOH, and the anolyte was 25 mM NaOH. Focusing was performed in a vertical tube format using 3.5% acrylamide, 0.095% bisacrylamide, 2% CHAPS, 0.4% Servalyte 3-5, 0.4% Servalyte 5-7, 0.8% Servalyte 8-10, and 100 mM dithiothreitol, 100 mM octyl glucoside (pH 7.4) to a final volume of 150 µl. After addition of 120 µl of [3H]taurocholate to the protein solution, the mixture was incubated for 15 min on ice under argon and then centrifuged at 100,000 × g for 30 min at 4 °C. The clear supernatant (200 µl) was subjected to gel filtration (23) using a Sephadex G-50 superfine column (1 × 35 cm) and eluted with 10 mM Tris, 250 mM sucrose, 100 mM octyl glucoside (pH 7.4) to remove octyl glucoside. Fractions (2.5 ml) containing canalicular vesicles were pooled and concentrated using a Centricon-10 centrifugal microconcentrator (Amicon Corp., Beverly, MA) at 3,000 × g. The final volume of the proteoliposome suspension used for transport studies was 200 µl (250 µg of protein).

**Preparation of Canalicular ATP-dependent Bile Salt Transporters**—Eighty µl of phosphatidylcholine in chloroform (25 mg/ml) was evaporated under vacuum. The lipid was dissolved in 600 µl of Tris/sucrose/octyl glucoside (10 mM Tris, 250 mM sucrose, 100 mM octyl glucoside; pH 7.4) by ultrasonication under argon. Canalicular plasma membranes (0.4 mg of protein) were solubilized in 120 µl of Tris/sucrose/octyl glucoside under stirring on ice. After addition of 120 µl of [3H]taurocholate to the protein solution, the mixture was incubated for 15 min on ice under argon and then centrifuged at 100,000 × g for 30 min at 4 °C. The clear supernatant (200 µl) was subjected to gel filtration (23) using a Sephadex G-50 superfine column (1 × 35 cm) and eluted with 10 mM Tris, 250 mM sucrose, 100 mM octyl glucoside (pH 7.4) to remove octyl glucoside. Fractions (2.5 ml) containing phosphatidylcholine vesicles were pooled and concentrated using a Centricon-10 centrifugal microconcentrator (Amicon Corp., Beverly, MA) at 3,000 × g. The final volume of the proteoliposome suspension used for transport studies was 200 µl (250 µg of protein).

**Isolation of the 112 kDa Glycoprotein and Its Functional Reconstitution into Proteoliposomes**—gp110 was isolated as described in detail (24, 26, 27) from Triton X-100-solubilized rat liver plasma membranes by immunoaffinity chromatography using the mAb Be2 (24–27) cross-linked to protein A-Sepharose. The glycoprotein was at least 95% pure on SDS-PAGE as detected by silver staining (27).

**Isoelectric Focusing (IEF) and Two-dimensional Electrophoresis**—The labeled membrane pellets (400 µg of protein) were washed in 1 M NaCl, 10 mM Tris buffer (pH 7.4) and then solubilized in 100 µl of lysis buffer containing 9.2 M urea, 2% CHAPS, 100 mM dithiothreitol, and 2% Pharmalyte 3-10 by sonication at 4 °C. Homogenates were centrifuged for 50 min at 100,000 × g. The composition of the gel for IEF in glass tubes was 3.5% acrylamide, 0.095% biacrylamide, 2% CHAPS, 0.4% Servalyte 3-5, 0.4% Servalyte 5-7, 0.8% Servalyte 8-10, 0.4% Pharmalyte 3-10, and 9.2 M urea. The catholyte was 50 mM NaOH, and the anolyte was 25 mM H3PO4. Focusing was performed without prefocusng of the gels at 2 mA/tube and constant voltage of 1500 for a total of 10 kV/h. For determination of the pH values the gels were cut into 5-mm pieces. Intact IEF gels were used for the separation in the second dimension by SDS-PAGE using a 4.5% stacking gel, a 8-15% resolving gel, and the buffer system of Laemmli (28) with 1% agarose.

**RESULTS**

**ATP-binding Proteins in Plasma Membrane Subfractions from Rat Liver**—Fig. 1A shows the autoradiographic analysis of the direct photoaffinity labeling of plasma membrane subfractions enriched in canalicular or sinusoidal membranes
ATP-dependent Transport of Taurocholate in Liver

Fig. 1. ATP-binding proteins in liver membrane subfractions enriched in sinusoidal or canalicular membranes. A, membrane subfractions (200 µg of protein) were incubated with 1.85 MBq of [35S]ATPγS (0.2 µM), photoaffinity-labeled, and subjected to SDS-PAGE (8–20% acrylamide gradient) as described under "Experimental Procedures." The lanes on the left show the protein stains (Coomassie Brilliant Blue R-250); the lanes on the right present the autoradiographic analysis of the dried gel. CM and SM correspond to canalicular membranes and sinusoidal membranes, respectively. B, canalicular membrane subfractions (200 µg of protein) were incubated with 3.7 MBq of [35S]ATPγS (1 µM). The samples were photoaffinity-labeled, subjected to SDS-PAGE, and autoradiographically analyzed as described under "Experimental Procedures." TR' corresponds to normal Wistar rat and TR to transport mutant rat. Arrows indicate the position of gp110.

Using [35S]ATPγS (0.2 µM). A marked difference was observed in the labeling pattern of both preparations, suggesting a domain-specific localization of ATP-binding proteins. In canalicular membrane subfractions the highest incorporation of radioactivity was found in proteins with apparent M₁ values of 130,000, 110,000, and 69,000. This labeling pattern differed from the one in subfractions enriched in sinusoidal membranes, where proteins with lower apparent M₁ values (24,000, 20,000, 16,000) were predominantly labeled. At higher concentration of [35S]ATPγS (up to 1 mM) a similar labeling pattern was obtained (Fig. 1B). Canalicular membrane subfractions prepared from livers of mutant rats (21), which lack the ATP-dependent transport of cysteinyl leukotrienes and related amphiphilic anions (8, 9), also showed the labeling of proteins in the M₁ range of 110,000 at low and high concentrations of [35S]ATPγS (Fig. 1B). In all photoaffinity labeling experiments with [35S]ATPγS the Mg²⁺ concentration was 10 mM since the extent of incorporation of radioactivity into proteins was dependent on Mg²⁺ at this optimal concentration. This indicates that the presence of a Mg²⁺-ATP complex is a prerequisite for ATP binding and labeling of the proteins.

Two-dimensional Electrophoresis and Identification of the Canalicular ATP-binding gp110 as the Bile Salt-binding Glycoprotein—Further characterization of ATP-binding membrane proteins in canalicular membranes was performed by two-dimensional electrophoresis (IEF followed by SDS-PAGE), which indicated at least 25 different ATP-binding proteins (Fig. 2). Table I summarizes the isoelectric points and apparent molecular weights of the autoradiographically detected proteins; actin and tubulin were assigned by comparison with reference data (29, 30). At least seven proteins in the M₁ range between 100,000 and 120,000 bind [35S]ATPγS. One of these was detected by Western blotting as well as by immunoprecipitation with the mAb Be 9.2 (Figs. 3 and 4). mAb Be 9.2 is directed against the transformation-sensitive glycoprotein gp110 of the canalicular plasma membrane of rat hepatocytes (24–27). gp110 had an isoelectric point of 3.7 (Figs. 2 and 4) and was degraded by digestion with peptide N-glycosidase F to its peptide moiety with an apparent M₁ of 48,000 (27).

Fig. 2. Separation of ATP-binding proteins in the canalicular membrane by two-dimensional electrophoresis. Membrane subfractions (400 µg of protein) were incubated with 3.7 MBq of [35S]ATPγS (0.4 µM), photoaffinity-labeled, and subjected to IEF and SDS-PAGE (8–15% acrylamide gradient) as described under "Experimental Procedures." The arrow indicates the position of gp110.

Table I
ATP-binding proteins in liver subfractions enriched in canalicular membranes identified by autoradiography after two-dimensional electrophoresis

<table>
<thead>
<tr>
<th>M₁ x 10⁻³</th>
<th>Isoelectric point</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>130</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>125</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td>115</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>115</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>115</td>
<td>4.6–5.5</td>
<td></td>
</tr>
<tr>
<td>110</td>
<td>3.7</td>
<td>gp110</td>
</tr>
<tr>
<td>100</td>
<td>4.2</td>
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<td>105</td>
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<td>68</td>
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<td></td>
</tr>
<tr>
<td>67</td>
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</tr>
<tr>
<td>67</td>
<td>5.5–6.0</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>4.6</td>
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<td>43</td>
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<td>43</td>
<td>5.0</td>
<td>Actin (30)</td>
</tr>
<tr>
<td>35</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>5.2</td>
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<td>32</td>
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<tr>
<td>24</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>4.2</td>
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</table>

In order to compare the molecular properties of this ATP-binding glycoprotein gp110 and the previously characterized bile salt-binding protein with a similar M₁ value (31–33) we used the photolabile bile salt derivative [3H]ATC and [35S]ATPγS for comparative photoaffinity labeling with membrane subfractions enriched in canalicular membranes. The photoaffinity-labeled gp110 was analyzed by SDS-PAGE (Fig. 3) as well as by IEF (Fig. 4) subsequent to immunoprecipitation with the mAb Be 9.2. The ATP-binding and the bile salt-binding proteins were identical with respect to M₁ and isoelectric point.

ATP-dependent Transport of Taurocholate into Canalicular Membrane Vesicles—In the presence of 1 mM ATP a substantial stimulation of taurocholate uptake was observed (Fig. 5). The rate of ATP-stimulated taurocholate uptake by vesicles enriched in canalicular membranes was 33 ± 5 pmol x mg
ATP-dependent Transport of Taurocholate in Liver

FIG. 3. SDS-PAGE after immunoprecipitation of gp110 with mAb Be 9.2 after photoaffinity labeling of canalicular membranes with [35S]ATPγS and [3H]ATC. Membrane subfractions (500 μg of protein) were incubated with 1.85 MBq of [35S]ATPγS (0.2 μM) or 370 kBq of [3H]ATC (2.5 μM). Photoaffinity labeling, immunoprecipitation, and SDS-PAGE (8–20% acrylamide gradient) was performed as described under "Experimental Procedures."

FIG. 4. IEF after immunoprecipitation of gp110 with mAb Be 9.2 after photoaffinity labeling of canalicular membranes with [35S]ATPγS and [3H]ATC. Membrane subfractions (500 μg of protein) were incubated with 1.85 MBq of [35S]ATPγS (0.2 μM) or 370 kBq of [3H]ATC (2.5 μM). For further details see "Experimental Procedures."

The ATP-stimulated taurocholate uptake was insensitive to EGTA and ouabain at concentrations up to 2 mM, whereas it was inhibited by vanadate in a dose-dependent manner (IC50 = 5 μM). The rate of ATP-dependent taurocholate uptake was saturable with respect to the ATP concentration with an apparent Kₘ value of 800 μM (Fig. 6). The concentration dependence of the ATP-dependent taurocholate uptake exhibited saturation kinetics with an apparent Kₘ value of 7.5 μM in the taurocholate concentration range of 0–100 μM. No ATP-dependent transport was observed at concentrations above 200 μM. This was possibly caused by the detergent effect of the bile salt, which accumulated rapidly in the inner space of the vesicles during incubation.

The stimulation of taurocholate uptake was specific for ATP. ATP-dependent uptake was 36 ± 6 pmol × min⁻¹ × mg protein⁻¹ (mean ± S.D., n = 3) at an ATP-concentration of 4 mM without creatine phosphate and creatine kinase. At the same concentration (4 mM) other nucleotides, i.e. GTP, UTP, and CTP, as well as ATP analogues, i.e. AMP-PCP and AMP-PNP, had no significant effect (less than 1% of the effect of ATP). P-glycoprotein substrates, such as doxorubicin and verapamil, did not inhibit the ATP-dependent taurocholate uptake at concentrations up to 200 μM. ATP-dependent transport of taurocholate was observed not only in canalicular membrane vesicles from normal Wistar rats but also from mutant rats lacking the canalicular ATP-dependent transport of cysteinyl leukotrienes and related anions (Table II).
proteoliposomes took up taurocholate in an ATP-dependent manner (34,35). Photolysis was avoided by cryofixation of the sample. The protein-ligand interactions were fixed by shock-freezing at defined time points. In this frozen state irradiation-induced highly reactive excited states and/or intermediates of the ligand can form covalent bonds with the respective binding sites (34,35).

Reconstitution of the ATP-dependent transport system for taurocholate into liposomes—Canalicular membrane proteins were solubilized with octylglucoside and reconstituted into phosphatidylcholine liposomes using gel filtration chromatography to remove the detergent (23). Such proteoliposomes were incubated with 5 μM [3H]taurocholate and 25 mM [3H]leukotriene C4 at 37 °C for 5 min as described under "Experimental Procedures." ATP-dependent transport was calculated from the difference in radioactivity taken up into vesicles in the presence or absence of 1 mM ATP. Data are expressed as mean values ± S.D.; n = 5.

Table II
ATP-dependent Transport of Taurocholate in Liver

<table>
<thead>
<tr>
<th>Membrane source</th>
<th>ATP-dependent transport</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Taurocholate</td>
</tr>
<tr>
<td>Normal</td>
<td>164 ± 30</td>
</tr>
<tr>
<td>Mutant</td>
<td>148 ± 21</td>
</tr>
</tbody>
</table>

Table III
Reconstitution of the ATP-dependent taurocholate transport system in proteoliposome vesicles using purified gp110 or canalicular membrane proteins

Proteoliposome vesicles containing all canalicular membrane proteins (50 μg) or purified gp110 (10 μg) were incubated with 5 μM [3H]taurocholate at 37 °C for 30 min as described under "Experimental Procedures." ATP-dependent transport was calculated from the difference in radioactivity taken up into proteoliposomes in the presence or absence of 1 mM ATP. Data are expressed as mean values ± S.D.; n = 5.

<table>
<thead>
<tr>
<th>Protein addition</th>
<th>ATP</th>
<th>- ATP</th>
<th>ATP-dependent transport</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol × mg protein^-1</td>
<td>pmol × mg protein^-1</td>
<td></td>
</tr>
<tr>
<td>Canalicular membrane proteins</td>
<td>239 ± 8</td>
<td>56 ± 5</td>
<td>183 ± 4</td>
</tr>
<tr>
<td>Purified gp110</td>
<td>569 ± 40</td>
<td>76 ± 34</td>
<td>493 ± 17</td>
</tr>
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</table>

Reconstitution of the Canalaricular ATP-dependent Transport System for Taurocholate into Liposomes—Canalicular membrane proteins were solubilized with octylglucoside and reconstituted into phosphatidylcholine liposomes using gel filtration chromatography to remove the detergent (23). Such proteoliposomes were incubated with 5 μM [3H]taurocholate in an ATP-dependent manner (Table III). gp110, which was purified by antibody-affinity chromatography to apparent homogeneity (26, 27), was functionally reconstituted into liposomes prepared from a phosphatidylcholine/cholesterol mixture. These gp110-containing proteoliposomes showed active ATP-dependent transport of taurocholate (Table III), whereas protein-free liposomes exhibited no ATP-dependent transport. The ATP-dependent transport of taurocholate was therefore mediated by gp110, which functioned as carrier.

Discussion
Membrane Domain-specific ATP-binding Proteins—Direct photoaffinity labeling with [35S]ATPγS has been used to identify ATP-binding proteins in plasma membrane subfractions from rat hepatocytes (Figs. 1 and 2; Table I). ATP as well as other nucleotides can serve without further modification for direct photoaffinity labeling if photolysis occurs at short UV wavelengths (18). This approach was previously used for the identification of nucleotide-binding sites in isolated enzymes (15-19). In this study unspecific labeling caused by long-lived intermediates generated during photolysis was avoided by cryofixation of the sample. The protein-ligand interactions were fixed by shock-freezing at defined time points. In this frozen state irradiation-induced highly reactive excited states and/or intermediates of the ligand can form covalent bonds with the respective binding sites (34,35).

Different ATP-binding proteins were detected in hepatocyte plasma membrane subfractions enriched in sinusoidal or canalicular membranes (Fig. 1). The three predominantly labeled ATP-binding proteins in the sinusoidal membrane fraction exhibited lower M5 values (24,000, 20,000, and 16,000) relative to the labeled proteins in canalicular membranes (i.e. 130,000 and 110,000). Differences in the protein pattern in both domains of the hepatocyte plasma membrane were established by several groups (4,36-38), particularly by the use of antibodies against domain-specific glycoproteins (24-27, 30,38-43).

Identification of gp110 as an ATP- and Bile Salt-binding Protein by Photoaffinity Labeling and Immunoprecipitation—Several ATP-binding proteins in the M5 range of 110,000 were identified by two-dimensional electrophoresis of the photoaffinity-labeled canalicular membrane subtraction (Fig. 2). The canalicular plasma membrane contains several glycoproteins in the M5, 110,000 range (4, 24, 25, 31). One of these has been described as a transformation-sensitive protein not detectable in plasma membranes of several hepatomas (24, 27). This glycoprotein with an isoelectric point near 3.7 showed ATP-binding properties as detected here by immunoprecipitation using the mAb Be 9.2 raised against this protein (Figs. 3 and 4). This protein is identical with the bile salt-binding protein, first identified in intact liver snips and canalicular membranes by photoaffinity labeling (32), as shown by the comparison of both the M5 and the isoelectric point (Figs. 3 and 4). The M5 of the protein moiety of the bile salt-binding protein (39) and of the ATP-binding protein was 48,000 after digestion with endo peptide N-glycosidase F.

Previous work has indicated that the canalicular bile salt-binding protein may be involved in carrier-mediated bile salt secretion (23,33). Functional reconstitution of the protein in proteoliposomes resulted in a membrane potential-sensitive bile salt transport (23). A recent purification with a glycocholate-affinity column yielded a single bile salt-binding protein (31). Except for a difference in the isoelectric point (5.3 versus 3.7) this glycoprotein (31) shares most properties with our glycoprotein binding ATP and bile salt.

A canalicular membrane glycoprotein in the M5, range of 110,000 was designated as HA4 (42). This protein was further characterized by means of a monoclonal antibody which recognizes two glycoproteins (42). One of them seems to be identical with gp120, one of the endogenous substrates for the hepatocyte insulin receptor tyrosine kinase (44), furthermore it is identical with Ca2+/Mg2+-dependent ecto-ATPase (45). This ecto-ATPase has been cloned recently and exhibits a high degree of homology with the human biliary glycoprotein 1 (46). HA4 was down-regulated during peroxisomal proliferator-induced alterations in the expression of rat hepatocyte plasma membrane proteins as well as during hepatocyte proliferation following two-third hepatectomy (47). The physiological function of the HA4 glycoprotein has not yet been elucidated; however, its localization at the apical plasma membrane domain of the hepatocyte suggests a potential role in transmembrane transport (45). The sequence comparison between the cell adhesion molecule (C-CAM 105) (39,40) indicates that it is related or identical with the canalicular Ca2+/Mg2+-dependent ecto-ATPase (48). It cannot be decided at present whether these proteins (HA4, C-CAM 105, pp120, Ca2+/Mg2+-ecto-ATPase) as well as the bile salt- and ATP-binding gp110 are closely related or in part identical.

ATP-dependent Transport of Taurocholate in Hepatocyte Canalaricular Membrane Vesicles—The kinetic properties of the canalicular transport system for bile salts have been extensively studied using plasma membrane subfractions enriched in canalicular membranes. This transport, mediated by a Na+-dependent uptake of taurocholate and leukotriene C4 into canalicular membrane vesicles prepared from Wistar (normal) and transport mutant (mutant) rats

Canalicular membrane vesicles (50 μg of protein) were incubated with 5 μM [3H]taurocholate or 10 mM [3H]leukotriene C4 at 37 °C for 5 min as described under "Experimental Procedures." ATP-dependent transport was calculated from the difference in radioactivity taken up into vesicles in the presence or absence of 1 mM ATP. Data are expressed as mean values ± S.D.; n = 3.
independent anion carrier, appeared to be driven by the physiological intracellularly negative membrane potential of approximately \(-30\) to \(-40\) mV (4, 49-53). The transport process was furthermore characterized by its apparent \(K_m\) value of about \(42\) \(\mu M\) for taurocholate and its preferential transport of trihydroxylated and conjugated dihydroxylated bile salts (50, 52). The question has been raised whether this membrane potential difference is sufficient to mediate the concentration process from blood \((-100\) \(\mu M\)) to bile \((-10\) mM). In this context other mechanisms, particularly vesicular transport, have been considered (3, 53, 54). Both mechanisms, the vesicular as well as the carrier-mediated transport, may contribute to the total secretion of taurocholate from the hepatocyte into bile.

Our data demonstrate for the first time the presence of an ATP-dependent bile salt transport system in the canalicular membrane (Fig. 5). Vanadate, an inhibitor of P-type ATPases (55), inhibits ATP-dependent taurocholate transport with an \(IC_{50}\) value of 5 \(\mu M\) which is comparable with the values reported for the other transport ATPases such as P-glycoprotein \(IC_{50} = 10\) \(\mu M\) (11) and the transport system for cysteinyl leukotrienes \(IC_{50} = 30\) \(\mu M\) (8). This vanadate inhibition suggests that \(\gamma\)-phosphate transfer from ATP to the carrier is involved in the process of bile salt transport. Several other ATPases, such as Ca\(^{2+}\)/Mg\(^{2+}\)-dependent ATPase, which is probably not directly involved in transport of organic solutes, are not vanadate-sensitive (55, 56).

For further characterization of the specificity of the ATP-dependent bile salt transport we used mutant rats with defective hepatobiary transport of cysteinyl leukotrienes but with normal taurocholate transport (8, 9, 21, 22, 57). Canalicular membrane vesicles from these mutant rats showed normal ATP-dependent taurocholate transport (Table II). Photoaffinity labeling of these membranes using \[^{35}S\]ATP\(_2^+\)S revealed the labeling of gp110 (Fig. 1B). It is of interest that a \(100,000\) protein in mutant rat canalicular membranes has also been labeled by the photolabile bile salt (58). No significant labeling of gp110 was detected in the canalicular membranes from normal and mutant rats by use of \(^3H\)-labeled leukotriene \(C_4\) as the photolabile ligand (8). Furthermore, ATP-dependent bile salt transport as well as leukotriene transport (8) were not inhibited by the P-glycoprotein substrates verapamil and doxorubicin. These data indicate that the taurocholate transport system differs from other previously characterized canalicular ATP-dependent transport systems. It is presently unknown whether the ATP-dependent transport system for taurocholate is distinct from the membrane potential-sensitive transport described earlier (23, 33, 49-53).

Functional Reconstitution of the ATP-dependent Taurocholate Transport System—Direct evidence that the ATP- and bile salt-binding glycoprotein gp110 functions as the ATP-dependent taurocholate carrier in the canalicular membrane was obtained by functional reconstitution of the protein into liposomes (Table III). This approach has recently been used to demonstrate the function of a 49-kDa protein as the sodium-dependent bile salt transport protein located in the sinusoidal membrane of the hepatocyte (59). Reconstitution into artificial liposomes also served to study the function of a 100-kDa glycoprotein as a potential-sensitive canalicular bile salt transport system (23). The properties of this 100-kDa glycoprotein appear to be similar as the ones of gp110; however, the driving force of the transporter (23) must be reestablished. Different methods, including gel filtration (23), biobead SM-2 (59), and dialysis, may be used to remove the detergent from the solubilization mixture prior to membrane protein reconstitution into liposomes. We have preferred gel filtration because of good reproducibility of the rapid liposome preparation. Total proteins solubilized from canalicular membranes and reconstituted into the liposomes also mediated ATP-dependent taurocholate transport (Table III). A significantly higher specific activity of transport was obtained with the purified gp110 reconstituted into liposomes. The latter were composed of a mixture of phosphatidylcholine and cholesterol in order to adjust the membrane fluidity to the canalicular membrane (60). During concentration and dialysis of the purified gp110 ATP was included in the medium in order to protect against denaturation or to facilitate renaturation of the protein to active function (61).

Comparison of the ATP-dependent Bile Salt Carrier with Other Primary-active Transport Systems in Canalicular Membranes—The driving forces for the transept membrane movement of the molecules transported across the sinusoidal and canalicular membrane seem to be domain-specific. Previously two primary-active transport systems were identified in the canalicular membrane which function as export carriers for molecules in the \(M_r\) range of 300-900: the P-glycoprotein as a multidrug export carrier (6), and the export carrier for cysteinyl leukotrienes and several other amphiphilic anions including glutathione \(S\)-conjugates and bromosulfophthalein (8-10, 62). In contrast, no primary-active carriers for such molecules have been identified in the sinusoidal hepatocyte membrane (2-5). In most cases, transport mediated by the uptake systems in the sinusoidal membrane domain is secondary-active (e.g. the Na\(^+\)-dependent bile salt transport system) or sensitive to the membrane potential (4, 50). The data presented in this and previous studies (6, 8, 10) indicate at least three different ATP-dependent export carriers in the canalicular membrane (Fig. 7): (i) the bile salt carrier corresponding to the ATP- and bile salt-binding glycoprotein gp110, (ii) the leukotriene export carrier (8-10, 62), and (iii) the multidrug export carrier which corresponds to the P-glycoprotein gp170 (6). These ATP-dependent unidirectional transport systems may belong to the recently defined superfamilies of ATP-binding proteins with homologous ATP-binding cassette in their sequence (63). Direct photoaffinity labeling with \[^{35}S\]ATP\(_2^+\)S using cellular fractions enriched in plasma membranes from both domains of a polarized cell like the hepatocyte will be useful for the further characterization of ATP-binding proteins which include transport systems.

![Fig. 7. ATP-dependent primary-active export carriers in the canalicular membrane involved in the hepatobiary elimination of bile salts, cysteinyl leukotrienes, and hydrophobic drugs. gp110 functions as the ATP-dependent bile salt export carrier (BSEC); the ATP-dependent leukotriene export carrier (LTEC) transports leukotriene \(C_4\) and other cysteinyl leukotrienes (8) as well as several amphiphilic anions including glutathione \(S\)-conjugates (8-10, 62), and bromosulfophthalein (9). The multidrug export carrier (MDOC), corresponding to gp170 (6), functions in the export of hydrophobic, mostly basic drugs, such as doxorubicin and verapamil, from the hepatocyte into bile.](image-url)
ATP-dependent Transport of Taurolcholate in Liver

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