Reconstitution and characterization of ATP-dependent bile acid transport in human and rat placenta

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Bile acid (BA) transport across the human microvillus maternal-facing trophoblast plasma membrane (mTPM) has been recently reported to be stimulated by the presence of ATP [Marin, Bravo, El-Mir and Serrano (1993) *J. Hepatol.* **18**, S41]. Reconstitution of BA transport activity in proteoliposomes from human mTPM is reported in this paper. Typical characteristics of BA transport in native mTPM vesicles, including a requirement for ATP hydrolysis and inhibition by other BA species, were preserved in proteoliposome preparations. BA transport into 20- and 14-day-

gestation rat mTPM vesicles was also stimulated by the presence of ATP as noted in human mTPM and in the rat liver canalicular membrane. Besides this functional similarity, these ATP-dependent carriers may share structural characteristics, as demonstrated by studies using an antibody (100Ab) raised against the 100 kDa BA carrier of the canalicular membrane from rat liver which recognized proteins in both human and rat brush-border trophoblast membranes.

INTRODUCTION

Bile acids (BAs) are steroids of great biological relevance. Besides their well-known role in bile formation and digestion/absorption of dietary fats, they have been reported to participate in many other physiological and pathophysiological processes, including mastocyte activation [1], stimulation of intestinal iron uptake [2], and, if present at high concentrations, modification of cellular proliferation [3,4]. The fetal liver is able to synthesize and conjugate BAs early in intra-uterine life. The existence of carrier systems for BAs in fetal- (basal) and maternal- (apical or brush-border) placental membranes has been reported previously [5–11]. The initial event in the transplacental transfer of BAs from the fetal to the maternal circulation is translocation across the trophoblast basal membrane. This carrier is sensitive to inversely directed bicarbonate gradients [6], but is insensitive to the presence of sodium and proton gradients, as well as to differences in electrical potential [5]. Trans-gradients of other inorganic (Cl^- , SCN^- , HPO_4^{2-} and SO_4^{2-}) and organic (taurine, glycine, acetate and lactate) anions fail to stimulate this BA transport [6]. Changes in the number and position of hydroxyl groups as well as in the amino acid moiety in amidated BAs modify the behaviour of the carrier [7]. Moreover, specificity is not restricted to BAs, because the presence of other non-BA cholephilic organic anions significantly affects the interaction of BAs with the carrier [8].

Intracellular BA molecules must traverse the trophoblast brush-border membrane in order to reach the maternal circulation. This transfer has been reported to occur via: (1) an electrogenic-facilitated diffusion system [11], as in the liver canalicular plasma membrane [12–14]; (2) a hydroxyl/BA exchange system [10]; and (3) an ATP-dependent transport system [9], as also occurs across the hepatocyte canalicular plasma membrane [15–17]. It is, at present, unclear whether some

or all of these systems are present on the trophoblast apical membrane. As an initial step in the clarification of these issues, we chose to solubilize and reconstitute trophoblast apical membrane proteins into proteoliposomes, thus allowing BA transport activity to be monitored and followed during future attempts at purification of the microvillus membrane BA carrier. Moreover, antibodies raised against the 100 kDa glycoprotein thought to be responsible for ATP-dependent BA transport across the rat hepatocyte canalicular membrane (100Ab [18]) were used in experiments on human and rat brush-border placental membranes to test the hypothesis that structurally similar proteins might be located in the apical membranes of both the syncytiotrophoblast and the hepatocyte [9,15–17].

MATERIALS AND METHODS

Materials

[^3H]Taurocholate (TCA) (2.1 Ci/mmol), [^3H]dihydroalprenolol (59.50 Ci/mmol) and [^3H]L-alanine (76.90 Ci/mmol) were obtained from Dupont–New England Nuclear (Boston, MA, U.S.A.). Unlabelled TCA, taurochenodeoxycholate (TCDCA), β , γ -imidoadenosine-5'-triphosphate (p[NH]ppA), Tris, Hepes, polyethylene glycol 8000 (PEG), EDTA, PMSF, dithiothreitol (DTT) and phosphatidylcholine were obtained from Sigma (St. Louis, MO, U.S.A.). SDS, acrylamide, bisacrylamide, ammonium persulphate and tetramethylethylenediamine (TEMED) were purchased from Bio-Rad (Richmond, CA, U.S.A.). Nitrocellulose sheets were purchased from Schleicher and Schuell (Keene, NH, U.S.A.). The rabbit anti-(rat polyclonal IgG) antibody against rat liver ecto-ATPase was a kind gift from Dr. Fred Suchy, New Haven, CT, U.S.A. Horseradish peroxidase-conjugated goat anti-(rabbit IgG) antibodies were

Abbreviations used: mTPM, maternal-facing (apical) trophoblastic plasma membrane; BA, bile acid; TCA, taurocholate; TCDCA, taurochenodeoxycholate; p[NH]ppA, β , γ -imidoadenosine 5'-triphosphate; PEG, polyethylene glycol 8000; DTT, dithiothreitol.

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obtained from Gibco BRL (Gaithersburg, MD, U.S.A.). ECL Western blotting detection reagents and hyperfilm-MP were from Amersham (Arlington Heights, IL, U.S.A.). The enzyme N-glycanase (EC 3.5.1.52) was purchased from Genzyme (Cambridge, MA, U.S.A.). Nitrocellulose filters were Gelman type GN-6 (0.45 μm). Labelled and unlabelled TCA were more than 95% pure as indicated by the manufacturer.

Animals

Timed pregnant Sprague-Dawley rat dams were obtained from Zivic-Miller (Zelienople, PA, U.S.A.). Animals were housed in temperature-controlled rooms with 12:12 h light-dark cycles and fed diet and water *ad libitum*. These studies were approved by the Laboratory Medical Ethics Committee of the University of Florida.

Preparation of human brush-border and rat maternal-facing (apical) plasma membrane (mTPM) vesicles

mTPM vesicles were purified from normal human placentas obtained at term from healthy pregnancies as well as from rat placentas at 14 and 20 days of gestation. These vesicle preparations were prepared by an adaptation of the method described by Booth et al. [19]. Briefly, within 30 min after delivery, the human placenta was stripped of the decidual tissue and the basal lamina. In the case of rat placentas, after anaesthetizing the animal, the placentas were obtained operatively and the decidua removed. The whole procedure was carried out at 4 °C. The tissue was washed four times with phosphate-buffered saline (PBS; 83 mM NaCl, 22.2 mM Na_2HPO_4 , 5.5 mM KH_2PO_4 , pH 7.4) and then it was minced. An aliquot was homogenized to be used as a reference when measuring the enrichment of membrane markers in the final vesicle preparations. The rest of the minced placenta was washed four times with PBS and stirred for 60 min in PBS. Afterwards, the mixture was filtered through nylon mesh and the supernatant centrifuged first at 800 *g* for 10 min and then at 10000 *g* for another 10 min. The pellets were discarded and the last supernatant was centrifuged again at 150000 *g* for 25 min. The resulting pellet was resuspended with 30 ml of Tris/mannitol buffer (300 mM mannitol, 2 mM Tris-base, pH 7.0) and homogenized by 12 strokes of a Potter-Elvehjem motor-driven Teflon homogenizer. Subsequently, 30 ml of Tris/mannitol buffer plus 0.6 ml of 1 M MgCl_2 was added to the suspension, which was again homogenized. This mixture was incubated on ice for 10 min (stirring every 2 min) and then centrifuged at 2200 *g* for 12 min. The supernatant was centrifuged again at 150000 *g* for 25 min. The pellet containing the mTPM was resuspended in the desired medium (normally buffer A: 250 mM sucrose, 100 mM KNO_3 , 10 mM MgCl_2 , 0.2 mM CaCl_2 , 10 mM Hepes/Tris, pH 7.4) and stored in liquid nitrogen. Before carrying out transport experiments, the vesicles in buffer A were first thawed and then vesiculated by six passes through a 25-gauge needle. Apical membrane vesicles derived from rat placenta were produced by an adaptation of the above method as described previously [20].

The purity and contamination of the preparations were assayed by dihydroprenolol binding as a marker for the basal plasma membrane and alkaline phosphatase activity (EC 3.1.3.1) as a marker for mTPM. Characteristics of the rat apical membrane preparation were as previously described [20]. Proteins were measured by the method of Lowry, as modified by Fafournoux et al. [21]. BSA was used as the standard.

Solubilization and reconstitution of plasma membrane proteins

The first step was carried out by using 100 mM octylglucoside in appropriate solubilization buffer (300 mM sucrose, 0.1 mM EDTA, 1 μM PMSF, 10 mM Hepes/Tris, pH 7.4). All procedures were carried out at 4 °C. The mixture with vesicles and detergent was incubated with stirring for 30 min and then centrifuged at 100000 *g* for 30 min. The pellet was discarded and the supernatant containing solubilized proteins was used for the reconstitution of BA carrier system(s) in proteoliposomes.

The method used in the reconstitution of the BA carrier protein(s) obtained from mTPM was that of Tamarappoo and Kilberg [22] with some modifications as described below. The whole protocol was carried out at 4 °C. First the detergent was removed by incubating the solubilized membrane proteins (1 vol.) with 2 vol. of 30% PEG (20% final concentration) for 15 min. The mixture was then centrifuged at 100000 *g* for 30 min and the supernatant was discarded. The pellet was washed three times with solubilization buffer to remove PEG and then it was resuspended (0.5–1.0 mg of protein/ml) in STAB buffer (0.05% Triton X-100, 20% glycerol, 2 mM EDTA, 2 mM DTT, 0.25 mg/ml phosphatidylcholine, 10 mM Tris, pH 7.4). The mixture was sonicated for 10 s in a bath-type sonicator (Laboratory Supplies, Hicksville, NY, U.S.A.) and centrifuged at 13600 *g* for 2 min. The protein suspension (1 vol.) was incubated again with 2 vol. of 30% PEG as before and centrifuged at 150000 *g* for 30 min. The pellet was washed three times with buffer A containing 1 μM PMSF (buffer B), resuspended in a small volume of buffer B and the amount of protein in this mixture was measured before its reconstitution into proteoliposomes.

Phosphatidylcholine stock solution was prepared by dissolving 40 mg of phosphatidylcholine in 1 ml of buffer B under N_2 . The tube was capped and sonicated in a bath-type sonicator for 15 min or until the solution became translucent. The phosphatidylcholine was then added to the solubilized proteins at a protein/lipid ratio of 1:20 (w/w), unless otherwise indicated. The mixture was frozen in liquid nitrogen and thawed at room temperature. Then it was diluted 1:10 with buffer B and sonicated for 10 s in a bath-type sonicator. The proteoliposomes obtained were pelleted by centrifugation at 100000 *g* for 30 min and resuspended in a minimal volume of buffer B by six passes through a 25-gauge needle. This suspension was then used in transport studies.

BA transport studies

[^3H]TCA transport by mTPM vesicles or by proteoliposomes, obtained as described above, was measured by an adaptation [5] of the rapid filtration technique as described by Hopfer et al. [23]. Experiments were initiated by adding 80 μl of incubation buffer to 20 μl of membrane vesicles (approx. 5 mg of protein/ml) in buffer A or proteoliposomes (0.5–1 mg of protein/ml) in buffer B. The compositions and conditions of different incubation buffers are indicated in the Table and Figure legends. In all cases, incubation was concluded by the addition of 4 ml of ice-cold stop solution (250 mM KCl, 25 mM MgSO_4 , 10 mM Hepes/Tris, pH 7.4) and immediate filtration through 0.45- μm -pore-size nitrocellulose filters prewashed with stop solution. The incubation test tube and subsequently the filters were rinsed once again with the same stop solution and then three additional times with a similar stop solution containing 0.1 mM unlabelled TCA. Values were corrected for the radioactivity found on the filters when vesicles or proteoliposomes were added once the ice-cold stop solution had already been mixed with the incubation medium. Typically

7×10^4 d.p.m. applied to the filters gave an average blank of 120 d.p.m.

In experiments carried out in proteoliposomes, the absolute TCA uptake rates showed some variation from one preparation to the other, though the numbers were of similar magnitude and, more importantly, qualitatively similar results were obtained. Therefore, in some cases only a single experiment is represented in the Figures, although each was repeated at least twice.

Protein deglycosylation

Protein deglycosylation was carried out with the enzymic method reported by Tarentino et al. [24] using N-glycanase. A solution of mTPM proteins (2 mg of protein/ml) in 0.5 M Tris/0.5% SDS/50 mM 2-mercaptoethanol, pH 7.5, was denatured by boiling for 5 min. A 20 μ l aliquot of this solution was then treated with 1.25% Nonidet P-40 and 10 units/ μ l of N-glycanase in a final volume of 60 μ l and incubated for 20 h at 37 °C. The reaction was stopped by adding PAGE loading buffer and the results analysed by immunoblotting.

Immunoblotting

SDS/PAGE and Western blot analyses were performed as follows. Protein samples were subjected to one-dimensional PAGE by the method of Laemmli and Favre [25] using a 7.5% acrylamide running gel and a 4% stacking gel. The gels were renatured by incubation for 1 h in 50 mM Tris/HCl containing 20% glycerol (pH 7.4) and the proteins were electroblotted to nitrocellulose sheets (0.45 μ m) in 20% methanol/10 mM NaHCO₃/4 mM Na₂CO₃ (pH 9.9) as reported by Dunn [26]. The blots were blocked in TBS buffer [0.1 M Tris/HCl, 0.15 M NaCl, 0.1% (v/v) Tween 20, pH 7.5] containing 5% (w/v) non-fat dry milk (blocking buffer). They were then incubated with immune or preimmune serum diluted 1:10000 in blocking buffer for 1 h. After rinsing with TBS, the blots were washed with TBS three times for 5 min. The rabbit primary antibody was detected by incubating the blots for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1:5000 in blocking buffer. The blots were rinsed and washed three times as before, and then the bands were detected by chemiluminescence (Amersham).

Data analysis

The results are expressed as means $\pm$ S.E.M. In some Figures where the S.E.M. bar is not seen, it is smaller than the symbol and lies within it. Significance was determined by Student's paired or unpaired *t*-test as appropriate. Data analysis was performed on a Macintosh-IIci Computer (Apple Computer, Cupertino, CA, U.S.A.).

RESULTS

Plasma membrane vesicle preparations

The results of marker enzyme determinations on mTPM preparations obtained from human and rat placenta revealed high enrichments of the brush-border plasma membrane marker alkaline phosphatase (Table 1), together with a relatively low degree of contamination with basal membrane as measured by enrichment of β -adrenergic receptors (dihydroalprenolol binding). These findings confirm that the preparations consisted predominantly of mTPM, as has been reported by us and others utilizing similar purification techniques [9,19,20].

Table 1 Marker enzyme studies of mTPM vesicle preparations from human and rat trophoblast

Results are means $\pm$ S.E.M. Numbers in parentheses represent the number of determinations in different preparations tested. Enrichment is the ratio of marker enzyme activity in the mTPM preparation to the activity in the homogenate. Abbreviation: DHAP, dihydroalprenolol.

Fraction	Homogenate	mTPM	Enrichment
Human mTPM			
Alkaline phosphatase (μ mol/h per mg of protein) (<i>n</i> = 5)	18.26 $\pm$ 3.93	567.1 $\pm$ 136.7	31.06
DHAP binding (pmol of DHAP/mg of protein) (<i>n</i> = 5)	0.066 $\pm$ 0.007	0.197 $\pm$ 0.007	2.97
Rat mTPM (20-day-gestation)			
Alkaline phosphatase (μ mol/h per mg of protein) (<i>n</i> = 3)	8.23 $\pm$ 1.38	231.8 $\pm$ 23.8	28.16
DHAP binding (pmol of DHAP/mg of protein) (<i>n</i> = 3)	0.046 $\pm$ 0.013	0.085 $\pm$ 0.016	1.84
Rat mTPM (14-day-gestation)			
Alkaline phosphatase (μ mol/h per mg of protein) (<i>n</i> = 3)	4.57 $\pm$ 0.26	169.0 $\pm$ 35.2	36.98
DHAP binding (pmol of DHAP/mg of protein) (<i>n</i> = 3)	0.029 $\pm$ 0.006	0.088 $\pm$ 0.003	3.03

Solubilization and reconstitution of human mTPM proteins into proteoliposomes

The procedure utilized involved considerable loss of membrane protein, with 67.7 ± 2.0 , 14.0 ± 0.5 , 9.5 ± 0.5 and 2.0 ± 1.0 % of initial protein remaining after protein solubilization, first PEG precipitation, sonication/centrifugation of proteins in STAB buffer and second PEG precipitation, respectively (*n* = 3). Interestingly, the protein/lipid ratio used in the reconstitution protocol had a significant influence upon the recovery of proteins in the final proteoliposome preparations as compared with that present prior to the addition of phosphatidylcholine (Table 2). Differential incorporation of proteins into the liposomes [27] may account for this finding. The highest protein recovery noted under our experimental conditions was obtained when a protein/lipid ratio of 1:20 (w/w) was utilized. When expressed per mg of protein, however, both the ATP-dependent and ATP-independent components of TCA uptake in all proteoliposome prepa-

Table 2 Protein recovery after incorporation of solubilized human mTPM proteins in the liposomes

Proteins were measured before and after adding lipids at differing protein/lipid (w/w) ratios. Results are means $\pm$ S.E.M. from data obtained on three different proteoliposome preparations at each protein/lipid ratio.

Protein/lipid ratio (w/w)	Protein recovered (%)
1:2	14.5 $\pm$ 4.3
1:10	29.4 $\pm$ 1.4
1:20	39.0 $\pm$ 2.7
1:40	29.4 $\pm$ 12

Figure 1 Time-course of TCA uptake in human mTPM proteoliposomes

This Figure demonstrates the effect of the presence of 3 mM ATP in the incubation medium (15 μ M taurocholate, 1 μ M PMSF, 250 mM sucrose, 0.2 mM CaCl_2 , 10 mM MgCl_2 , 100 mM KNO_3 , 10 mM Hepes/Tris, pH 7.4) on the time-course of taurocholate uptake at 37 °C by human mTPM proteoliposome preparations. Values shown are for a representative experiment, repeated on four different preparations. O, -ATP; ●, +ATP.

Table 3 Effect of ATP and β , γ -imidoadenosine 5'-triphosphate (p[NH]ppA) on TCA uptake by human mTPM vesicles and proteoliposomes

Taurocholate (TCA) uptake was measured after incubation for 2 min at 37 °C of vesicle or proteoliposome preparations (20 μ l) with 80 μ l of buffer A (250 mM sucrose, 0.2 mM CaCl_2 , 10 mM MgCl_2 , 100 mM KNO_3 , 10 mM Hepes/Tris, pH 7.4) for vesicles and buffer B (buffer A plus 1 μ M PMSF) for proteoliposomes, containing 15 μ M taurocholate and in the absence or the presence of 3 mM ATP or 3 mM p[NH]ppA. Results are means $\pm$ S.E.M. from data obtained on three to six different placenta preparations. Results were compared by Student's *t*-test. **P* < 0.025.

	TCA uptake (pmol/mg of protein per 2 min)		
	Control	ATP	p[NH]ppA
Vesicles	91 $\pm$ 9.0	130 $\pm$ 12.4*	95 $\pm$ 19
Proteoliposomes	549 $\pm$ 79	792 $\pm$ 63.4*	497 $\pm$ 67.4

rations were similar, regardless of the protein/lipid ratios utilized (results not shown).

Characteristics of BA transport by proteoliposomes from human mTPM

TCA uptake by proteoliposomes prepared with proteins obtained from human mTPM was measured in the presence or absence of ATP at incubation times ranging from 30 s to 40 min (Figure 1). In the presence of ATP, TCA retention by proteoliposomes was markedly increased (+37%) compared with the retention found in incubations without ATP. No 'overshoot' phenomenon was observed. These characteristics of BA transport were also observed in native mTPM vesicle preparations [9]. The highest difference (+68%) between TCA retention in the presence and absence of ATP in these experiments was found at the 2 min incubation time. Thus, this incubation time was used in the following experiments, unless otherwise stated. TCA retention

Table 4 Inhibition by TCDCA of TCA transport by human mTPM vesicle and proteoliposome preparations

TCA uptake was measured after incubation for 2 min at 37 °C of vesicle or proteoliposome preparations (20 μ l) with 80 μ l of buffer A (250 mM sucrose, 0.2 mM CaCl_2 , 10 mM MgCl_2 , 100 mM KNO_3 , 10 mM Hepes/Tris, pH 7.4) for vesicles and buffer B (buffer A plus 1 μ M PMSF) for proteoliposomes, containing 15 μ M TCA and in the absence (-ATP) or the presence (+ATP) of 3 mM ATP and in the absence (-TCDCA) or the presence (+TCDCA) of 200 μ M TCDCA. Results are means from data obtained in duplicate or triplicate on one placenta preparation. Total number of experiments was three.

	TCA uptake (pmol/2 min per mg of protein)		Inhibition (% of -TCDCA)
	-TCDCA	+TCDCA	
Vesicles			
-ATP	74.8	56.7	24
+ATP	117.5	75.0	36
ATP-dependent	42.6	18.3	57
Proteoliposomes			
-ATP	366.3	244.3	33
+ATP	712.0	284.2	60
ATP-dependent	370.9	39.9	89

by liposomes without proteins was not affected by the presence of ATP in the incubation medium (results not shown).

Stimulation of TCA uptake in vesicles and proteoliposomes obtained from human mTPM was dependent on the presence of hydrolysable ATP. Substitution of ATP by a non-hydrolysable ATP analogue, such as p[NH]ppA, resulted in the abolition of stimulation in both mTPM vesicles and proteoliposomes (Table 3).

Another characteristic of BA uptake into human mTPM vesicles is inhibition of both ATP-dependent and ATP-independent TCA transport by TCDCA [9]. This effect was preserved in proteoliposome preparations; in fact, the degree of inhibition appeared to be qualitatively greater in proteoliposomes (Table 4). In both preparations, ATP-dependent TCA transport was affected to a greater degree than was ATP-independent transport. The existence in human mTPM of a sodium-independent amino acid transport system with a preference for L-alanine as a substrate has been reported [28]. Therefore L-alanine uptake was selected to test for the possibility of TCDCA-induced non-specific effects upon membrane transport. The presence of TCDCA (200 μ M) had no significant effect on L-alanine (100 μ M) uptake by human mTPM proteoliposomes (960.3 $\pm$ 15.4 pmol/mg of protein per h), compared with the amino acid uptake measured in the absence of this BA (1013.9 $\pm$ 60.8 pmol/mg of protein per h). It is likely, therefore, that the observed TCDCA-induced inhibition of ATP-dependent and ATP-independent TCA transport is a specific effect on BA transport rather than a non-specific effect on the proteoliposomes due to TCDCA detergent properties.

Finally, as denoted in Table 3, an enrichment of BA transport activity in mTPM proteoliposome preparations compared with vesicle preparations was noted. ATP-dependent and ATP-independent TCA uptakes were increased approx. 6-fold in proteoliposomes compared with vesicles. The native vesicles were enriched 31-fold in mTPM as compared with homogenate (as measured by alkaline phosphatase activity); therefore our proteoliposome preparations were enriched in BA carrier activity approx. 190-fold over the initial homogenate.

Figure 2 Western blot analysis of human and rat mTPM proteins with or without deglycosylation

Rabbit antiserum raised against the 100 kDa bile acid transport protein of the rat canalicular hepatocyte plasma membrane was used. SDS/PAGE and transblotting to nitrocellulose filters (10 μ g of protein) were done as described in the Materials and methods section. The blots were incubated with immune serum (1:10000 dilution) as the primary antibody and then with horseradish peroxidase-conjugated goat anti-(rabbit IgG) (1:5000 dilution) as the secondary antibody. Detection of bands was by enhanced chemiluminescence. mTPM protein samples are from: H, human; 20R, 20-day-gestation rat; 14R, 14-day-gestation rat; N, non-deglycosylated; and D, deglycosylated. Experiments were repeated on at least two membrane preparations.

Immunoblot assay of human mTPM proteins

Plasma membrane proteins previously obtained from human mTPM were subjected to electrophoresis and immunoblotting. Both preimmune serum and immune serum (100Ab [18]) were used in these experiments. These studies revealed an approx. 64 kDa protein which was able to bind 100Ab (Figure 2). No bands were noted when preimmune serum was used. Incubation of plasma membrane proteins with N-glycanase failed to change the calculated molecular mass of the immunoreactive protein (Figure 2).

BA retention by rat mTPM vesicles

TCA retention by mTPM vesicles derived from rat placenta at term (20 day gestation) and from 14-day-gestation placenta was stimulated, +30% and +43% respectively, in the presence of ATP (Table 5), similar to that noted above in human mTPM. Stimulation of transport by ATP was significant ($P < 0.05$) at each time point tested in vesicles derived from 20-day-gestation rat placenta.

Immunoblot assay of rat mTPM proteins

Brush-border membrane proteins from rat placenta at 20- and 14-day gestation were also subjected to electrophoresis and immunoblotting using the above-mentioned sera. A protein of approx. 130 kDa was found to bind the 100Ab in 20-day-gestation placenta membranes, while in 14-day-gestation preparations two proteins of 140 kDa and 100 kDa had affinity for this antibody (Figure 2). Treatment of rat-derived proteins with preimmune serum resulted in the absence of bands on Western blot. Prior treatment of rat proteins with N-glycanase induced a marked reduction in the value of molecular mass measured for the immunoreactive proteins, approx. 50 kDa in both preparations, although the presence of another protein in 14-day-gestation membranes with an approximate molecular mass after deglycosylation of 56 kDa is also shown (Figure 2).

Table 5 Time-course of TCA retention by rat mTPM vesicles

Effect of the presence of 3 mM ATP plus an ATP-regenerating system (3 mM creatine phosphate plus 100 μ g/ml creatine kinase) in the incubation medium (25 μ M TCA, 250 mM sucrose, 0.2 mM CaCl_2 , 10 mM MgCl_2 , 100 mM KNO_3 , 10 mM Hepes/Tris, pH 7.4) on the time-course of TCA retention at 37 $^\circ\text{C}$ by rat mTPM vesicle preparations from 20- and 14-day-gestation placentas. Values are means $\pm$ S.E.M. obtained from experiments carried out on one (14-day) and three (20-day) different placenta preparations.

	TCA uptake (pmol/mg of protein per 2 min)	
	Control	ATP
20-day gestation		
30 s	52 $\pm$ 2	69 $\pm$ 4
2 min	119 $\pm$ 5	160 $\pm$ 16
40 min	267 $\pm$ 9	325 $\pm$ 20
14-day gestation		
30 s	29 $\pm$ 1	50 $\pm$ 2
2 min	91 $\pm$ 10	115 $\pm$ 10
40 min	173 $\pm$ 6	224 $\pm$ 3

DISCUSSION

The present paper shows that the ATP-dependent and ATP-independent BA transport systems present in the brush-border membrane of the human trophoblast [9] can be solubilized utilizing octylglucoside and then reconstituted in phosphatidylcholine proteoliposomes after removal of detergent with PEG. Octylglucoside has frequently been used for solubilization of membrane proteins, and, at least in the hepatocyte sinusoidal plasma membrane [27], smooth endoplasmic reticulum [29] and canalicular plasma membrane [30,31], does not appear to induce functional or structural alterations in BA transport systems. In preliminary studies using different detergents, we found that octylglucoside did not abolish BA transport activity. An additional advantage of octylglucoside was that it solubilized apical membrane proteins efficiently as compared with other detergents tested (results not shown).

Protein precipitation with PEG allows rapid detergent removal and, therefore, shorter exposure of solubilized proteins to detergent than do other frequently used methods including gel filtration, column chromatography or dialysis. Moreover, proteoliposomes obtained in the presence of PEG show a decrease in non-specific permeability [32]. In order to ensure maximal protein recovery, a final concentration of 20% PEG was used. This PEG concentration does not have adverse effects on a variety of tested transport systems [21,22,33] and, as shown in this paper, BA transport activity can be recovered in proteoliposomes obtained by this method. The use of STAB buffer plus sonication permits adequate solubilization of the PEG-precipitated proteins [21]. Another characteristic of the reconstitution method is that the buffer used during the freeze-thaw/sonication procedure contains K^+ instead of Na^+ as reported by McCormick et al. [34].

The general characteristics of the reconstituted BA transport system(s) from human placental brush-border plasma membrane are the same as those of the transporter(s) in the native mTPM vesicles. ATP-dependent stimulation of TCA uptake without an 'overshoot' phenomenon is observed in both preparations [9]. Additionally, the non-hydrolysable ATP analogue p[NH]ppA is incapable of stimulating BA transport activity, suggesting that the requirement for hydrolysis of the γ -phosphate of ATP, believed to be mandatory for ATP-induced stimulation of TCA transport in mTPM, is preserved in proteoliposomes derived

from this membrane. Finally, TCDCA inhibited both ATP-dependent and ATP-independent TCA transport in human mTPM and proteoliposome preparations. These results further support the hypothesis that this transport system(s) is not specific for TCA [9]. Moreover, these data are consistent with the existence of carrier protein(s) involved in ATP-independent BA transport by this membrane [10,11]. Whether both components of BA transport across mTPM are accounted for by a single or multiple carrier protein(s) is currently uncertain.

BA transport was increased approx. 6-fold in proteoliposomes compared with native vesicles. Results obtained by others using human mTPM proteoliposomes [35,36] showed no significant enrichment of taurine or 5-hydroxytryptamine transport activities during the solubilization and reconstitution procedure compared with that in native mTPM vesicles. There were, however, significant differences in their reconstitution procedure as compared with ours. By contrast, other authors have shown a 19-fold enrichment in hepatic system N amino acid transport activity in proteoliposomes obtained by a procedure similar to the one used here [22].

Human fetal BAs have been detected in a variety of biological fluids including meconium, amniotic fluid, urine, gall-bladder bile and serum [37–39]. Concentrations in fetal serum have been reported to be higher than those in maternal blood. Therefore a net transfer of these molecules from the fetus to the mother is believed to occur in order to protect the fetus from potential deleterious effects of these compounds including teratogenicity caused by some species of secondary BAs [40]. As noted previously, BA transport across the human basal trophoblast membrane occurs via anion exchange, driven probably by an inversely directed bicarbonate gradient [5–8], while BA excretion across the apical membrane occurs via an ATP-dependent BA transporter [9]. Serum BA levels in fetal rats are not known, although synthesis and conjugation of these molecules by rat fetal liver has been reported [41,42]. Nevertheless, there is a lack of information concerning BA transport across the haemotrichorial rat placenta. The results in this paper demonstrate that BA transport in rat mTPM is stimulated by ATP as observed for human mTPM and rat liver canalicular membrane.

Several glycoproteins related to BA transport have been characterized in the rat liver canalicular membrane. A 110 kDa glycoprotein that can be photoaffinity-labelled with ATP and photolabile bile salt derivatives has been reconstituted into liposomes and ATP-dependent TCA uptake subsequently demonstrated [31]. The amino acid sequence of this BA carrier is identical to the rat liver canalicular ecto-ATPase implicated in ATP-dependent BA transport [43], which, in turn, shares identity with the rat cell-adhesion molecule CAM-105 [44]; moreover, it has been reported that these glycoproteins have two isoforms [44,45]. These proteins are also identical to a substrate for the insulin-receptor tyrosine kinase, pp120/HA4 [46]. Transfection of ecto-ATPase cDNA conferred BA efflux capacity and ecto-ATPase activity on heterologous cells [43,47,48]. Functional BA transport required phosphorylation of Ser⁵⁰³ and was regulated by phosphorylation of Tyr⁴⁸⁸, both in the cytoplasmic tail of the ecto-ATPase protein [48]. Ecto-ATPase/cell CAM-105 mRNA has been demonstrated in a variety of rat extrahepatic tissues including intestine, kidney, lung, muscle and spleen [49], though the function of this protein in those tissues is unknown. Antibodies raised against the 100 kDa BA carrier of the rat canalicular hepatocyte membrane [18] recognize rat mTPM proteins of similar, although not identical, structural characteristics. Specifically, ecto-ATPase/cell CAM-105 recognized by 100Ab has, in rat liver, an apparent molecular mass of 100–110 kDa, with a calculated molecular mass of 57 kDa, while the proteins

recognized by 100Ab in rat mTPM have molecular masses of 100–140 kDa and deglycosylated masses of 50–56 kDa. Like other glycoproteins, these rat proteins exhibit heterogeneity of apparent molecular mass by SDS/PAGE analysis. These differences are highlighted by differential patterns of glycosylation in placental apical membrane vesicles derived from 14- as compared with 20-day-gestation pregnancies. When deglycosylated, this heterogeneity disappears and a unique form is present. In 14-day-gestation preparations there is an additional deglycosylated protein, which, we speculate, may be an isoform of the same family of glycoproteins. 100Ab also recognized a human mTPM protein, although its molecular mass (64 kDa) and glycosylation characteristics were quite different from those of the rat. Ecto-ATPase/cell CAM-105 shares structural and, perhaps, immunological characteristics with a variety of human proteins [44]. Of these, carcinoembryonic antigen and biliary glycoprotein I seem unlikely candidates given their molecular masses of 180 and 85 kDa respectively, as well as their extensive glycosylation profiles [44,50]. Non-specific cross-reacting antigen (NCA) also shares homology but differs in molecular mass (55 and 95 kDa) as well as in tissue distribution [51]. A more likely candidate protein may be human pregnancy-specific β_1 -glycoprotein, which has been shown to be present in human placenta and to have an apparent molecular mass of 64 kDa [52,53]. These proteins are, however, glycosylated and are thought to be predominantly cytoplasmic. A possible explanation for the lack of glycosylation noted in our study may be that N-glycanase catalyses hydrolysis at the amide bond of asparagine in N-linked oligosaccharides [24]; although of broad specificity, this enzyme's hydrolytic capability may be limited if a normally susceptible oligosaccharide is associated with a sequence in which the asparagine residue is on the amino or carboxyl end of the peptide [54].

In summary, we have demonstrated a method for the reconstitution of ATP-dependent BA transport in proteoliposomes derived from human mTPM. We have further demonstrated the presence of ATP-dependent BA transport in apical membrane vesicles derived from rat placenta. Finally, we have shown, utilizing an antibody against the rat liver ecto-ATPase, the presence of an immunologically similar protein in mTPM derived from both human and rat placenta.

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