Reconstitution of Bile Acid Transport in a Heterologous Cell by Cotransfection of Transporters for Bile Acid Uptake and Efflux*

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The rat liver canalicular bile acid transporter/ecto-ATPase/cell CAM 105 (CBATP) is a 110-kDa transmembrane phosphoglycoprotein that is thought to have bile acid efflux, ecto-ATPase, and cell adhesion properties. Its extracellular amino-terminal domain is highly homologous to carcinoembryonic antigen (CEA), a glycoprophosphatidyl inositol-anchored membrane protein with cell adhesion properties and a marker for adenocarcinoma. In the current study, we examined the possibility of more clearly defining the role of CBATP in bile acid efflux by cotransfecting a heterologous cell, the COS cell, with cDNAs for a bile acid importer, the ileal bile acid transporter (IBAT), as well as for CBATP. The results show that when IBAT mediates uptake of [3H]taurocholate to a level 20-fold higher than that achieved previously by nonspecific pinocytosis, CBATP mediates time-, temperature- and concentration-dependent efflux. Efflux of [3H]taurocholate mediated by CBATP in the cotransfected COS cells is saturable and has curvilinear kinetic characteristics (Vmax = 400 pmol/mg protein/min, Kc = 70 μM). It is inhibited by 4,4′-diisothiocyanostilbene-2,2-disulfonic acid and dependent on ATP but not dependent on membrane potential. Although CEA could not mediate bile acid efflux in COS cells cotransfected with IBAT and CEA, efflux of [3H]taurocholate was detected in COS cells cotransfected with IBAT and a chimeric molecule having the carboxyl-terminal tail and membrane spanning domain of CBATP and the amino-terminal extracellular tail of CEA. Taken together, these data provide further evidence that CBATP confers bile acid efflux properties on heterologous cells and that its cytoplasmic tail and membrane spanning segment are integral to this property. The data also establish a model system for more clearly defining the molecular determinants of bile acid transport mediated by this molecule. The net vectorial transport of bile acids into the biliary drainage system is the major determinant of bile secretion. Transport across the canalicular domain of the hepatocyte represents the rate-limiting step in this system. Studies in canalicular membrane vesicles have indicated that canalicular bile acid efflux is predominantly driven by ATP, but electrochemical membrane potential may also drive canalicular bile acid transport to a certain extent. Most data suggest that several distinct transporters, or transport systems, are involved (1–6).

The rat liver canalicular bile acid transport protein/ecto-ATPase/cell CAM 105 (CBATP) is one candidate for bile acid efflux activity at the canalicular membrane of hepatocytes. It is a ~110-kDa phosphoglycoprotein localized to the canalicular domain of hepatocytes. It was purified by bile acid affinity chromatography from detergent-solubilized rat liver canalicular membrane vesicles (7). Internal amino acid sequence analysis revealed it to be identical to the rat liver ecto-ATPase and to the cell adhesion molecule cell CAM 105. It has a carboxy-terminal cytoplasmic tail of 71 amino acids, a single membrane-spanning domain, and a large extracellular amino-terminal tail of 423 amino acids, which has extensive homology with carcinoembryonic antigen (CEA) and the immunoglobulin supergene family. Transfections studies have shown that CBATP mediates ecto-ATPase, cell adhesion, and bile acid efflux activities in heterologous COS cells (8, 9). Mutagenesis studies have shown that R98 within an ATPase consensus sequence at the extreme amino terminus is required for ecto-ATPase activity and that the 108 amino acid amino-terminal domain is required for cell adhesion activity (10, 11). Deletion of the cytoplasmic tail is associated with loss of bile acid efflux, ecto-ATPase, and cell adhesion activities, even though the protein is appropriately localized to the external surface of the plasma membrane and can bind ATP (8, 9). Site-directed mutagenesis of phosphorylation consensus sequences in the cytoplasmic tail shows that protein kinase C-dependent phosphorylation of Ser503 is required for bile acid efflux activity, that tyrosine kinase-dependent phosphorylation of Tyr488 regulates bile acid efflux activity, but neither phosphorylation is necessary for ecto-ATPase activity. Finally, bile acid efflux activity of CBATP was found to be dependent on ATP, particularly extracellular ATP, but not on its own ecto-ATPase activity (12).

In these previous studies, the bile acid efflux activity of CBATP was demonstrated in transfected COS cells by first loading the cells with [3H]taurocholate via nonspecific pinocytosis using the approach that had been pioneered in studies of efflux of chemotherapeutic agents by the multidrug resistance gene products (MDR) (8). To get sufficient nonspecific uptake, the membrane potential of the transfected COS cells had to be clamped pharmacologically with valinomycin (8). Although this system allowed us to show that CBATP had bile acid efflux activity and that this could be proven by a number of genetic

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* The abbreviations used are: CBATP, canalicular bile acid transport protein; CEA, carcinoembryonic antigen; DMEM, Dulbecco’s modified Eagle’s medium; IBAT, ileal bile acid transport protein; DIDDS, 4,4′-diisothiocyanostilbene-2,2-disulfonic acid; ATPγS, adenosine 5′-O-(thiotriphosphate).

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criteria, the assay was cumbersome and was not amenable to studying the role of membrane potential in bile acid efflux. To develop a more physiological system and a system that could be more easily manipulated pharmacologically, we have established a model system in which uptake of \(^{[3]H}\)taurocholate is mediated by transfection of a cloned bile acid importer, IBAT, and efflux is assayed by cotransfection of CBATP cDNA.

**MATERIALS AND METHODS**

**Plasmid Constructs**—Wild-type and mutant rat CBATP constructs have been described previously (8, 10, 11). This includes pExp3, R98A-CBAP, Y488S-CBAP, T502S, S503A-CBAP, and truncated CBATP. The hamster IBAT construct (IBAT-44 final) has also been described previously (13). The CEA cDNA (gpc3-humcea; Ref. 14), kindly provided by Thomas Barnett, was subcloned into the HindIII-XhoI site of pCMDS. Three new constructs, R98A, CEA, CBATP-CEA chimera, and CBATP-R98ACEA chimera, were generated using the polymerase chain reaction overlap extension technique (15) and then subcloned into the pCMDS vector.

For R98ACEA, the outside primers corresponded with nucleotides 85–100 and 993–1012 of CEA together with a spacer of four nucleotides, a new EcoRI restriction site, and a new BamHI restriction site (5'-TCATGAAATCAGAACACAGACG-3'; 5'-CGCACTATTAGATGATGATGACAGC-CA-3'). These primers corresponded to nucleotides 932–1148 of CEA (5'-GGCATACCTGTCGTAAGCAGATATG-ACT-3', 5'-ATAGTCTCTGTGACCTGATCTGGCG-3'). The resulting polymerase chain reaction fragment was subcloned into wild-type CEA in the pCMDS vector, which had been digested previously with EcoRI/BamHI and purified away from the wild-type internal fragment.

For the CEA-CBAP chimera, the outside primers corresponded to nucleotides 1563–1585 of CEA and nucleotides 1628–1651 of CBATP with new EcoRI and XhoI restriction sites, and the inside primers corresponded to nucleotides 2122–2136 of CEA (five amino acids just external to the membrane insertion site of CEA) and nucleotides 1316–1330 of CBATP (transmembrane domain of CBATP): outside primers (5'-CAGTGGCCGACACAGCAGACAGC-3'; 5'-GATTTCATGATGGGACAGACAGACAGA-3'); inside primers (5'-TGTGGCGACACAGCAGACAGACAGA-3'; 5'-AAAGCATCACAGTCATCTGGCCTGTTCCA-3'). The resulting 0.95-kilobase polymerase chain reaction fragment was digested with BglII and XhoI and cloned into pBluescript together with a 1.5-kilobase BalI/EcoRI partial digest of wild-type CEA. The resulting insert was removed at HindIII/XhoI to be subcloned into pCMDS. A similar strategy was used for the CBATP-R98ACEA chimera; using the R98ACEA construct described above. In each case, the constructs were characterized by restriction map analysis (16, 17) and by dideoxynucleotide sequencing (18) to confirm the construct and to exclude polymerase chain reaction sequence artifacts.

**Cell Culture and Transfection**—COS 1 cells were transfected by the DEAE-dextran method and used for experimental purposes 48 h after transfection (19). In specific experiments, cellular ATP was depleted by incubating transfected COS cells for 20 min at 37 °C in DMEM without glucose but supplemented with 20 mM 2-deoxyglucose and 10 mM sodium azide. Under these conditions, cellular concentrations of ATP could be lowered from 800–1200 \(\mu\)M to 6–8 \(\mu\)M, as determined by the ATP luciferase assay (20). Results were normalized for protein concentration as determined by the Lowry assay (21). Under these conditions, cell viability as determined by trypan blue exclusion (22) was not significantly different between these transfected cells and control cells (data not shown).

**Bile Acid Uptake Assay**—Transfected COS cells were incubated with DMEM supplemented with \(^{[3]H}\)taurocholate in excess. At the end of the uptake period, cell monolayers were lysed in 1 N NaOH, and the cell lysates were subjected to scintillation counting. In separate experiments, the time, temperature, and concentration of \(^{[3]H}\)taurocholate were varied to determine the optimal conditions for uptake.

**Bile Acid Efflux Assay**—Transfected COS cells were incubated with \(^{[3]H}\)taurocholate under optimal conditions for uptake. At the end of the uptake period, monolayers were washed extensively in PBS and incubated for specified time intervals in DMEM alone for the efflux period. In some experiments, 5 \(\mu\)M ATP, 1 mM DIDS, and/or unlabeled taurocholate were added. Extracellular medium was harvested, and cell monolayers were lysed in 1 N NaOH. The extracellular medium and cell lysates were then subjected to scintillation counting. Counts in the extracellular medium were converted to picomoles on the basis of the specific activity of the initial \(^{[3]H}\)taurocholate and then plotted as pmol/milligram protein/minute. Kinetic data \((K_m\) and \(V_{max}\)) were determined on the basis of the resulting curves.

**Ecto-ATPase Assay**—Ecto-ATPase activity was measured by a method described previously (23) 48 h after transfection.

**Analytical Techniques**—For Western blot analysis, antibody to CBATP was used in a protocol described previously (7). Methods described previously were used for studies of biosynthesis (24), cell surface iodination (25), and immunoprecipitation followed by SDS-polyacylamide gel electrophoresis/fluorography (24).

**RESULTS**

**Cotransfection of COS Cells with IBAT and CBATP**—We determined the conditions under which uptake of \(^{[3]H}\)taurocholate was saturated in COS cells transfected with IBAT alone or IBAT and CBATP cDNA together. To determine the duration of time necessary for saturation of uptake, transfected COS cells were incubated at 22 °C with \(^{[3]H}\)taurocholate, 400 \(\mu\)M, for several different time intervals (Fig. 1A). Cells were lysed in 1 N NaOH and subjected to scintillation counting. Uptake was time-dependent, reaching a plateau within 20 min. There was no significant difference between cells transfected with IBAT alone and cells transfected with IBAT and CBATP together. Then the transfected COS cells were incubated for 20 min with \(^{[3]H}\)taurocholate, 400 \(\mu\)M, at several different temperatures (Fig. 1B). Uptake was temperature-dependent, reaching a plateau at 22 °C. There was no significant difference between cells transfected with IBAT alone and those transfected with IBAT and CBATP together. Next, the transfected COS cells were incubated for 20 min at 22 °C in \(^{[3]H}\)taurocholate in several different concentrations (Fig. 1C). The results show that uptake was concentration-dependent, reaching a plateau between 200 and 400 \(\mu\)M. In COS cells transfected with IBAT and CBATP, there was a minimal decrease in the amount of uptake as compared with COS cells transfected with IBAT alone. This is probably due to efflux mediated by cotransfected CBATP. Efflux mediated by cotransfected CBATP does not have a more significant effect in Fig. 1C or any effect in Fig. 1, A and B, because these experiments were done in the absence of exogenous ATP. Because efflux mediated by CBATP is stimulated by exogenous ATP (see Fig. 3), this means that there will be minimal efflux mediated by CBATP during uptake studies done in the absence of exogenous ATP. Even when exogenous ATP is present, CBATP pumps \(^{[3]H}\)taurocholate out of cells less efficiently than IBAT pumps \(^{[3]H}\)taurocholate into cells. For uptake mediated by IBAT, \(K_m=23 \mu M\) and \(V_{max} = 396 \text{ pmol/mg protein/min}\) (13), and for efflux mediated by CBATP in the presence of exogenous ATP, \(K_m=70 \mu M\) and \(V_{max} = 400 \text{ pmol/mg protein/min}\) (Fig. 2A). When uptake of \(^{[3]H}\)taurocholate was assayed in the presence of exogenous ATP (5 min), there was −40–50% reduction in uptake after 60 min in COS cells cotransfected with IBAT and CBATP as compared with COS cells transfected with IBAT alone (Fig. 1D).

Taken together, the experiments in Fig. 1 (A–C) show that uptake reaches a plateau at 200–400 \(\mu\)M in the cotransfected cells and establish a time of 20 min, temperature of 22 °C, and concentration of \(^{[3]H}\)taurocholate of 400 \(\mu\)M as optimal for the subsequent studies. In each case, the uptake of \(^{[3]H}\)taurocholate was 20-fold or more greater in COS cells transfected with IBAT alone or both IBAT and CBATP than untransfected COS cells and COS cells transfected with CBATP alone and pharmacologically clamped with valinomycin (8).

Next, we examined the possibility that CBATP mediated bile acid efflux under these conditions. For Fig. 2A, COS cells were transfected with IBAT alone, cotransfected with IBAT and CBATP, or cotransfected with IBAT and a mutant CBATP (T502A, S503A-CB-ATP). Our previous studies had shown that this mutant CBATP did not undergo protein kinase C-mediated phosphorylation and lacked bile acid efflux activity, even
though it was appropriately targeted to the external surface of the plasma membrane (12). After 48 h, the cells were incubated at 22 °C for 60 min with [3H]taurocholate in several different concentrations. At the end of this time interval, the cells were washed extensively and incubated at 22 °C for several different time intervals up to 60 min. B, temperature dependence. Cells were incubated at 22 °C for 60 min with [3H]taurocholate in several different concentrations. At the end of these incubations, the cells were rinsed extensively, homogenized, and subjected to scintillation counting. D, effect of ATP. Cells were incubated at 22 °C with 400 μM [3H]taurocholate for several different time intervals up to 60 min. This incubation was done in the absence or presence of 5 mM ATP. The results of three replicate samples at each data point are reported as mean ± 1.0 S.D. (bars). □, IBAT; ●, IBAT + ATP; ○, IBAT + CBATP; ●, IBAT + CBATP + ATP.

Next, we examined the time course of efflux mediated by CBATP (Fig. 2B). COS cells transfected with IBAT alone or cotransfected with IBAT and CBATP were incubated for 60 min at 22 °C with [3H]taurocholate 400 μM. At the end of this time interval, the cells were washed extensively and then incubated at 22 °C for several different time intervals in medium supplemented with unlabeled taurocholate (400 μM) and 5 mM ATP in the absence or presence of 1 mM DIDS. At the end of each time interval, the extracellular medium was harvested, and the cell monolayers were lysed for analysis by scintillation counting. Results are reported as a relative percentage using counts present in the cell lysate at time 0 as arbitrarily designated 100%. There was no difference in the counts present in the cell lysates at time 0 for cells transfected with IBAT alone or cells cotransfected with IBAT and CBATP. For cells cotransfected with IBAT and CBATP, the results show that there is time-dependent disappearance of radioactivity from the cells between time 0 and 5 min, coincident with the appearance of radioactivity in the extracellular medium. Almost 80% of the initial radioactivity has disappeared from the cells by 5 min of the chase period, and a similar percentage has appeared in the extracellular medium. The majority of this disappearance from cell monolayers and appearance in extracellular medium is DIDS-sensitive. There is, however, some radioactivity that leaks from the cells in the presence of DIDS. Interestingly, this DIDS-insensitive fraction has different kinetics, reaching a
plateau within 3 min. For cells transfected with IBAT alone, there is some time-dependent disappearance of radioactivity from the cells and appearance of radioactivity in the extracellular medium. It is much less than that observed in the cotransfected cells. Only 30% of the initial radioactivity disappears, even by 10 min. This disappearance is completely insensitive to DIDS and is identical in magnitude and kinetics to the DIDS-insensitive fraction of the cotransfected cells, therefore providing evidence that it represents nonspecific diffusion. Unlabeled taurocholate was used in this experiment to optimize the chase effect, but similar results have been observed without unlabeled taurocholate (data not shown). Taken together, these studies show that CBATP can mediate specific, facilitated efflux of taurocholate in cotransfected COS cells and that its efflux properties can be detected more easily, over a longer duration of time and in the absence of the pharmacologic agent valinomycin originally used to promote nonspecific uptake of taurocholate by clamping the membrane potential.

Effect of ATP and Membrane Potential on Bile Acid Efflux in Cotransfected COS Cells—Now we could use this assay to examine the possibility that bile acid efflux mediated by CBATP is driven by ATP and/or membrane potential differences. COS cells were cotransfected with IBAT and CBATP and then, 48 h later, the cotransfected cells were incubated in 2-deoxyglucose...
Fig. 3. Effect of ATP on bile acid efflux in cotransfected COS cells. Cells were cotransfected with IBAT and CBATP cDNA and studied 48 h later. At that time, the cells were incubated for 20 min at 22 °C with 400 μM [3H]taurocholate to load the cells as well as 20 mM 2-deoxyglucose and 10 mM sodium azide to deplete cellular ATP. Previous studies have shown that these conditions severely deplete cellular ATP levels but do not affect cell viability or one function of CBAT, its ecto-ATPase activity (20). These conditions did not significantly alter uptake of [3H]taurocholate (data not shown). The cells were then washed and incubated for 5 min at 22 °C with fresh unlabeled medium in the absence or presence of 1 mM DIDS and in absence or presence of ATP in several different concentrations. Results are reported as mean ± 1.0 S.D. (bars). $K_m = 10 \mu M$; $V_{max} = 300$ pmol/mg protein/min.

To determine whether the electrochemical potential of the membrane drives bile acid efflux activity mediated by CBATP, we examined the effect of clamping the membrane potential with valinomycin. Cells were cotransfected with IBAT and CBATP and then, 48 h later, incubated with 150 μM valinomycin, an ionophore that clamps the membrane potential (8, 10, 11). Cells were then washed and incubated for 5 min at 22 °C with fresh unlabeled medium and sodium azide under conditions associated with depletion of cellular ATP but without affecting cell viability. During this same 20-min interval, 400 μM [3H]taurocholate were also added to the medium for uptake studies. The cells were then washed extensively, and separate monolayers were incubated in the absence or presence of 1 mM DIDS and in the absence or presence of exogenous ATP in several different concentrations for an efflux assay of 5 min (Fig. 3). The results show that there is no efflux in the absence of ATP. It also shows that efflux is absolutely dependent on ATP. The effect of extracellular ATP is concentration-dependent and saturable with a $K_m \approx 10 \mu M$ and $V_{max}$ of 300 pmol/mg protein/min.

To establish the validity of the cotransfection assay, we examined a series of positive and negative controls in the assay. In each case, these controls, shown schematically on the basis of their presumed relationship to the plasma membrane in Fig. 4, were cotransfected with IBAT. The negative controls included a truncated CBATP construct in which 66 of the 71 amino acids in the cytoplasmic tail have been deleted and two constructs in which specific amino acids required for phosphorylation have been deleted, Y488F-CBATP and T502A, S503A-CBATP. These three constructs did not mediate bile acid efflux in our previous assay system (12). The R98A-CBATP construct represented a positive control. It has a mutation in the ectoplasmic ATPase consensus sequence, which abrogates ecto-ATPase activity but not bile acid efflux activity. We also used several new controls based on the structure of CEA. CBATP shares extensive homology with CEA in its amino-terminal extracellular tail, particularly the extreme amino-terminal half of CEA. However, CEA lacks a membrane-spanning domain and cytoplasmic tail. It is linked to the membrane by a phosphatidylinositol-glycan moiety (29). Because our previous studies had shown that the bile acid efflux activity of CBATP depended on its cytoplasmic tail, we doubted that CEA would have bile acid efflux activity. We also generated a chimeric construct in which the cytoplasmic tail and membrane-spanning domain of CBATP were fused to the extracellular domain of CEA, a construct we called the CBATP-CEA chimera, predicting that it would now possess bile acid efflux activity. Finally, we generated two additional constructs that were based on the observation that CEA has an ATPase consensus sequence almost identical to that of CBATP and at the exact same distance from the amino terminus as in CBATP. For the last two constructs, we subjected the CEA construct and the CBATP-CEA chimeric construct to site-directed mutagenesis of Arg98 to alanine, the mutation that abrogated ecto-ATPase activity in CBATP. These constructs are referred to as R98A-CEA and CBATP-R98A-CEA.

We examined the expression of all of these mutants after transfection of COS cells by Western blot analysis, immunoprecipitation after metabolic labeling, and immunoprecipitation after cell surface labeling using antibodies to CBATP and CEA. In Western blot analysis (Fig. 5A), we detected equivalent amounts of an $\sim$180-kDa polypeptide for CEA, R98A-CEA, CBATP-CEA, and CBATP-R98A-CEA and $\sim$110-kDa polypeptide for R98A-CBATP, Y488F-CBATP, T502A, S503-CBATP, and wild-type CBATP. There was an equivalent amount of a $\sim$100-kDa polypeptide for truncated CBATP but no polypep-
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Fig. 5. Expression of wild-type, mutant, and chimeric CEA and CBATP molecules in transfected COS cells. Cells were analyzed by Western blot analysis (A), biosynthetic labeling (B), and cell surface labeling (C). Left, relative electrophoretic migration of molecular mass markers. We could not detect a decrease in electrophoretic migration of the chimeric CEA molecules as compared with the wild-type molecule on these 10% gels, but there was a decrease in electrophoretic migration of ~8 kDa for the chimeric CEA molecules on 6.5% gels in which proteins of 180 kDa were resolved in the middle of the gel (data not shown).

Fig. 6. Bile acid efflux in COS cells cotransfected with IBAT and wild-type, mutant, or chimeric CBATP or CEA genes. Cells were subjected to bile acid efflux assays 48 h after transfection. Cotransfected cells were incubated for 60 min at 22 °C in medium supplemented with 400 μM [3H]taurocholate. The cells were then washed and incubated for 5 min at 22 °C in medium supplemented with ATP in the absence or presence of 1 mM DIDS.

Results showed that there were similar levels of synthesis of wild-type, chimeric, and mutant CEA molecules. Our previous studies have shown that the truncated CBATP, R98A-CBATP, CEA, R98A-CEA, and CBATP-CEA chimera confer ecto-ATPase activity on the transfected COS cells but control vector alone, IBAT and R98A-CBATP, R98A-CEA, and CBATP-R98A-CEA-CBA therefore have these properties.

We now examine the bile acid efflux activity in these cotransfected COS cells. Separate monolayers that had been cotransfected 48 h earlier were incubated for 1 h at 22 °C in DMEM supplemented with 400 μM [3H]taurocholate acid. The cells were then washed extensively and then incubated in DMEM supplemented with 5 μM ATP in the absence or presence of DIDS for 5 min. The resulting cell culture medium samples were subjected to scintillation counting (Fig. 6). The results show that there is significant efflux of taurocholate by COS cells cotransfected with IBAT and CBATP, with IBAT and R98A-CBATP, and to a lesser extent, by IBAT and Y488F-CBATP but not by COS cells transfected with IBAT alone or by COS cells cotransfected with IBAT and truncated CBATP or IBAT and T502A, S503A-CBATP. These results are similar to
The results of the studies reported here establish the validity of a new system for assaying carrier-mediated bile acid efflux. In our previous studies, we used a system in which transfected cells were labeled with \(^{3}H\) taurocholate by nonspecific pinocytosis, which required pharmacologic clamping of the membrane potential with valinomycin (8, 10). By cotransfecting the genes for a transporter that mediates uptake of bile acids and for a candidate efflux transporter, there is an ~20-fold increase in loading of the cells and no need for clamping of the membrane potential. Furthermore, the efflux capacity of candidate gene products can be detected for a much longer period of time. In the studies reported here, we could see the effects of CBATP for as long as the studies were done, 60 min, whereas the effects of CBATP were only apparent for several minutes in the previous assay system. Results of the study provide further evidence that CBATP can mediate bile acid efflux. The effect of CBATP is time-dependent, concentration-dependent, saturable, and has kinetic characteristics that are similar to those reported previously in rat liver canalicular membrane vesicles (7). The effect of CBATP on efflux of \(^{3}H\) taurocholate is modulated by one pharmacologic state (ATP depletion) but not by another (valinomycin). This effect is abrogated by truncation of the cytoplasmic tail and by site-directed mutagenesis of one phosphorylation site (S503A) in the cytoplasmic tail, is decreased but not eliminated by site-directed mutagenesis of another phosphorylation site (Y488F), and is unaffected by site-directed mutagenesis of an ATP consensus sequence in the extracellular tail. This effect can be conferred on a related protein, CEA, by fusing its cytoplasmic tail and membrane-spanning domain onto that protein. Site-directed mutagenesis of the ATPase consensus sequence in the extracellular tail of the chimera does not alter bile acid efflux activity, even though it abrogates ecto-ATPase activity. The reduction in bile acid efflux activity mediated by Y488F-CBATP and the abrogation of bile acid efflux activity mediated by T502A, S503A-CBATP are again notable, in that neither of these mutations affected ecto-ATPase activity. None of the alterations in bile acid efflux activity or ecto-ATPase activity, resulting from pharmacologic or genetic manipulation, could be attributed to alterations in biosynthesis, half-life, or targeting to the external surface of the plasma membrane.

One particularly interesting result of this study is the bile acid efflux activities of the CBATP-CEA chimera. Our previous studies had shown that bile acid efflux activity was lost upon deletion of the cytoplasmic tail of CBATP (8). Here the studies show that cytoplasmic tail and membrane-spanning segment of CBATP can confer bile acid efflux properties on the extracellular amino-terminal tail of a related molecule, CEA. Presumably the cytoplasmic tail, with or without the membrane-spanning segment, plays an essential role in binding of the bile acid substrate. It was also interesting to find that the cytoplasmic tail and membrane-spanning segment of CBATP could confer ecto-ATPase activity on the same CBATP-CEA chimeric molecule. Our previous studies had shown that CBATP lost its ecto-ATPase activity when its cytoplasmic tail was deleted, but we could not exclude the possibility that this was simply explained by a failure of the truncated CBATP to be in the appropriate conformation (8). Taken together with the current studies, however, it is now apparent that the cytoplasmic tail and membrane-spanning domains transmit information that is essential for ecto-ATPase activity and can even transmit that information to CEA, a molecule which has an ectoplasmic ATPase consensus sequence and can bind ATP but does not ordinarily possess ecto-ATPase activity.

Although the results of this study show that CBATP can mediate bile acid efflux in a model system, they do not establish the role or contribution of CBATP to bile acid transport at the canalicular membrane under physiologic conditions. The results do show that CBATP cannot account for bile acid efflux driven by the electrochemical potential differences at the membrane. Bile acid transport mediated by CBATP is absolutely dependent on ATP. However, our previous studies have suggested that it is extracellular ATP that induces bile acid efflux mediated by CBATP (8, 10). Perhaps CBATP provides a mechanism for bile acid efflux in response to canalicular ATP or in response to ATP released during liver cell injury and that there are other molecules/systems responsible for canalicular bile acid transport in response to intracellular ATP (classical P-
type ATPase transporter) as well as canalicular bile acid transport in response to the electrochemical membrane potential.

In contrast to most conventional transport proteins that have multiple transmembrane domains (30), CBATP has only two, or perhaps one, transmembrane domain. This may mean that it is a member of a nonconventional class of transporters that only have a single transmembrane domain and includes the minK potassium channel (31, 32) and an influenza virus M2 proton pump (33, 34). However, our recent studies of the cell adhesion properties of CBATP in infected S9 cells (39) have indicated that CBATP molecules bind to each other. Clustering of CBATP molecules could, therefore, bring two or more CBATP molecules and their transmembrane domains into close proximity to potentially form a pore in the membrane for bile acid efflux.

Although it was not the major focus of this study, the results do indicate that CEA can bind extracellular ATP. Because the ATPase consensus sequence responsible for ATP binding is highly conserved among members of the CEA family, it is likely that ATP binding is a property of many of these molecules. There is apparently a substantial amount of ATP and other adenine nucleotides in bile (35). However, whether CEA molecules sticking out into the lumen of the biliary canalculus can bind ATP or other nucleotides under physiologic conditions or whether binding of ATP by CEA plays a specific physiologic role is not known.

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