The Rat Liver Ecto-ATPase Is Also a Canalicular Bile Acid Transport Protein* 

C. Jeffrey Sippell, Frederick J. Suchy, M. Ananthanarayanan, and David H. Perlmutter

From the Departments of Pediatrics, Cell Biology, and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110 and the Department of Pediatrics, Yale University School of Medicine, New Haven, Connecticut 06510

The production of bile is proportional to the net vectorial translocation of bile acids from the sinusoidal blood into the canalicular lumen. This requires the participation of specific bile acid transport mechanisms at both the sinusoidal and canalicular membranes, but transport across the canalicular membrane represents the rate-limiting step in this physiologic process. It is also known that the driving forces for canalicular bile acid transport include the negative electrochemical potential and an ATP-stimulated transport mechanism (1). With these considerations in mind, we recently purified to homogeneity a ~110-kDa glycoprotein from detergent-solubilized rat liver canalicular membrane vesicles by bile acid affinity chromatography. Monospecific polyclonal antibodies raised against this protein inhibited bile acid transport in canalicular membrane vesicles (2). Two other groups have independently labeled a protein with similar characteristics using photo-reactive bile acid probes and have shown that the protein, reconstituted in synthetic proteoliposomes, possesses bile acid transport properties (3-5). In order to further define the structure of the protein that we had purified from canalicular membranes, the amino acid sequences of internal chymotryptic fragments were determined. The sequences (2) were identical to a rat liver canalicular ecto-ATPase (6). Moreover, the molecular mass, isoelectric point, and carbohydrate composition of the protein purified by bile acid affinity chromatography were identical to that of the ecto-ATPase (2, 6). This raised the possibility that a single polypeptide was responsible for both bile acid transport and ecto-ATPase activities. To explore this possibility, we studied biosynthesis, bile acid transport, and ecto-ATPase activity in COS cells transiently transfected with cDNA for the ecto-ATPase (6). We also studied expression of an ecto-ATPase cDNA construct in which the coding region of the 3'-terminus had been truncated. This construct was designed to delete the carboxyl-terminal intracytoplasmic tail of the gene product without disturbing its appropriate insertion within the membrane. Because bile acid efflux presumably requires binding of the bile acid within the cytoplasm and then translocation across the canalicular membrane, we reasoned that the cytoplasmic tail of the putative transporter would be essential for bile acid efflux.

EXPERIMENTAL PROCEDURES

Materials—Na125I, [35S]ATPyS, [3H]taurocholate, and [3H]taurocholate were obtained from Du Pont-New England Nuclear. Nitrocellulose sheets, horseradish peroxidase-conjugated goat anti-rabbit IgG, and horseradish peroxidase color development reagent containing 4-chloro-1-napthol, were purchased from Bio-Rad. Triton X-100, Coomassie Blue R-250, ATP, ADP, adenosine-5'- (y-thio)triphasphate, ADP, AMP, GTP, AMP-PNP, and trypsin were obtained from Sigma. DMEM and fetal calf serum were obtained from Whittaker Bioproducts (Walkersville, MD). Sulfosuccinimidyl-3'(4-hydroxyphenyl)propionate (sulfo-SH-PP) was obtained from Pierce Chemical Co.

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transport protein was isolated by bile acid affinity chromatography as described previously (2). The band containing the affinity-puriﬁed canalicular bile acid transport protein was puriﬁed on SDS-PAGE preparative gels and visualized by Coomassie Blue staining. This band was then excised, eluted, and digested with chymotrypsin as described (7). The resultant digest was then lyophilized, and the amino acid sequence was determined by Edman degradation in the Protein Chemistry Laboratory at Yale University School of Medicine.

**Plasmid Constructs**—The full-length ecto-ATPase cDNA (pExp3) was kindly provided by S. H. Lin, Houston, TX (6). A truncated cDNA missing the 66 carboxyl-terminal amino acids, but leaving 5 cytoplasmic amino acids, the transmembrane domain, and the entire amino-terminal extracellular tail intact was generated by polymerase chain reaction ampliﬁcation of pExp3.

**Cell Culture and Transfection**—COS 1 cells were transfected by the DEAE-dextran method and used for experimental purposes 48 h after transfection (8).

**Western Blot Analysis**—Cells were homogenized in PBS with 1% Triton X-100, 0.5% deoxycholic acid, 10 mM EDTA, 2 mM phenylmethylsulfonyl ﬂuoride, and the cell homogenates subjected to Western blot analysis with antibody to the puriﬁed canalicular bile and transport protein and horseradish peroxidase anti-IgG (2).

**Metabolic Labeling, Immunoprecipitation, and Analytical Gel Electrophoresis**—Cells were labeled for 3 h at 37°C with [35S]methionine 250 µCi/ml DMEM lacking methionine or for 2.75 h at 37°C with [32P]orthophosphate 0.5 µCi/ml DMEM lacking phosphate. The cells were then washed, homogenized, and subjected to immunoprecipitation with antibody to the canalicular bile acid transport protein (2) and Staphylococcus protein A as described previously (9). Immunoprecipitates were analyzed by SDS-PAGE followed by ﬂuorography or autoradiography (9).

In separate experiments transfected cells were also subjected to cell surface radioiodination by the Bolton Hunter method (10) and then analyzed by immunoprecipitation with antibody to the puriﬁed canalicular bile acid transport protein and antibody to actin. Immunoprecipitates were again analyzed by SDS-PAGE/autoradiography.

**Bile Acid Efflux and Ecto-ATPase Assays**—Transfected cells were incubated at 15°C for 60 min in DMEM with valinomycin (100 µM), [3H]taurocholate in several different concentrations, in the absence or presence of DIDS (1 mM).

The cells were then washed twice in PBS and efflux of [3H]taurocholate into DMEM measured 20 s later. ATPase activity was measured by a previously described method (6). The results were reported in terms of protein concentration as determined by the Lowry assay (11).

**RESULTS**

**Amino Acid Sequencing of the Canaliculal Bile Acid Transport Protein**—Amino acid sequencing of the puriﬁed canalicular bile acid transport protein was performed as described in the methods. Three peptide fragments were analyzed, and their sequences were as follows: QNVNKTDERAY, LSVDQFQPNIQTSQVF, and NVT, which correspond to sequences 110–120, 121–138, and 148–150 of the cloned rat liver canalicular ecto-ATPase (6). This sequence identity prompted a review of the chemical characteristics of the canalicular ecto-ATPase and the bile acid transport protein. In fact, previous studies had shown that the molecular weight, carbohydrate content, and isoelectric point of the two proteins were similar, if not identical (2, 6). Therefore, we examined the possibility that bile acid transport and ecto-ATPase activities represented two functions of a single polypeptide.

**Detection of the Protein Encoded by the Ecto-ATPase cDNA in Transfected COS Cells**—The full-length and truncated ecto-ATPase cDNAs (Fig. 1) were cloned into the mammalian expression vector pCDM8 to allow for expression at high levels in COS cells (12). First, we determined whether COS cells transfected with these plasmid constructs expressed a polypeptide which could be recognized by antibody to the puriﬁed bile acid transport protein (2). In Western blot analysis (Fig. 2A), we detected a ~110-kDa polypeptide in COS cells transfected with the full-length plasmid construct. This polypeptide was not detected in COS cells transfected with vector alone. A polypeptide which migrated more rapidly was detected in COS cells transfected with the truncated plasmid construct. Transfected COS cells were also pulse-labeled with [35S]methionine and the resulting radiolabeled cell lysates subjected to immunoprecipitation and then SDS-PAGE/autoradiography (Fig. 2B). COS cells transfected with full-length and truncated ecto-ATPase cDNA constructs synthesized several

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polypeptides which could be recognized by antibody to the purified bile acid transport protein and which could not be detected in COS cells transfected with vector alone. For the full-length plasmid construct, the predominant newly synthesized polypeptide was ~110 kDa in relative molecular mass. At least three other faster migrating polypeptides were also demonstrated. The time-dependent appearance of these latter polypeptides (data not shown) suggests that they are under-glycosylated precursors, but we have not yet excluded the possibility that they are proteolytic degradation products. For the truncated plasmid construct there are also four polypeptides, in each case migrating to a relative molecular mass ~8–10 kDa smaller than the corresponding polypeptides for the full-length gene product. Next, we examined whether the same polypeptides could be detected at the cell surface of transfected cells by immunoprecipitation after cell surface iodination with the sulfated Bolton-Hunter reagent (Fig. 2C). A ~110-kDa radiiodinated polypeptide and a faster migrating radiiodinated polypeptide were detected in COS cells transfected with the full-length and truncated plasmid constructs, respectively, but no radiiodinated polypeptide was detected in cells transfected with vector alone. In this experiment there was not as large a difference as expected between the electrophoretic mobilities of the truncated and full-length protein probably due to use of a 10% gel. In other experiments, on a 7.5% gel, the difference in electrophoretic mobility is greater. In addition, the ubiquitous submembranous protein actin was not detected by sequential immunoprecipitation of all these cell lysates with antibody to actin (data not shown). Thus, this method for cell surface radiiodination could not detect protein on the internal aspect of the cell membrane or in the cytosol. The data show that the ecto-ATPase cDNA encodes a polypeptide that is immunochemically identical to the purified bile acid transport protein and that this polypeptide is targeted to the external surface of the plasma membrane in transfected COS cells even when the carboxy-terminal cytoplasmic tail is deleted.

Bile Acid Efflux and ATPase Activity in Transfected COS Cells—In order to develop an assay for bile acid efflux, we adapted methodology which had originally been used to demonstrate daunomycin efflux by cells which expressed the multidrug resistance transport system (13). First, we examined the possibility that [3H]taurocholate could be internalized by COS cells. Cells were incubated at 15 °C with [3H]taurocholate for time intervals up to 60 min. Valinomycin (100 μM) was always included in the incubation medium to abrogate the electrochemical potential. Uptake was determined by liquid scintillation counting of cell homogenates. The results indicate that there is time-dependent and saturable uptake of [3H]taurocholate. Equilibrium was reached by 20 min and maintained until the final time point of 60 min. Uptake was not significantly different for cells which had been transfected with vector alone or the truncated or full-length constructs. Uptake was not significantly affected by the anionic transport inhibitor DIDS. In separate experiments we also showed that uptake was similar for COS cells transfected with vector alone or truncated or full-length constructs when concentrations of [3H]taurocholate from 100 to 600 μM were used (data not shown).

Next, we examined the possibility of measuring the efflux of [3H]taurocholate which had been internalized by transfected COS cells. Transfected cells were incubated for 60 min at 15 °C with medium containing [3H]taurocholic acid in several different concentrations, in the presence of valinomycin (100 μM), and in the absence or presence of DIDS (1 mM), an inhibitor of several anionic transport processes including canalicular bile acid transport (Fig. 3a). Cells were then washed extensively and incubated in medium alone for 20 s at 15 °C. Measurement of the appearance of radioactivity in the extracellular medium (Fig. 3a) and disappearance from the cells (data not shown) demonstrated that there was saturable and DIDS-sensitive efflux of bile acids by cells transfected with the full-length construct but not by those transfected with the truncated construct or vector alone. Efflux by COS cells transfected with the full-length ecto-ATPase cDNA construct had kinetic characteristics for bile acid efflux (Km = 100 μM; Vmax = 200 pmol/mg of protein/20 s) that are similar to those reported for rat liver canalicular membrane vesicles (14). When four separate experiments were compiled (Fig. 3b), characteristics of bile acid efflux in COS cells transfected with the full-length ecto-ATPase were very similar to that in the single experiment shown in Fig. 3a, albeit with greater variation at each point. The results in Fig. 3, a and b, were representative of five separate experiments.

Next, we examined the possibility that efflux of [3H]taurocholate by transfected COS cells was time-dependent (Fig. 3c). COS cells which had been transfected with the full-length construct were incubated for 1 h at 15 °C with [3H]taurocholate in subsaturating concentrations using the same conditions as described for kinetic experiments above. The cells were then rinsed and incubated in medium lacking taurocholate for several different time intervals. At the end of each interval, extracellular medium was harvested and cell monolayers homogenized for analysis by scintillation counting. The results show that there is disappearance of [3H]taurocholate from the cells within 40 s coincident with its appearance in the extracellular medium. The time-dependent disappearance from the cells and appearance in medium was essentially saturated within 2 min. All of the radioactivity that was originally internalized could be recovered in either cells or medium at each time point. It is not known why approximately 40–50% of the initial radioactivity remains in the cell, but almost identical results have been described for daunomycin efflux as mediated by the MDR transporter of ascites tumor cells (13). It may be that some of this radioactivity is bound nonspecifically to matrix or cell surface.

We also determined ecto-ATPase activity in transfected COS cells (Fig. 4). This activity was detected in COS cells transfected with the full-length cDNA construct but not in those transfected with the truncated construct or vector alone. The ecto-ATPase activity detected in COS cells transfected with the full-length cDNA was almost completely abrogated by antibody to the purified bile acid transport protein but not by preimmune serum. Therefore, the polypeptide encoded by the ecto-ATPase cDNA possessed bile acid efflux and ecto-ATPase activities in transfected COS cells, but only when its carboxy-terminal cytoplasmic tail was intact. The results of the bile acid efflux and ecto-ATPase activity measurements could not be explained by effects of the transferred gene product on cell adhesion or cell aggregation. Cells were transfected at 90% confluence and studied 48 h later at complete confluence. At the end of the assays there was no difference in morphology, cell counts, or total protein concentration between monolayers transfected with vector alone or truncated or full-length constructs. Furthermore, efflux mediated by the cells transfected with the full-length construct was curvilinear and saturable (Fig. 3b), indicating a carrier-mediated mechanism, as opposed to the linear efflux which would be expected of a nonspecific diffusion mechanism associated with changes in cell adhesion.

Pharmacological Manipulations of Bile Acid Efflux and Ecto-ATPase Activity in Transfected COS Cells—In order to further establish that the ~110-kDa glycoprotein encoded by the ecto-
**FIG. 3.** The kinetics of taurocholate transport in transfected COS cells. a, the kinetics of DIDS-sensitive taurocholate efflux. Cells were transfected with the full-length construct (closed circle), truncated construct (open circle), or vector alone (open triangle). Cells were studied 48 h later, incubated with specified concentrations of \[^{3}H\]taurocholate, and then rapidly washed in PBS. Efflux of \[^{3}H\]taurocholate into 1 ml of DMEM was measured at 20 s. DIDS-sensitive initial velocity was then determined as the difference between the velocity measured in the presence and in the absence of DIDS and expressed as picomoles of taurocholate efflux/mg of cellular protein \[^{14}\]. Protein concentration was determined by the method of Lowry \[^{11}\]. b, kinetics of DIDS-sensitive taurocholate efflux in four separate experiments. Data from four separate experiments done exactly as described for the single experiment in Fig. 3a were compiled. Each point represents the mean and standard deviation for duplicate samples. c, the time-dependent kinetics of taurocholate efflux in COS cells transfected with the full-length construct. Cells transfected with the full-length construct were incubated and washed as in a and then were incubated with medium alone for several different time intervals. Extracellular medium and cell homogenates were then subjected to scintillation counting. The results were expressed as the relative percentage of the total counts (counts/min/mg of protein) in either the intracellular (IC) or extracellular (EC) compartment at each time point.

**FIG. 4.** Ecto-ATPase activity in transfected COS cells. ATPase activity was determined as described in the methods for cells transfected with vector alone, truncated construct, or full-length construct. In addition cells transfected with the full-length construct were preincubated with a 1:1000 dilution of either preimmune or immune serum to the bile acid transport protein \(\text{Anti-BATP}\) for 2 h at 37 °C, washed in PBS × 2, and assayed for ATPase activity as above.
and ecto-ATPase activity, we next examined whether DIDS, a known inhibitor of bile acid transport (as shown in Fig. 3b), would also inhibit ecto-ATPase activity (Fig. 6). COS cells transfected with the full-length ecto-ATPase construct were incubated in the absence or presence of DIDS under conditions identical to those used for efflux measurements. DIDS completely inhibited ecto-ATPase activity conferred on the cells by DNA-mediated gene transfer. Similar concentrations of DIDS were necessary to inhibit taurocholate efflux ($K_i = 60 \mu M$) and ecto-ATPase activity ($K_i = 50 \mu M$).

In addition, we examined the effect of vanadate on bile acid efflux and ecto-ATPase activity in transfected COS cells (Fig. 7). The results indicate that vanadate completely inhibits basal and ATP-stimulated bile acid efflux (Fig. 7a) but has no effect on ecto-ATPase activity (Fig. 7b). Previous independent studies have shown that vanadate, in the same concentrations, inhibits bile acid transport (5, 15) but has no effect on ecto-ATPase activity in canalicular vesicles (16, 17). These results, therefore, provided further evidence that the bile acid transport properties conferred on COS cells by transfection of the ecto-ATPase cDNA are similar to those described previously in canalicular membrane vesicles.

**Photoaffinity Labeling of the Bile Acid Transport/Ecto-ATPase Protein by [35S]ATPyS**—An ATP-dependent bile acid transport protein has been demonstrated in rat liver canalicular vesicles by photoaffinity labeling with nonhydrolyzable ATP analogue [35S]ATPyS (5). Taking the data into consideration, we examined the possibility that the protein expressed in our transfected COS cells and recognized by antibody to the purified canalicular bile acid transport protein could be cross-linked by the same photoprobe. Transfected COS cells were incubated for 45 min at 37°C in the dark with the photoprobe, transferred to an ice bath, and subjected to a 15-min pulse of UV light at 254 nm (Fig. 8). A ~110-kDa polypeptide was labeled in cells transfected with the full-length construct, and a ~100-kDa polypeptide was labeled in cells transfected with the truncated construct. There was no photoaffinity labeling in cells transfected with vector alone. The photoaffinity labeling was specific in that it was almost completely blocked by unlabeled ATPγS. The photoaffinity labeling was specific for the transfected gene product as shown by its apparent molecular mass and recognition by antibody to the purified canalicular bile acid transport protein. Since it is unlikely that ATPγS binds to the intramembranous domain or exceedingly short cytoplasmic tail (5 residues) of the truncated protein, these results also show that the ATP binding site of the ecto-ATPase/bile acid transport protein is in its extracellular domain and, therein, provides further evidence that the ecto-ATPase is a functional bile acid transporter.
cells were photoaffinity-labeled by modification of the methods of Muller et al. (5). Briefly, 48 h after transfection, COS cells were rinsed twice and then for 45 min in DMEM lacking phosphatases. Cell monolayers were then washed and incubated at 37°C for 2 min in medium supplemented with [35S]ATPγS (1 mBq (0.8 μM)) in the absence (−) or presence (+) of 200-fold molar excess of unlabeled ATPγS. The cell monolayers were then transferred to an ice bath and subjected to UV irradiation at 254 nm for 15 min. The cell homogenates were then subjected to immunoprecipitation and autoradiography as described previously. This autoradiogram represents an exposure of 7 days. Additional bands were not detected by exposure of 21 days (data not shown).

FIG. 8. Photoaffinity labeling of the bile acid transport/ecto-ATPase protein in transfected COS cells. Transfected COS cells were photoaffinity-labeled by modification of the methods of Harrison and Mobley (38). Briefly, 48 h after transfection, COS cells were phosphatase-starved by incubation in DMEM lacking phosphate for 45 min at 37°C. The cells were then incubated for 15 min at 37°C with 0.5 mCi of [35S]orthophosphate in 1 ml of medium. Cells were then washed vigorously in PBS and resuspended in cell lysis buffer containing 50 mM sodium fluoride and 10 mM sodium pyrophosphate to prevent chemical dephosphorylation. The lysates were then subjected to immunoprecipitation with antibody to purified bile acid transport protein (+) or preimmune serum (−) and immunoprecipitates analyzed by SDS-PAGE/autoradiography as described previously.

FIG. 9. Phosphorylation of the bile acid transport/ecto-ATPase protein in transfected COS cells. COS cells were transfected with the full-length construct, truncated construct, or vector alone and 48 h later were examined for phosphorylation by the methods of Harrison and Mobley (38). Briefly, 48 h after transfection, COS cells were phosphatase-starved by incubation in DMEM lacking phosphate for 45 min at 37°C. The cells were then incubated for 165 min at 37°C with 0.5 mCi of [35S]orthophosphate in 1 ml of medium. Cells were then washed vigorously in PBS and resuspended in cell lysis buffer containing 50 mM sodium fluoride and 10 mM sodium pyrophosphate to prevent chemical dephosphorylation. The lysates were then subjected to immunoprecipitation with antibody to purified bile acid transport protein (+) or preimmune serum (−) and immunoprecipitates analyzed by SDS-PAGE/autoradiography as described previously.

Finally, we examined the effect of activation or inhibition of protein kinase C, and presumably phosphorylation, on the functional activities of the ecto-ATPase/bile acid transport protein. The transfected COS cells were incubated for 45 min with 50 μM [3H]taurocholate. PMA or staurosporine was then added to the incubation for an additional 15 min. The cells were then assayed for bile acid transport as described previously. The results show that the protein kinase C stimulator, PMA, enhanced bile acid efflux, whereas the protein kinase C inhibitor, staurosporine, completely inhibited bile acid efflux (Fig. 11a). These modulators did not alter or confer bile acid efflux properties on COS cells transfected with the truncated construct or vector alone (data not shown). In contrast, neither PMA nor staurosporine affected ecto-ATPase activity of transfected COS cells (Fig. 11b). Neither activation of protein kinase C by PMA nor inhibition of protein kinase C by staurosporine affected the bile acid transport protein.

Phosphorylation of the Bile Acid Transport/Ecto-ATPase Protein in Transfected COS Cells—A sequence KRPTS at amino acids 499–503 within the cytoplasmic tail of the ecto-ATPase is very similar to consensus sequences for protein kinase C- and protein kinase A-dependent phosphorylation (17). We therefore examined the possibility that the ecto-ATPase/bile acid transport protein expressed in transfected COS cells was phosphorylated (Fig. 9). Transfected cells were pulse-labeled with [32P]orthophosphate and the resulting radiolabeled cell lysates subjected to immunoprecipitation with antisera to the purified bile acid transport protein (+) or preimmune sera (−). SDS-PAGE/autoradiographic analysis demonstrated incorporation of phosphate into a ~110-kDa protein in cells transfected with the full-length construct. This protein was not detected by preimmune sera nor was it present in cells transfected with truncated construct or vector alone. These results demonstrate that the ecto-ATPase/bile acid transport protein is phosphorylated and that the phosphorylation site is probably within the carboxyl-terminal 66 amino acids of its cytoplasmic tail. Next, we examined the effect of protein kinase C activation and inhibition of protein kinase C on phosphorylation of the ecto-ATPase/bile acid transport protein in transfected COS cells (Fig. 10). Cells were pulse-labeled with [32P]orthophosphate for 2.75 h. In the last 15 min of the incubation, either 2.5 μM PMA, 10 μM staurosporine, or control vehicle was added to the incubation. The results indicate that there is an increase in phosphorylation mediated by PMA and a marked decrease in phosphorylation in the presence of staurosporine (Fig. 10). These effects were also apparent in transfected COS cells which had been preincubated with DIDS. Densitometric analysis of duplicate experiments indicated that PMA induced a 2.0–2.5-fold increase and that staurosporine mediated a 3.0-fold decrease (Fig. 10b). There was no change in phosphorylation in response to 8-bromo-CAMP in concentrations and under conditions which activate protein kinase A-dependent phosphorylation (data not shown).

Finally, we examined the effect of activation or inhibition of protein kinase C, and presumably phosphorylation, on the functional activities of the ecto-ATPase/bile acid transport protein. The transfected COS cells were incubated for 45 min with 50 μM [3H]taurocholate. PMA or staurosporine was then added to the incubation for an additional 15 min. The cells were then assayed for bile acid transport as described previously. The results show that the protein kinase C stimulator, PMA, enhanced bile acid efflux, whereas the protein kinase C inhibitor, staurosporine, completely inhibited bile acid efflux (Fig. 11a). These modulators did not alter or confer bile acid efflux properties on COS cells transfected with the truncated construct or vector alone (data not shown). In contrast, neither PMA nor staurosporine affected ecto-ATPase activity of transfected COS cells (Fig. 11b). Neither activation of protein kinase C by PMA nor inhibition of protein kinase C by staurosporine affected the bile acid transport protein.
activation by staurosporine affected ATPase activity in transfected COS cells which had been preincubated with DIDS, a condition which inhibits ATPase activity (Fig. 6). These results suggest that phosphorylation, which is probably dependent on protein kinase C activation, is essential for bile transport properties but not for the ecto-ATPase activities of this single polypeptide.

**DISCUSSION**

Taken together, these results indicate that a single polypeptide, originally purified from rat liver canalicular vesicles, can by itself, or as a component of a multimeric protein complex, bind bile acids and mediate bile acid efflux and ecto-ATPase activity when expressed in heterologous cells. Several lines of evidence from our previous studies as well as this current study show that bile acid efflux and ecto-ATPase activities are separate properties of the same polypeptide. First, a ~110-kDa protein which was purified from rat liver canalicular vesicles on the basis of its ability to bind to immobilized bile acid (2) was shown here to have an amino acid sequence that was identical to the sequence of a cloned rat liver canalicular ecto-ATPase. The protein conferred bile acid transport activities on reconstitution of synthetic proteoliposomes, and antibody to this protein selectively inhibited bile acid transport in rat liver canalicular vesicles (2). Second, here we show that transfection of COS cells with cDNA encoding the ecto-ATPase conferred the expression of a ~110-kDa protein that was specifically recognized by antibody to the purified bile acid transport protein and conferred both ecto-ATPase and bile acid efflux activities. Efflux of [H]taurocholate by transfected cells was time-dependent, concentration-dependent, and had the kinetics characteristics of a carrier-mediated process, similar to the carrier-mediated process described previously in rat liver canalicular vesicles (14). Expression of a correctly targeted but deleted form of the ecto-ATPase did not confer bile acid efflux properties on the transfected cells, thus providing further evidence that these properties are attributable to the transferred intact gene product and not to a nonspecific effect. Third, the bile acid efflux properties conferred on transfected cells had pharmacological characteristics that were very similar to those of bile acid efflux in the standard rat liver canalicular vesicle experiments. These included the effects of ATP, nonhydrolyzable ATP analogs and vanadate, and photolabeling by an ATP analog (5, 15, 16). Fourth, vanadate and staurosporine, which are known to inhibit protein phosphorylation in a variety of systems, inhibited phosphorylation and bile acid efflux properties, but not ecto-ATPase activities that had been specifically conferred on the heterologous cell by DNA-mediated gene transfer. Thus, the two activities of this single polypeptide were selectively modulated by alteration in its structure and pharmacological manipulation of its function.

Although other transport proteins have ATPase activities and, in fact, depend on ATP hydrolysis to drive transport (Na-K-pump (18); sarcoplasmic reticulum Ca-pump (19, 20)), to our knowledge this is the first transport protein for which the ATPase is on the external face of the plasma membrane. Extracellular ATP has been shown to stimulate the chloride channel of cells containing the CFTR gene (21). However, the mechanism of action for ATP in this system is still not understood. The canalicular bile acid transport protein described here is also unusual in that it has only two, or perhaps only one, transmembrane domain (6). Many other transport proteins have multiple transmembrane domains (22). This may mean that the canalicular bile acid transporter is similar to the Na-K-pump, also called Na-K-ATPase, a heterodimeric molecule requiring only its catalytic subunit for functional activity in transfected cells (18). Alternatively this bile acid transporter may be a member of a novel class of transporters which have only a single transmembrane domain, such as the minK potassium channel (23, 24), an influenza virus M2 proton pump (25, 26), and a rabbit renal cortical neutral amino acid transporter (27).

Results of this study also indicate that the relatively long carboxyl-terminal cytoplasmic tail is essential for bile acid efflux and for ecto-ATPase activity. This almost certainly means that there is a bile acid-binding region within the cytoplasmic tail. However, in a preliminary computer search we have not identified any domains within this cytoplasmic tail that are also found in the sequence of other bile acid-binding proteins, including the sinusoidal sodium-dependent bile acid transport protein (28, 29) and a hepatic cytosolic fatty acid/bile acid-binding protein (30). Computer analysis has indicated the presence within the cytoplasmic tail of a sequence which is similar to the consensus sequences for protein kinase C- and protein kinase A-dependent phosphorylation. The results of our phosphorylation experiments are consistent with the notion that this sequence is important in bile acid transport. There is incorporation of [32P]orthophosphate into the full-length ecto-ATPase/bile acid transport protein but not into the ecto-ATPase/bile acid transport protein which has had its cytoplasmic tail deleted (Fig. 9). Inhibition of phosphorylation is associated with inhibition of bile acid efflux but not of the other functional activity of the polypeptide, its ecto-ATPase activity (Fig. 11).

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2 C. J. Sippel, F. J. Suchy, M. Ananthanarayanan, unpublished data.

![Fig. 11. The effect of protein kinase C modulators on bile acid transport and ecto-ATPase activity in transfected COS cells.](image-url)
it will be necessary to identify the specific site that is phosphorylated and to subject it to site-directed mutagenesis to establish the relationship between phosphorylation, bile acid binding, and transport activity.

Although our data establish that bile acid transport and ecto-ATPase properties are separate activities of the same polypeptide, the data do not establish, or even necessarily imply, that the two activities are functionally related. The bile acid efflux and ecto-ATPase activities were similarly affected by nucleotides and by DIDS, but these two functions could be dissociated by the effects of vanadate and staurosporine. Furthermore, one could argue that nucleotides, such as ATP and nonhydrolyzable ATP analogs, affect bile acid efflux and ecto-ATPase activities by completely different mechanisms and that DIDS inhibits ecto-ATPase activity in a relatively nonspecific manner by covalent modification of the polypeptide. Our data show that ecto-ATPase activity is not dependent on bile acid transport activity but definitive determination of whether bile acid transport and ecto-ATPase activity is dependent on ATP, but further studies need to be done to establish whether these activities are attributable to the molecule being studied in this report and whether this means that these cells may have the capacity to pump out relatively low concentrations of bile acids, or other organic anions, that may accumulate in these cells by passive diffusion. This would mean that the specialized function of the liver in excretion of bile acids is predominantly related to the presence of a sinusoidal bile acid transporter which permits uptake of bile acids in high concentration. Alternatively, the ecto-ATPase activity on many cell membranes could be attributed to the alternate isoform recently suggested by Lin et al. (34). Third, expression of the rat liver canalicular bile acid transporter is developmentally regulated with the antigen being initially detectable in whole liver homogenates by prenatal day 21, in canalicular vesicles by postnatal day 7, but with functional activity only detectable by postnatal day 14 (14). It will be important to determine whether ecto-ATPase activity is also developmentally regulated and whether there is a lag between the initial expression of the protein and its ecto-ATPase activity. Fourth, Bartles et al. (35) have shown that canalicular membrane proteins are transported to the basolateral (sinusoidal) membrane prior to reaching the ultimate localization at the apical (canalicular) membrane. It would, therefore, be of great interest to determine whether ecto-ATPase activity can be initially detected at the sinusoidal surface of hepatocytes before reaching the canalicular membrane. Fifth, the canalicular bile acid transport protein/ecto-ATPase is highly homologous with biliary glycoprotein 1 (BGP1), carboxyamino acid (CEA) and a number of related molecules (36, 37). The functions of polypeptides in the BGP1/CEA family are not known. Thus, further examination of the structural basis for bile acid transport and ecto-ATPase activities of the canalicular bile acid transport protein/ecto-ATPase described in this report may also shed some light on the physiologic roles of the BGP1/CEA polypeptides.

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REFERENCES

1. Nathannson, M. H., and Boyer, J. (1991) Hepatology 14, 551-558.
2. Sippel, C. J., Anathanarayanan, M., and Suchy, F. J. (1990) Am. J. Physiol. 251, G728-G737.
3. Ruets, S., Pichler, G., Hugentobler, G., Winterhalter, K., Kurz, G., and Meier, P. J. (1987) J. Biol. Chem. 262, 13324-13330.
4. Ruets, S., Hugentobler, G., and Meier, P. J. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6147-6151.
5. Muller, M., Ietsukawa, L., Berger, U., Kliinemann, C., Lucka, L., Schreyer, A., Kannicht, C., Reutter, W., Kurz, G., and Kappell, D. (1991) J. Biol. Chem. 266, 18920-18926.
6. Lin, S. H., and Guidotti, G. (1989) J. Biol. Chem. 264, 14009-14141.
7. Margolis, R. N., Schell, M. J., Taylor, S. L., and Hubbard, A. L. (1980) Biochem. Biophys. Res. Commun. 166, 582-586.
8. Cullen, B. R. (1987) Methods Enzymol. 152, 892-893.
9. Perlmuter, D. H., and Funsal, P. J. (1986) J. Biol. Chem. 261, 16499-16503.
10. Lederkremer, G. Z., and Lodish, H. F. (1991) Cell 66, 1237-1244.
11. Lowery, O. H., Rosebrough, N. J., Farr, A. L., and Randall, J. J. (1951) J. Biol. Chem. 193, 265-275.
12. Taylor, B. (1984) Nature 329, 840-942.
13. Skorngard, T. (1979) Cancer Res. 38, 1755-1791.
14. Novack, D. A., Sippel, C. J., Anathanarayanan, M., and Suchy, F. J. (1991) Am. J. Physiol. 260, G743-G751.
15. Adachi, Y., Kobayashi, H., Kurumi, Y., Shoryo, M., Kitono, M., and Yamamoto, T. (1991) Hepatology 14, 655-659.
16. Lin, S. H. (1980) J. Biol. Chem. 260, 10976-10980.
17. Lin, S. H. (1989) J. Biol. Chem. 264, 14403-14407.
18. Kennelly, P. J., and Krebs, E. G. (1980) J. Biol. Chem. 266, 15555-15558.
19. Shull, G., Schwartz, A., and Lingrel, J. B. (1986) Nature 316, 691-695.
20. MacLeenen, D. H. Brand, C. J., Korczak, B., and Green, N. M. (1986) Nature 326, 686-709.
21. Stutta, J. M., Cimini, T. C., Mason, S. J., Fulton, J. M., Clarke, L. L., and Boucher, R. C. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1621-1625.
22. Jennings, N., and Wong, A. H. (1989) Ann. Rev. Biochem. 58, 999-1027.
23. Rauscher, K. (1991) New Biol. 3, 315-322.
24. Goldstein, S. A. N., and Miller, C. (1991) Nature 353, 403-408.
25. Pinto, L. H., Holsinger, L. J., and Lamb, R. A. (1992) Cell 69, 517-528.
26. Skehel, J. J. (1992) Nature 358, 110-111.
27. Bertran, J., Werner, A., Moore, M. L., Stange, G., Markovich, D., Biber, C. J. Sippel, unpublished data.
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28. J., Testar, X., Zorzano, A., Palacin, M., and Murer, H. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5601-5605
29. Hagerbauch, B., Steiger, B., Foguet, M., Lubbert, H., and Meier, P. J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10629-10633
29. Ananthanarayanan, M., Bucavalus, J. C., Schneider, B. L., Sippel, C. J., and Suchy, F. J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10629-10633
30. Sweetser, D. A., Lowe, J. B., and Gordon, J. J. (1980) J. Biol. Chem. 261, 6788-6795
31. Ocklind, C., and Obrink, B. (1982) J. Biol. Chem. 257, 6788-6795
32. Hixson, D. C., McEntire, K. D., and Obrink, B. (1986) Cancer Res. 45, 3742-3749
33. Aurivillius, M., Hanson, O. C., Lazrek, M. B., Bock, E., and Obrink, B. (1990) FEBS Lett. 264, 267-269
34. Lin, S. H., Huang, Q. H., Fannagen, D., Hixson, D., and Culic, O. (1992) FEBS J. 6, 253
35. Barten, J. R., Ferraci, H. M., Steiger, B., and Hubbard, A. (1987) J. Cell Biol. 106, 1241-1251
36. Thomas, P., Toth, C. A., Saini, K. S., Jessup, M., and Steele, G. (1990) Biochim. Biophys. Acta 1032, 177-189
37. Hindo, Y., Neumaier, M., Hefta, S. A., Drzeniek, Z., Wagener, C., Shivley, L., Hefta, L., Shivley, J. F., and Paxton, R. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 86, 6959-6963
38. Harrison, B. C., and Mobley, P. L. (1991) J. Neurochem. 56, 1723-1730