Evidence for an ATP-independent long-chain phosphatidylcholine translocator in hepatocyte membranes

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Fuchs, Michael, Martin C. Carey, and David E. Cohen. Evidence for an ATP-independent long-chain phosphatidylcholine translocator in hepatocyte membranes. Am. J. Physiol. 273 (Gastrointest. Liver Physiol. 36): G1312–G1319, 1997.—Transport of phosphatidylcholine (PC) molecules across canalicular plasma membranes of the liver is essential for their secretion into bile. To test for evidence of protein-mediated translocation of natural long-chain PCs, we investigated whether hepatocyte membrane fractions reconstituted into proteoliposomes promoted transmembrane translocation of radiolabeled PCs. Translocation of PC molecules in proteoliposomes was measured by an assay that employed multilamellar acceptor vesicles and the specific PC transfer protein purified from liver. As inferred from the percentage of radiolabel removed from proteoliposomes, facilitated PC translocation occurred in microsomes and canalicular and basolateral plasma membranes from rat liver but not in erythrocyte ghosts, microsomes, homogenates of COS and H35 cells, or Xenopus laevis oocytes. Heat denaturation in the presence of 2-mercaptoethanol and Pronase digestion of solubilized membrane proteins inhibited translocation. In contrast to the mdr2 gene product (Mdr2), which promotes ATP-dependent, verapamil-inhibitable PC translocation, ATP did not enhance and verapamil failed to block PC translocation. These data support the possibility that an ATP-independent PC translocator, possibly distinct from Mdr2, may be present in hepatocyte canalicular plasma membranes.

Biliary lipid secretion; canalicular plasma membrane; “flip–flop”; proteoliposomes; P-glycoprotein

As suggested previously (33) but only visualized recently (15), bile salts promote biliary phosphatidylcholine (PC) secretion by inducing vesiculation of the outer leaflet of the canalicular plasma membrane (cLPM). To preserve the steady-state composition of the cLPM, an efficient mechanism must replenish PC molecules after vesicle formation and detachment. Suggested mechanisms for intracellular delivery of PC molecules from the endoplasmic reticulum to the inner leaflet of the cLPM are cytosolic PC transfer protein (PC-TP) (12) and/or vesicular transport (2). Because the extensively hydrated zwitterionic head group of PC presents an appreciable energy barrier that prohibits “flip–flop” of PC molecules across biological membranes (16), evidence for a transmembrane translocating protein (“flip–flop”) has been sought by a number of investigators (summary in Ref. 19).

Berr, Meier, and Stieger (6) first demonstrated ATP-independent uptake of short-chain PCs into vesicular spaces of isolated cLPM. Thereafter, Smit and colleagues (30) demonstrated the apparent total absence of PC in bile of mice with homozygous disruption of the mdr2 gene and hypothesized that the mdr2 gene product (Mdr2), a 170-kDa P-glycoprotein, plays a key role in transmembrane translocation of PC molecules across the cLPM. Indeed, expression of Mdr2 in yeast secretory vesicles (28, 29) and the human homologue (MDR3 gene product) in fibroblasts (31) as well as kidney epithelial cells (32) confers ATP-dependent PC-translocating activity. In direct support for a role of Mdr2 in translocation of biliary PCs, Nies, Gatmaitan, and Arias (26) demonstrated ATP-dependent translocation of an NBD-labeled fluorescent PC analog 1-acyl-2-(6-((7-nitro-2-1,3-benzoxadiazol-4-yl)amino)caproyl)-sn-glycero-3-phosphocholine in purified cLPM. Notwithstanding these findings, Kullack-UBLick and co-workers (22) have expressed ATP-independent translocating activity for short-chain PCs after injection of Xenopus laevis oocytes with size-fractionated rat liver mRNA too small to encode Mdr2.

To further explore transmembrane translocation of PCs by proteins of isolated cLPM, we adapted a model system that permitted the use of radiolabeled long-chain PCs, which are trivially modified compared with native PCs, to detect evidence for translocating activity (4, 21). These studies provide direct evidence for protein-mediated PC translocation in plasma membrane subfractions of hepatocytes. Because this protein neither requires ATP hydrolysis nor is inhibitable by verapamil, an inhibitor of P-glycoprotein, it may be distinct from Mdr2.

MATERIALS AND METHODS

Chemicals

Grade I egg yolk PC and phosphatidylethanolamine (PE) were purchased from Lipid Products (S. Nutfield, Surrey, UK). Egg yolk sphingomyelin, bovine liver phosphatidylsterol, bovine brain phosphatidylserine, and bovine heart cardiolipin were obtained from Avanti Polar Lipids (Birmingham, AL). Lipids were judged to be 98% pure by high-performance liquid chromatography (HPLC) (12) and/or thin-layer chromatography (TLC). L-α-1-Palmitoyl-2-[linoleoyl-1-14C]PC (50 mCi/mmole) and [cholesteryl-1,2,6,7-3H(N)]oleate (98.5 Ci/mmol) were obtained from Du Pont-New England Nuclear (Boston, MA) and were 98% radiochemically pure by TLC. Sodium taurocholate (Sigma Chemical, St. Louis, MO) was recrystallized and found to be 99% pure by HPLC (12). Protease (Pronase E from Streptomyces griseus) was obtained from Sigma. ACS-grade NaCl was roasted at 600°C for at least 6 h to remove organic impurities. Pyrex brand glassware was alkali-alcohol (EtOH-4 M KOH 1:1 vol/vol) and acid (1 M HNO3) washed and rinsed with purified water before use. Water was filtered, ion exchanged, glass distilled (Corning Glass Works, Corning, NY), and finally purified with a MilliQ filtration system (Millipore, Bedford, MA). All other chemicals were of the highest purities available.
Animals and Cells

Male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and housed in a constant-temperature and-humidity environment with alternating illumination (light: 0600–1800) and fed ad libitum with free access to water. Frogs of the species X. laevis from Nasco (Fort Atkinson, WI) and obtained as a generous gift from Dr. Seth Alper (Beth Israel Hospital, Boston) were maintained in deionized water at room temperature in accordance with Harvard University’s “Handbook for Use of Animals in Research and Teaching.” African green monkey kidney fibroblast-like (COS) cells and rat hepatoma (Reuber H35) cells from American Type Culture Collection (Rockville, MD) were kindly provided by Dr. Carl L. Berg (Brigham and Women’s Hospital, Boston). COS cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum, penicillin (50 U/ml), and streptomycin (50 µg/ml) under an atmosphere of 95% O2-5% CO2. H35 cells were cultured in minimal essential medium with 1% nonessential amino acids, 10% fetal bovine serum, 10% calf serum, penicillin (50 U/ml), and streptomycin (50 µg/ml).

Isolation of Membranes and Tissues

Rat liver plasma membrane fractions. Livers of fasted rats were harvested immediately after decapitation. Microsomes were prepared by serial centrifugation and stored at −70°C in 0.25 M sucrose, 10 mM tris(hydroxymethyl)aminomethane (Tris, pH 7.4) (3). Pooled liver plasma membrane fractions of six to eight fed rats were isolated by sucrose density gradient centrifugation and stored at −70°C in buffer containing 0.25 M sucrose, 2 mM CaCl2, 5 mM MgSO4, 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), and 10 mM Tris (pH 7.4) for no longer than 3 mo before use (23). To delineate the degree of membrane purification, we employed measurements of standard marker enzymes that permitted assessments of membrane cross-contamination as well as contamination with intracellular organelles. In all membrane subfractions, microsomes were enriched 4- to 5-fold with glucose-6-phosphatase (3), cLPM were enriched 35- to 45-fold with leucine aminopeptidase, and basolateral plasma membrane (bLPM) vesicles were enriched 25- to 30-fold with Na+K+-adenosinetriphosphatase (23). Liver plasma membranes were only slightly contaminated with glucose-6-phosphatase (0.2- to 0.5-fold enrichment; Ref. 3) and acid phosphatase (0.8- to 1.5-fold enrichment; Ref. 23), suggesting minimal admixture with microsomes and lysosomes, respectively. The respective contributions to whole liver homogenate by microsomes (24%) and lysosomes (2%) (25) were employed to estimate the degree to which liver plasma membrane subfractions were contaminated with these organelles. Contamination percentages (5–12% for microsomes and 2–3% for lysosomes) were calculated as the products of organelle enrichment in plasma membranes times the percentage of total protein. By comparison with the findings of Meier and Boyer (23) in which a 4-fold enrichment of bLPM with leucine aminopeptidase was found to account for ~10% cross-contamination with cLPM, the 0.5- to 2.5-fold enrichment of the same marker enzyme suggested minimal cross-contamination with cLPM in our preparations.

Rat bile and serum. Two rats were anesthetized with pentobarbital (50 mg/kg body wt), and after laparotomy, polyethylene catheters (Clay Adams, Parsippany, N J) were introduced into their common bile ducts. Surgical intervention lasted 10–15 min, during and after which body temperature was kept constant at 37°C with a heating lamp. Catheters were exteriorized, abdominal cavities were closed, and animals were placed in restraining cages with free access to water and chow. After 2-h recovery from anesthesia, bile samples were collected during 20-min intervals for 1 h. Extrahepatic cholestasis was induced in a rat by ligating the common bile duct at two places along its length, followed by transection of the duct between ligatures and closure of the abdominal cavity. After 72 h, the rat was reanesthetized with pentobarbital, the abdominal cavity was reopened, and blood for preparation of serum was obtained by puncture of the abdominal aorta.

Frog oocytes. To harvest oocyte homogenate and microsomes, one frog was anesthetized with 0.17% tricaine, and portions of the ovary were removed. The ovarian tissue was immediately placed in a petri dish containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, 2.5 mM sodium pyruvate, and 0.1 U gentamicin/ml (pH 7.4). Oocytes dissected from their surrounding tissues were defolliculated by collagenase digestion (2 mg/ml) for 1 h. Twenty to thirty oocytes were then homogenized by 30 strokes in a tight Dounce homogenizer in 83 mM NaCl, 1 mM MgCl2, 10 mM HEPES (pH 7.9), 0.5 mM phenylmethylsulfonyl fluoride, 5 µg pepstatin, and 5 µg leupeptin (25 µl/oocyte). Microsomes were prepared according to the procedure of Geering et al. (20).

COS and H35 cells. Homogenates of COS and H35 cells were prepared by minor modifications of the method of Clark et al. (9). Briefly, cells were washed at room temperature with 10 ml per plate of phosphate-buffered saline (PBS), harvested in 5 ml of 5 mM EDTA, and scraped into centrifuge tubes. All subsequent steps were performed at 4°C. The cells were twice washed in PBS, pelleted by centrifugation at 800 g for 5 min, and swollen for 10 min in hypotonic solution containing 10 mM Tris and 0.5 mM MgCl2 (pH 7.5). This was followed by homogenization with 30 strokes of a tight Dounce homogenizer. The homogenate was then diluted with an equal volume of a solution composed of 0.5 M sucrose, 6 mM 2-mercaptoethanol, 300 mM KCl, and 10 mM Tris (pH 7.5) and again homogenized with 20 strokes. For preparation of COS microsomes (9), the homogenate was centrifuged at 10,000 g for 20 min at 4°C. After the addition of 2.5 M KCl (0.9 ml) to the supernatant, microsomes were pelleted by centrifugation at 40,000 g for 1 h. Microsomes were then resuspended in 0.25 M sucrose, 0.15 M KCl, 3 mM 2-mercaptoethanol, and 10 mM Tris (pH 7.5), centrifuged again at 40,000 g, resuspended in the same solution (1–2 mg protein/ml), and stored at −70°C until use.

Erythrocyte ghosts. Erythrocyte ghosts were prepared by hypotonic lysis. Briefly, freshly drawn blood from a healthy human donor was collected into a syringe containing 0.2 M EDTA as antiocoagulant. All subsequent steps were performed at 4°C. Plasma and “buffy coat” were removed after centrifugation at 1,500 g for 10 min. Packed cells were washed three times in 5 mM Na2HPO4 and 150 mM NaCl, then lysed by addition of 5 mM Na2HPO4 and pelleted by centrifugation at 20,000 g for 15 min. After three additional washes with 5 mM Na2HPO4 ghosts were stored until use at −70°C at a protein concentration of 3–4 mg/ml.

Assay of PC Transmembrane Translocation

Principle. Transmembrane translocation of [3H]PC from inner to outer leaflets of proteoliposomes was measured employing a modification of the techniques of Johnson, Hughes and Zilversmit (21) and Backer and Dawidowicz (4). Proteoliposomes were prepared with [3H]PC distributed at the inner and outer leaflets (see below), PC-TP from bovine liver is a soluble lipid transfer protein that catalyzes intermembrane transfer of PC molecules exclusively and is membrane imper-
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mimetable (21). When incubated together with proteoliposomes and multilamellar vesicles (see below), PC-TP exchanged radiolabeled PC molecules of the outer leaflet of proteoliposomes with unlabeled PCs from the outer leaflet of multilamellar vesicles. In the absence of PC translocation and through use of this assay, it has been shown that approximately one-third of the radiolabeled PC in unilamellar model membranes is nonexchangeable, reflecting the fraction of PC molecules that resides on the inner membrane leaflet (21). When PC molecules are translocated from inner to outer leaflets, they became available for exchange by PC-TP, and this was reflected by a further decrease of the [14C]PC content to a value significantly less than one-third (21).

Proteoliposomes and unilamellar vesicles. Proteoliposomes were prepared from isolated membrane subfractions according to Backer and Dawidowicz (4). Unless otherwise stated, 3.5 mg egg yolk PC, 1.2 mg PE, 0.4 µCi [14C]PC, 5 µCi [3H]cholesterol oleate, and 15 mg sodium taurocholate were coprecipitated from chloroform-methanol (1:1, vol/vol). Aliquots of isolated membranes containing 500 µg of protein were added and the final volume adjusted to 0.5 ml with 20 mM NaCl, 5 mM Tris, 0.5 mM EDTA, and 5 mM 2-mercaptoethanol (pH 6.5). Lipid films were resuspended by vortex mixing for 15 min at 4°C, incubated on ice for 30 min and then centrifuged at 100,000 g for 30 min at 4°C. The absence of a pellet indicated complete micellar solubilization of membrane proteins and lipids. As verified by HPLC (12) in preliminary experiments (n = 3), sodium taurocholate was completely removed after exhaustive dialysis (SpectroPor 2 dialysis tubing, Spectrum, Houston, TX) at 4°C for 3 days with six changes (2 liters each) of 20 mM NaCl, 5 mM Tris, 0.5 mM EDTA, and 5 mM 2-mercaptoethanol (pH 6.5). Proteoliposomes prepared in this manner contained radiolabeled lipids on both membrane hemileaflets, and native membrane lipids contributed 5–7% to the total lipid content. Quasi-elastic light scattering (12) demonstrated that proteoliposomes created from each membrane source were of similar size (mean hydrodynamic radius 862 ± 292 Å).

To demonstrate a role for membrane proteins in PC translocation, liver microsomes and cLPM and bLPM subfractions containing 500 µg protein were heated for 5 min at 100°C in the presence or absence of 2-mercaptoethanol (1 vol%) before preparation of proteoliposomes. In separate experiments, membranes were exposed to Pronase (300 µg/mg protein) before and after solubilization with taurocholate. To explore the influence of membrane lipid composition on PC translocation, isolated membranes were reconstituted into proteoliposomes (20 mM NaCl, 5 mM Tris, 0.5 mM EDTA, and 5 mM 2-mercaptoethanol, pH 6.5) were prepared by bath sonication (Special Ultrasonic Cleaner, Fullerton, CA) as previously described (35) or by freeze thawing (18). This was accomplished by addition of 5 mM ATP with a regenerating system (1 mM MgCl2, 10 mM phosphocreatine, and 100 U creatine kinase) alone or together with 20 µM verapamil (29). Proteoliposomes were then frozen in liquid nitrogen for 5 min and then allowed to thaw on ice for 1 h before study.

Unilamellar vesicles composed of pure PC (3.5 mg) and PE (1.2 mg) dispersed in 0.5 ml of the same buffer used for preparation of proteoliposomes (20 mM NaCl, 5 mM Tris, 0.5 mM EDTA, and 5 mM 2-mercaptoethanol, pH 6.5) were prepared by bath sonication (Special Ultrasonic Cleaner, Laboratory Supply, Hicksville, NY) as previously described (12). Quasi-elastic light scattering confirmed that sizes of the unilamellar vesicles fell within the same range observed for proteoliposomes.

Acceptor vesicles. Multilamellar vesicles consisting of PC and cardiolipin (95:5 molar ratio) were prepared according to DiCorleto and Zilversmit (17). Briefly, egg yolk PC (104.6 mg), cardiolipin (10.4 mg) and the antioxidant butylated hydroxytoluene (0.1 mol%) were coprecipitated from chloroform in a round-bottom flask. The organic solvent was removed under nitrogen followed by resuspension in diethyl ether and drying to form a thin lipid film, which was rehydrated with 10 ml of buffer (5 mM EDTA and 50 mM Tris; pH 7.2). Multilamellar vesicles were formed by shaking for 1 h at room temperature. To isolate a population of multilamellar vesicles that could be separated readily from proteoliposomes by centrifugation, suspensions were centrifuged at 55,000 g for 30 min, the supernatant was discarded, and the pellet was gently resuspended in 6 ml of the same buffer to yield a final PC concentration of 39 mg/ml.

PC-TP. PC-TP was purified 508-fold (~11% purity) from bovine liver (obtained fresh from a local slaughterhouse) through the carboxymethyl cellulose step described by Westerman, Kamp, and Wirtz (34) and stored in 50% glycerol at −20°C. Before use, glycerol was removed by overnight dialysis at 4°C against at least 1,000 vol of 150 mM NaCl, 20 mM Tris, 5 mM EDTA, 3 mM NaN3, and 10 mM 2-mercaptoethanol (pH 7.4) using SpectroPor 2 dialysis tubing. After dialysis, the protein concentration was 13 ± 2 µg/ml.

Assay. Donor proteoliposomes (50 µl) were incubated at 37°C in a shaking water bath together with multilamellar acceptor vesicles (200 µl), PC-TP (200 µl), and buffer (50 mM Tris and 5 mM EDTA; pH 7.2; 50 µl). Control samples contained buffer (200 µl) but no added PC-TP. Preliminary experiments established that an incubation period of 30 min was sufficient to reach steady-state [14C]PC exchange. To facilitate calculation of the remaining fraction of [14C]PC, intervesicular PC transfer was arrested and multilamellar vesicles pelleted by centrifugation (55,000 g) for 4°C for 30 min. The [14C]PC and [3H]cholesterol oleate contents of a 100-µl aliquot of proteoliposomes that remained in suspension after centrifugation were determined by liquid scintillation counting (Liquid Scintillation System LS 5000TD, Beckman, Fullerton, CA). The remaining 350 µl of proteoliposomes were incubated with an additional 200-µl aliquot each of fresh multilamellar vesicles and of PC-TP. The incubation and sampling protocol was then repeated until the [14C]PC content of proteoliposomes reached a constant value designated as residual [14C]PC content. Preliminary experiments demonstrated that fourfold variations in PC-TP concentrations did not influence the residual [14C]PC content of proteoliposomes. As assessed by TLC no degradation of PC occurred during the time course of the exchange assay (~300 min). Employing similar experimental conditions, Backer and Dawidowicz (4) have demonstrated no compromise of proteoliposomal integrity as evidenced by their complete retention of entrapped aqueous phase markers.

Calculation of [14C]PC Content of Proteoliposomes

To quantify the proportion of [14C]PC in proteoliposomes that was replaced with unlabeled PC from multilamellar vesicles during one incubation period, proteoliposomes and multilamellar vesicles were separated by centrifugation. To account for small losses of proteoliposomes during centrifugation, proteoliposomes also contained [3H]cholesterol oleate,
which is not exchanged by PC-TP (21). Exchange of \(^{14}\text{C}\)PC with unlabeled PC resulted in a decrease of the \(^{14}\text{C}\)PC-to-\(^{3}\text{H}\)cholesteryl oleate ratio in proteoliposomes. With each successive time period of incubation, additional \(^{14}\text{C}\)PC in proteoliposomes was replaced with unlabeled PC, further decreasing the \(^{14}\text{C}\)PC-to-\(^{3}\text{H}\)cholesteryl oleate ratio of proteoliposomes incubated without and with PC-TP, respectively. The residual \(^{14}\text{C}\)PC content of proteoliposomes after completion of each incubation, expressed as percentage of initial value, was calculated as 100 − [(A − B)/A] × 100, where A and B represent the \(^{14}\text{C}\)PC-to-\(^{3}\text{H}\)cholesteryl oleate ratio of proteoliposomes incubated without and with PC-TP, respectively. The residual \(^{14}\text{C}\)PC content was defined as the asymptotic \(^{14}\text{C}\)PC content, which no longer decreased with further incubation.

Evidence for PC-translocating activity was inferred from residual contents of \(^{14}\text{C}\)PC. Residual \(^{14}\text{C}\)PC contents significantly lower than those obtained with control proteoliposomes (i.e., derived from erythrocyte membrane proteins that lack PC translocate activity; Ref. 4) or unilamellar vesicles were consistent with transmembrane translocation of PCs from inner to outer membrane hemileaflet of proteoliposomes. In this context, the term translocating activity refers solely to the presence or absence of a reconstituted PC translocator that promotes a decrease in residual \(^{14}\text{C}\)PC content and provides no information regarding the specific activity of the responsible molecule(s).

Analytical Procedures

Protein concentrations were assayed by the method of Bradford (see Ref. 12) using bovine serum albumin as a standard. Phospholipid concentrations were determined from lipid phosphorus (12). Phospholipid classes and cholesterol in proteoliposomes were extracted, separated by TLC on silica plates (chloroform-methanol-water, 65:25:4, vol/vol/vol), and phospholipids were visualized using a molybdenum spray. Concentrations of ATP were determined enzymatically using the phosphoglycerate kinase method (1).

Statistical Analysis

Data presented in RESULTS represent means ± SD of two to three experiments performed in triplicate. Statistical comparisons were performed using a two-tailed Student’s t-test.

RESULTS

\[^{14}\text{C}\]PC Exchange Between Proteoliposomes and Acceptor Vesicles

Figure 1 illustrates the \(^{14}\text{C}\)PC content of proteoliposomes after serial incubations with multilamellar acceptor vesicles. After the first incubation period, 69–72% of \(^{14}\text{C}\)PC remained in proteoliposomes composed from erythrocyte ghosts, bLPM, cLPM, and liver microsomes. During the first three incubations the \(^{14}\text{C}\)PC content decreased linearly, and this was most pronounced for liver microsomes followed in rank order by cLPM > bLPM > erythrocyte ghosts. After four changes of acceptor vesicles, the \(^{14}\text{C}\)PC content of erythrocyte ghost proteoliposomes was 36 ± 1.0% and remained constant thereafter. The same residual \(^{14}\text{C}\)PC content was obtained employing pure phospholipid unilamellar vesicles (data not displayed), confirming the absence of PC translocase activity in proteoliposomes of erythrocyte ghosts (4). In contrast, with bLPM, cLPM, or liver microsomal proteoliposomes, the \(^{14}\text{C}\)PC contents leveled off at 23 ± 1.4%, 19 ± 1.2%, and 16 ± 1.6%, respectively.

Figure 2 plots the residual \(^{14}\text{C}\)PC content of proteoliposomes for each membrane type studied. For cells other than hepatocytes, the highest residual \(^{14}\text{C}\)PC content (35 ± 1.2%) was observed for erythrocyte ghosts, which were used as controls (see below), and the lowest residual \(^{14}\text{C}\)PC content (29 ± 2.0%) was observed for COS cell microsomes. Residual \(^{14}\text{C}\)PC contents of proteoliposomes of other membrane types gave intermediate values (31–33%). Proteoliposomes composed of rat bile and serum yielded a 36–37% residual \(^{14}\text{C}\)PC content. The residual \(^{14}\text{C}\)PC contents of bLPM (23 ± 1.6%; \(P < 0.001\) vs. erythrocyte ghosts),
cLPM (18 ± 3.1%; P < 0.001), and liver microsomes (15 ± 3.0%; P < 0.001) were much lower, consistent with transmembrane translocation of PC molecules, i.e., from inner to outer leaflets of proteoliposomes, where they became available for exchange by PC-TP.

Effects of Heat Denaturation and Pronase Digestion on Residual [14C]PC Content of Proteoliposomes

To evaluate the hypothesis that PC-translocating activity was mediated by the protein fraction, we employed standard procedures for denaturing membrane proteins. We found that heat treatment alone failed to eradicate the translocating activity in cLPM as evidenced by a 19 ± 1.9% residual [14C]PC content in proteoliposomes compared with 18 ± 2.5% for non-heated controls (P > 0.05). In contrast, Fig. 3 illustrates that, when denatured by heating to 100°C in the presence of 2-mercaptoethanol, the residual [14C]PC content was increased from 14 ± 2 to 31 ± 2.5% (P < 0.01), 18 ± 2.5 to 36 ± 3% (P < 0.01) and 23 ± 1.9 to 27 ± 2.1% (P < 0.05) in proteoliposomes composed of liver microsomes, cLPM, and bLPM, respectively. Exposure of similar membrane subfractions to Pronase after the bile salt solubilization step during preparation also ensured similar membrane subfractions to Pronase after the bile salt solubilization step during preparation also decreased the residual [14C]PC content to 22 ± 0.01, 18 ± 0.01, 15 ± 0.01, and 15 ± 0.01, respectively. Translocation was observed when intact membrane subfractions, respectively. In contrast no effect on PC contents of proteoliposomes. Isolated liver microsomes, cLPM, and bLPM, respectively. Exposure of similar membrane subfractions to Pronase after the bile salt solubilization step during preparation also decreased the residual [14C]PC content to 22 ± 0.01, 18 ± 0.01, 15 ± 0.01, and 15 ± 0.01, respectively. Translocation was observed when intact membrane subfractions were treated with Pronase. We also found that combining erythrocyte ghosts and liver microsomes (250 µg protein of each membrane type) during the preparation of proteoliposomes decreased the residual [14C]PC content to 22 ± 0.7% compared with 35 ± 1.2% using erythrocyte ghosts alone (P < 0.01). Taken together, these results strongly suggest that transmembrane translocation of PC was protein mediated.

Effect of Membrane Lipid Composition on Transmembrane Translocation of PC

The influence of the PE-to-PC molar ratio was investigated using proteoliposomes created from erythrocyte ghosts and cLPM. As shown in Fig. 4, increasing the PE-to-PC molar ratio from 0.4 to 3.0 decreased the residual [14C]PC content, regardless of whether proteoliposomes had been prepared from erythrocyte ghosts or cLPM. However, at each PE-to-PC ratio, the residual [14C]PC content was twofold lower for cLPM compared with erythrocyte ghosts (P < 0.001), consistent with transmembrane PC translocation in cLPM. When proteoliposomes contained cLPM proteins in a lipid bilayer representative of the microsomal membrane, the residual [14C]PC content decreased by only 15% (P > 0.05) compared with control proteoliposomes composed of PC and PE. In contrast, when rat liver microsome were reconstituted into proteoliposomes with lipids modeling the cLPM, the residual [14C]PC content increased from 15 ± 3 to 21 ± 2.8% (P < 0.01).

Effects of ATP and Verapamil on Transmembrane Translocation of PC

Enzymatic measurements after exhaustive dialysis of proteoliposomes confirmed the absence of ATP and demonstrated that ATP is not required for reconstitution of PC-translocating activity. However, to explore whether PC translocation could be enhanced by ATP hydrolysis, isolated cLPM were reconstituted into proteoliposomes, which were then loaded with ATP. Compared with non-ATP-containing controls, we observed no significant decrease of the residual [14C]PC content (20 ± 2.4 to 21 ± 3.1%; P > 0.05). Moreover, determinations of ATP concentrations after loading of proteoliposomes either in the presence or absence of a regenerating system demonstrated no appreciable decrease in ATP concentrations during the time course of our experiments. This finding excluded the possibility that...
proteoliposomal membranes hydrolyzed added ATP and thereby masked an ATP-dependent contribution to PC-translocating activity. Because others have shown that verapamil in micromolar concentrations inhibits PC-translocating activity of Mdr2 expressed in yeast secretory vesicles (29), we investigated the effect of 20 μM verapamil on translocation of PCs in cLPM. When proteoliposomes were loaded with this concentration of verapamil along with an ATP-regenerating system, we observed only a minimal increase in the residual [14C]PC content (19 ± 1.7 to 21 ± 2.3%; P > 0.05).

DISCUSSION

The coupling of intracellular and extracellular events during biliary phospholipid secretion requires transmembrane translocation of PCs from the inner to the outer leaflet of the cLPM (10). As evidenced by in vitro experiments employing a short-chain PC (6) and a fluorescent PC analog (26), together with in vivo studies of biliary lipid secretion in the mdr2−/− “knock-out” mouse (30), a canalicular transmembrane translocator appears to be present in rodents. Because transmembrane translocation of natural long-chain PCs had not been evaluated in isolated cLPM, we adopted a technique that utilized radiolabeled long-chain PCs (4, 21). In this assay, unilamellar vesicles and proteoliposomes prepared from erythrocyte ghosts yielded a residual [14C]PC content of 35–40%, which is consistent with the absence of PC transmembrane translocating activity (8). Similarly, transmembrane translocation of PC was absent in homogenates and microsomes of COS, H35 cells, and frog oocytes (Fig. 2). The significantly smaller (15–23%) residual [14C]PC contents observed in rat liver microsomes, cLPM and bLPM strongly suggest that PC molecules were translocated across these membrane bilayers (Fig. 1).

Despite frequent changes of acceptor vesicles with the current experimental conditions (Fig. 1), the putative PC translocator from liver cell membranes failed to remove all [14C]PC from proteoliposomes. Possible explanations for the nonexchangeable fraction of membrane PCs include the following. First, inactivation of PC-TP during incubation; this was excluded by frequent replenishment of PC-TP. Second, the PC translocator could have been inactivated during the exchange assay, but this is unlikely for the following reasons. Figure 1 demonstrates that most PC was removed during the first three incubation periods (90 min). Zilversmit and Hughes (36), employing a similar assay system to examine translocation of PCs in rat liver microsomes, demonstrated that transmembrane PC translocation activity persisted during a 6-h period. This is further supported by a study of purified cLPM vesicles by Nies, Gatmaitan and Arias (26) in which transmembrane translocation of a fluorescent PC analog was detected by its rapid chemical reduction with loss of fluorescence on the outer membrane hemileaflet. These authors (26) observed no decline in PC-translocating activity for cLPM after incubation periods of up to 30 min. Moreover, the same authors also found that up to 40% of translocated fluorescent PC had become inaccessible to the reducing agent, even after addition of Triton X-100, which solubilized the membrane and exposed all PC molecules in mixed micelles. Third, our proteoliposomes might have contained a fraction of vesicles without PC translocators (or with PC translocators oriented incorrectly), and thus [14C]PCs on the inner membrane leaflet of some proteoliposomes would not be accessible for exchange. This possibility was eliminated by experiments in which the membrane protein content of proteoliposomes was decreased by a factor of two. In this case after serial incubations (data not shown), no increase in the residual [14C]PC content of proteoliposomes was observed, which would have been expected if the PC translocator concentration was rate limiting. Finally, submicellar bile salts, which were rigorously excluded in our studies (see MATERIALS AND METHODS), might be required for optimal PC translocation as suggested by Ruetz and Gros (28) and Nies et al. (26) for Mdr2. Due to the excess PC present in the form of multilamellar vesicles in our experimental design, this hypothesis could not be directly evaluated because the high partition coefficient of submicellar bile salts, when added to PC membranes (11), has been demonstrated to induce both rapid exchange of PCs and even phase transitions (11).

Our finding that PC translocation was inhibited by protein denaturation and Pronase digestion is in agreement with previous observations for ATP-independent PC translocation (4, 6, 7). The requirement for 2-mercaptoethanol in addition to heat to inactivate protein PC-translocating activity is presumably due to the presence of one or more strategic disulfide bonds. In the current work, treatment with proteolytic enzymes before membrane solubilization did not eliminate PC translocation, an observation also made by Backer and Dawidowicz (4) employing liver microsomes. This suggests that the proteolytic enzyme did not have access to the active site of the transporter or, alternatively, that proteolysis was incomplete. The likelihood that PC translocation is protein mediated in our work was further supported by experiments in which we were able to confer PC-translocating activity on erythrocyte proteoliposomes by the addition of liver microsomes. Although our collective findings strongly suggest that a protein derived from hepatocyte membranes mediates PC translocation in proteoliposomes, it is important to consider that this protein may not function physiologically as a PC flippase. Formation of proteoliposomes by detergent solubilization followed by dialysis presumably results in at least partial loss of microdomains and molecular organization of the native membrane (16). We cannot therefore exclude the possibility that artificial lipid-protein interactions in proteoliposomes permit facile flip-flop of PCs reconstituted from hepatocyte membranes but not membranes from other cell types (Fig. 2).

Because we demonstrated absence of PC-translocating activities in both normal rat bile and serum of cholestatic rats, it is apparent that the flippase we have assayed is a tightly bound transmembrane protein which cannot easily be removed from liver plasma...
membranes. This stands in contrast to glycosylphosphatidylinositol-linked canalicular membrane proteins such as glutamyl transpeptidase, alkaline phosphatase, or 5'-nucleotidase, which are released into bile or into serum under pathophysiological conditions.

In contrast to ATP-dependent PC-translocating activity, which is believed to be confined to clPM vesicles (26), we observed ATP-independent long-chain PC transmembrane translocation in clPM and blPM as well as in microsomes. Because the principal mechanism for targeting some plasma membrane proteins appears to require their initial delivery to blPM before clPM (5), it is not unexpected that PC-translocating activity might be detectable in each of the membrane subfractions. In the case of ATP-independent PC translocation, Berr, Meier and Stieger (6) excluded substantial microsomal contamination as a source of translocating activity in clPM by demonstrating 60% enrichment of PC-translocating activity in clPM compared with microsomes. An alternative explanation is that, since our experimental design permitted inferences on PC-translocating activity only at equilibrium, even small degrees of microsomal contamination could conceivably have yielded spurious results.

Because the lipid compositions of microsomes, blPM, and clPM differ substantially (24, 35), we varied PE-to-PC ratios and pure lipids of clPM (Fig. 4) to alter the phospholipid asymmetry and fluidity of the proteoliposomal membranes. With increasing PE-to-PC ratios, more [14C]PC was exchanged, consistent with asymmetric distribution of PC and PE (i.e., PE enriched in the inner membrane hemileaflet) (16). Because proteoliposomes composed of clPM lipids had higher residual [14C]PC contents, it appears that membrane lipid composition may also play a role in modulating protein-mediated translocation of PC. Moreover, these results demonstrate that the same PC translocator appears to be functionally active in both the more fluid lipid bilayer of microsomes as well as the more rigid membranes typical of clPM.

Persuasive evidence has accumulated to suggest that Mdr2 in clPM may function as an ATP-dependent PC transmembrane translocator essential for bile formation (19). To test for ATP dependence, we carried out experiments in the presence of ATP concentrations reported by others to stimulate Mdr2-mediated PC-translocating activity (26, 28, 29). Although we failed to observe any stimulatory effects of ATP either after the initial incubation periods of proteoliposomes with multilamellar vesicles (data not shown) or on residual [14C]PC contents (see RESULTS), these concentrations may have been insufficient to enhance PC translocation in the current studies. Whereas we cannot exclude the possibility in our experiments that the ATP binding site of Mdr2 was disrupted during membrane solubilization and that we were observing a residual ATP-independent function of Mdr2, our findings are consistent with emerging evidence that a second, ATP-independent PC transmembrane translocator is present on liver plasma membranes, which is distinct from Mdr2 (6, 13, 19, 22). Current evidence supporting this possibility includes the finding that size-fractionated liver mRNA that is too small to encode Mdr2 confers ATP-independent PC-translocating activity in X. laevis oocytes (22) and that simultaneous expression of antisense RNA to Mdr2 fails to block the enhancement (13). In addition, preliminary findings suggest that there is translocating activity for short-chain PCs in cLPM vesicles isolated from mdr2 (−/−) knockout mice (27). On the basis of the marked reduction in biliary phosphatidylcholine secretion in mdr2 (−/−) knockout mice, an ATP-independent PC translocator in clPM presumably plays only a minor role in bile formation under physiological conditions. Nevertheless, the presence of such a transport protein would help to explain the observation of reduced but not completely absent phospholipid vesicles in bile canaliculi of mdr2 (−/−) mice (14).

In conclusion, this study provides evidence for ATP-independent translocation of long-chain PCs in hepatocyte plasma membrane subfractions and supports the likelihood that at least two different translocators participate in transmembrane PC translocation in rat liver. Although in bile formation the ATP-independent process appears to be ancillary, isolation and characterization of the responsible protein(s) are now required to elucidate the physiological function of this PC translocator.

The authors thank the Harvard Digestive Diseases Center Molecular Biology Core (National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-34854–12, Dr. Seth Alper, Director) for assistance with harvesting and preparation of X. laevis oocyte membranes.

This work was performed while M. Fuchs was a Deutsche Forschungsgemeinschaft Postdoctoral Fellow (Grant Fu 288/1–1) and a recipient of an American Digestive Health Foundation-Astra Merck Senior Postdoctoral Fellowship. A stipend grant-in-aid provided by the Falk Foundation, Freiburg, Germany, is gratefully acknowledged. D. E. Cohen was supported by a Pfizer-American Geriatrics Society Postdoctoral Fellowship and an American Liver Foundation Liver Scholar Award. Additional support was from National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-36588 and DK-34654 to M. C. Carey.

Portions of this work were presented at the Annual Meeting of the American Gastroenterological Association in San Diego, CA, 1995, and at the United European Gastroenterology Week in Berlin, Germany, 1995, and published in abstract form (Gastroenterology 108: A144, 1995; Gut 37: A1744, 1995).

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Received 8 April 1997; accepted in final form 27 August 1997.

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