ATP-dependent phosphatidylcholine translocation in rat liver canalicular plasma membrane vesicles

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Abstract Phosphatidylcholine (PC) translocation was studied in rat liver canalicular plasma membrane vesicles using a fluorescent PC analogue that permitted the quantitation of asymmetric PC distribution in the outer and inner leaflet of the vesicles. PC translocation to the outer leaflet of the canalicular membrane was stimulated by ATP and an ATP-regenerating system in a time- and temperature-dependent manner resulting in 200 pmol PC translocated/mg protein per 30 min. A non-hydrolyzable ATP analogue did not support translocation. Translocating activity was observed with PC but not with phosphatidylethanolamine and was specific for inside-out oriented canalicular membrane vesicles. Addition of taurocholate (10 μM), a micelle-forming bile acid, enhanced ATP-dependent PC translocation 1.5 ± 0.1-fold, whereas addition of taurodehydrocholate (10 μM), a non-micelle-forming bile acid, did not. These results indicate the presence of an ATP-dependent transporter that "flips" phosphatidylcholine from the inner to the outer leaflet of the rat bile canalicular plasma membrane from where it can become associated with bile acids in the canalicular lumen, thereby enhancing ATP-dependent flipping activity. Several lines of evidence suggest that the transporter is Mdr2 P-glycoprotein.—**Nies, A. T., Z. Gatmaitan, and I. M. Arias.** ATP-dependent phosphatidylcholine translocation in rat liver canalicular plasma membrane vesicles. *J. Lipid Res.* 1996. **37:** 1125–1136.

Supplementary key words flippase activity • bile salts • P-glycoprotein • Mdr2

Bile formation is an important function of the liver in all vertebrates. Hepatic bile is formed by excretory pathways for lipophilic exogenous (e.g., drugs, environmental chemicals) and endogenous compounds, such as phospholipids, bile salts, cholesterol, and anionic conjugates (e.g., bilirubin glucuronide, leukotriene C_4GSH). Specific transport systems for the active secretion of bile salts (1–3) and non-bile acid organic anions (4, 5) into bile have been described. The processes by which cholesterol and phospholipids (mainly phosphatidylcholine) enter bile are incompletely understood (6–8). A main focus of investigation is to understand how biliary phosphatidylcholine (PC) molecules are continuously secreted without affecting function and structure of the bile canalicular membrane.

Biliary PC is synthesized intracellularly in the endoplasmic reticulum (9) and is proposed to be delivered to the canalicular membrane by a cytosolic PC-translocating protein (10) or by vesicular transport (11). Having reached the canalicular membrane, PC is postulated (12) to be translocated from the inner to the outer leaflet by a transmembrane translocator (13, 14). The next step in bile formation involves bile salt-induced vesiculation of lipid microdomains in the outer leaflet of the canalicular membrane, as recently shown by Crawford et al. (15). Two proteins have been postulated to be involved in transmembrane PC translocation. *1)* Recent investigations of multidrug resistance demonstrated an important role for Mdr2 P-glycoprotein in PC translocation. Mdr2 is the dominant P-glycoprotein expressed in bile canalicular plasma membrane (16). Whereas the function of Mdr1 P-glycoprotein is well described as an ATP-dependent transporter for chemotherapeutic drugs and other hydrophobic cations across the canalicular membrane (for review see 17), the function of Mdr2 does not involve multidrug resistance. Important studies by Smit et al. (18) showed that mice that bear a null allele at the mdr2 locus have normal biliary concentrations of bile acids but lack PC in bile. These mice became jaundiced and developed inflammation, ne-

Abbreviations: CMV, canalicular membrane vesicles; SMV, sinusoidal membrane vesicles; ySV, yeast secretory vesicles; γ-GT, γ-glutamyltransferase; C_6-NBD-PC, 1-acyl-2[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylcholine; C12-NBD-PC, 1- acyl-2[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl] phosphatidylcholine; NBD-PE, 1-acyl-2[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylethanolamine; PC, phosphatidylcholine; F_I, initial fluorescence; F_D, fluorescence after dithionite treatment; F_T, fluorescence after dithionite and Triton treatment; TC, taurocholate; diC_4PC, L-dibutyroyl-glycero-3-phosphatidylcholine.

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crosis, and fibrosis of small bile ductular epithelium. The liver pathology is proposed to be due to the lack of PC that can no longer form mixed micelles with TC which can act as a potent detergent, affecting small bile ducts. In mice transgenic for human MDR3, the human homologue of murine mdr2, PC translocation across the plasma membrane of fibroblasts was enhanced compared to results in control fibroblasts (19). Furthermore, transfection of cells with mdr2 results in expression of Mdr2 (170 kDa) in the plasma membrane but does not confer multidrug resistance (20). In secretory vesicles of yeast (ySV) transfected with murine mdr2, ATP-dependent PC translocation was observed (21). Collectively, these findings support the hypothesis that Mdr2 is involved in ATP-dependent translocation of PC across the canalicular membrane. The second candidate PC translocator is an ATP-independent process which was observed in canalicular membrane vesicles by Berr, Meier, and Stieger (14).

Using a water-soluble, fluorescent acyl-caproyl-PC analogue and assay conditions described by Ruetz and Gros (21), we investigated whether a PC translocating protein which is functionally similar to the murine Mdr2 protein expressed in ySV (21), is present in rat liver canalicular membrane vesicles.

MATERIALS AND METHODS

Animals and materials

Male Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). [^{3}H(G)]TC (2 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA). Polyclonal antibody against the extracellular domain of γ-glutamyltransferase (γ-GT) was kindly provided by Dr. Masayasu Inoue (Osaka City Medical School, Japan). NBD-labeled phospholipids, C_6-NBD-PC, 1-acyl-2[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylcholine, C_{12}-NBD-PC, 1-acyl-2[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]phosphatidylcholine, and NBD-PE, 1-acyl-2[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylethanolamine, were obtained from Avanti Polar Lipids Inc. (Alabaster, AL) with "acyl" representing the fatty acid content of egg lysophosphatidylcholine (62% palmitic acid, 29% stearic acid, 9% unsaturated fatty acids). The Bio-Rad dye reagent was from Bio-Rad Laboratories (Hercules, CA). PC-specific phospholipase C (E.C. 3.1.4.3) and alkaline phosphatase (E.C. 3.1.3.1) were purchased from Boehringer Mannheim (Mannheim, Germany). Pronase E (protease type XXV from *Streptomyces griseus*) and all other chemicals were obtained from Sigma (St. Louis, MO) and were of highest purity available.

Subcellular fractionation

Canalicular membrane vesicles (CMV, 22) and sinusoidal membrane vesicles (SMV, 23) were prepared and purified as described previously. Total microsomes were isolated as described by Saucan and Palade (24). All subcellular fractions were resuspended in buffer A (10 mM Tris-HEPES, pH 7.4, 250 mM sucrose, 0.2 mM $CaCl_2$) and kept at -80°C.

Protein concentrations were measured according to Bradford (25) using the Bio-Rad dye reagent and bovine serum albumin as standard.

In some experiments, PC content of the vesicles was enzymatically measured using PC-specific phospholipase C and alkaline phosphatase in borate buffer (26). Released inorganic phosphate was measured according to Taussky and Schorr (27). Total PC content of CMV was 2.7 ± 0.3 μmol/mg protein (n = 7).

Separation of inside-out and right-side-out vesicles

The CMV preparation yields about 90% right-side-out and 10% inside-out oriented vesicles (1, 22, 28). When measuring LAP activity in the absence and presence of 0.1 mg SDS/mg protein, specific LAP activity was 7.2 ± 2.9% (n = 5) higher than that observed in the absence of detergent confirming that about 10% of the vesicles were inside-out. In some experiments, inside-out oriented vesicles were separated from right-side-out oriented vesicles by antibody-induced density perturbation through a continuous 20–50% sucrose gradient as described previously (22, 28). Briefly, CMV were incubated with anti-γ-GT antiserum which recognizes the extracellular domain of γ-GT (22). After 20 min incubation at 37°C and subsequently at 4°C, anti-rabbit IgG antiserum was added. After incubation for 30 min at 37°C, membrane samples were layered on a continuous sucrose density gradient (20–50% sucrose in 10 mM Tris-HEPES, pH 7.4, with a 60% sucrose cushion). After centrifugation for 60 min at 17,000 *g*, vesicles were recovered from the low density fractions (fractions 2–4) and from the high density fractions (fractions 5–8 of the sucrose gradient), resuspended in a small volume of buffer A and kept at -80°C until labeling with NBD-PC. Vesicles recovered from the low density fractions were mainly oriented inside-out and vesicles collected from the high density fractions were mainly right-side-out (1, 22, 28) (**Table 1**).

Transport studies

TC transport was measured by rapid filtration (29). The transport incubation medium contained 10 μM [^{3}H]TC, 5 μM TC, 1.2 mM ATP, and an ATP-regenerating system (3 mM phosphocreatine, 3.6 μg creatine phosphokinase) in buffer B (10 mM Tris-HEPES, pH 7.4, 250 mM sucrose, 0.2 mM $CaCl_2$, 10 mM $MgCl_2$). Trans-

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TABLE 1. PC translocation in inside-out and right-side-out oriented canalicular membrane vesicles

Preparation, Fraction	LAP Activity - Detergent	LAP Activity + Detergent	% Inside-Out Oriented Vesicles	ATP-Dependent PC Translocation[a] pmol/ml/30 min	ATP-Dependent PC Translocation[a] per Unit LAP Activity + Detergent	Factor
Preparation 1						
Inside-out vesicles	2.1	8.8	76	3.2	0.36	17.9
Right-side-out vesicles	13.1	14.2	8	0.3	0.02	
Preparation 2						
Inside-out vesicles	nd	1.3	nd	8.1	6.21	11.6
Right-side-out vesicles	nd	13.6	nd	7.3	0.54	
Preparation 3						
Inside-out vesicles	nd	1.9	nd	0.8	0.40	9.3
Right-side-out vesicles	nd	8.9	nd	0.4	0.07	

Inside-out and right-side-out oriented vesicles were separated as described in Experimental Procedures. Inside-out vesicles were recovered from low density fractions and right-side-out vesicles from high density fractions.

[a]ATP-dependent PC translocation is given in pmol C_6-NBD-PC/ml per 30 min. Because antibody had bound to the right-side-out vesicles, PC translocation activity could not be expressed per mg protein. Therefore, PC translocation is expressed per unit LAP activity (1 unit LAP activity = 1 μmol hydrolyzed L-leucine-p-nitroaniline/ml per 30 min). PC concentration was measured in preparation 1 and was 2.2 and 3.3 μmol/ml in inside-out and right-side-out vesicles, respectively; nd, not determined.

port was initiated by addition of 20 μl vesicle suspension in buffer A (30–70 μg protein) to 0.1 ml of incubation medium. Aliquots were removed after 75 sec, diluted with 1 ml ice-cold buffer B, and immediately filtered through a glass microfiber filter (GF/C, 0.45 μm pore size, Whatman, Maidstone, UK). The filters were washed with 5 ml ice-cold buffer B. Radioactivity trapped on the filters was measured in a liquid scintillation counter (LS 1801, Beckman, Palo Alto, CA). All values were corrected for radioactivity bound to the filters in the absence of membrane vesicles.

The asymmetric distribution of PC across the canalicular membrane was measured using a fluorescence assay adapted from Ruetz and Gros (21) based on the method of McIntyre and Sleight (30).

Preparation of donor liposomes containing fluorescent phospholipid

Donor liposomes containing C_6-NBD-PC were prepared by mixing 1 mg C_6-NBD-PC with 1 mg PC and 1 mg 2× recrystallized cholesterol in a total volume of 1.32 ml and subsequent removal of the storage solvent by vacuum desiccation. The dried PC/cholesterol mixture was resuspended in 0.29 ml ethanol and injected at room temperature with a Hamilton syringe into 3.5 ml of buffer C (100 mM NaCl, 50 mM sucrose, 10 mM Tris-HEPES, pH 7.4). C_6-NBD-PC containing donor liposomes were dialyzed at 4°C for 16 h against buffer A. Donor liposomes containing NBD-labeled PE and C_{12}-NBD-PC were similarly prepared after mixing 1 mg NBD-PE and 1 mg C_{12}-NBD-PC, respectively, with PC and cholesterol. In some experiments, donor liposomes that were labeled with C_6-NBD-PC only in the inner leaflet were used. These "only inside-labeled donor liposomes" were prepared by incubating donor liposomes for 30 min with 40 mM dithionite and subsequent dialysis at 4°C for 16 h against buffer A.

Preparation of vesicles containing fluorescent phospholipid

Because C_6-NBD-PC has a higher water solubility than unlabeled PC, the fluorescent PC analogue is easily transferred between membranes whereas the unlabeled PC is not (30). Transfer was initiated by mixing CMV with donor liposomes at a concentration of 17 nmol C_6-NBD-PC/mg protein. After incubating the mixture for 30 min at 4°C, free donor liposomes were separated from NBD-labeled CMV by centrifugation of the mixture at 100,000 *g* for 30 min in a swinging bucket rotor through 16% sucrose (16% sucrose in 10 mM Tris-HEPES, pH 7.4, 0.2 mM $CaCl_2$). The sedimented NBD-labeled CMV were washed once with buffer A and used immediately for the phospholipid translocation assay. The same procedure was used for preparation of NBD-PE-labeled CMV, C_6-NBD-PC-labeled SMV, and C_6-NBD-PC-labeled microsomes.

Phospholipid translocation assay

Equal volumes of C_6-NBD-PC-labeled CMV (0.5–1.0 mg/ml) and buffer D (buffer A supplemented with 5 mM Na_2ATP, 10 mM Mg-gluconate, 20 mM phosphocreatine, and 3 μg/ml phosphocreatine kinase) were mixed and lipid translocation was initiated by incubating the mixture at 37°C. After the indicated time periods, 100 μl was removed and immediately placed into a microcuvette containing 400 μl buffer A. NBD-PC fluorescence emission was measured (LS-5B fluorescence spectrophotometer, Perkin-Elmer, Norwalk, CT) at an excitation wavelength of 470 nm (10 nm slit width) and emission wavelength of 540 nm (5 nm slit width). The initial fluorescence (F_I) was recorded until a stable baseline was achieved. Then, 25 μl of sodium hydrosulfite (= dithionite; 1 M in 1 M Tris, pH 10) was added and the decrease in fluorescence due to chemical reduction of NBD-PC (nitrobenzoxadiazol-PC) to non-fluorescent

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ABD-PC (aminobenzoxadiazol-PC) was recorded for 6–7 min until a new stable baseline was achieved (F_D). As dithionite is a water-soluble and membrane-impermeable anion (30), the decrease in fluorescence probably reflects reduction of NBD-PC molecules associated with the outer leaflet of the vesicular membrane. Upon addition of detergent (80 μl 10% Triton X-100), the vesicles were disrupted making the inner leaflet accessible to dithionite which resulted in a further decrease in fluorescence (F_T) reflecting the amount of C_6-NBD-PC associated with the inner leaflet and the intravesicular space.

The percentage of fluorescence associated with the outer and inner leaflet was calculated as

$$\% \text{ out} = 100\ [(F_I - F_D)/(F_I - F_T)] \text{ and } \% \text{ in} = 100 - \% \text{ out}$$

Preparation of calibration curve

For the calibration curve, liposomes containing 0.7% C_6-NBD-PC were prepared as described above. The relative fluorescence of increasing amounts of these liposomes was measured in buffer A. Fluorescence emission was linear within a range of 0–38 pmol/ml C_6-NBD-PC. The standard curve was used to convert changes in fluorescence associated with the inner leaflet (typically 5–25 pmol/ml) into pmol C_6-NBD-PC/mg protein and pmol NBD-PE/mg protein, respectively.

Protease treatment of C_6-NBD-PC-labeled CMV

In some experiments, C_6-NBD-PC-labeled CMV were treated with protease (Pronase E). The amount of C_6-NBD-PC associated with the inner leaflet of C_6-NBD-PC-labeled CMV was measured before and after incubation of vesicles in 10 mg/ml Pronase E for 30 min. Because identical amounts of vesicle protein were added to the phospholipid translocation assay, values are given in pmol C_6-NBD-PC/mg vesicle protein.

Intrinsic phospholipase A_2 activity of C_6-NBD-PC-labeled CMV

Because C_6-NBD-PC might be degraded by an intrinsic phospholipase A_2 activity of CMV to a fluorescent product, NBD-caproic acid, fluorescence emission of 25 μl C_6-NBD-PC-labeled CMV in 475 μl buffer A at 25°C was monitored. As observed by Meyuhas et al. (31), a decrease in fluorescence emission was observed. This is probably due to partitioning of the degradation product NBD-caproic acid into lipidic compartments of CMV where fluorescence is only partly quenched. Intrinsic phospholipase A_2 activity of CMV resulted in 4.5 ± 2.0%/30 min (n = 3) loss of initial fluorescence. However, after reduction with dithionite, no phospholipase A_2 was observed. Therefore, changes in Triton-dependent fluorescence were most likely due to fluorescence emission of NBD-PC.

Statistics

Values are given as means ± standard error of the means (SEM) derived from at least three independent experiments performed in duplicate. When Student's *t*-test was used to determine statistical significance, *P* values are given.

RESULTS

Preparation of NBD-labeled CMV

Because donor liposomes have an average diameter of 30 nm (32) and are considerably smaller than CMV, which have diameters ranging from 400 to 900 nm (22), both were separated by centrifugation through 16% sucrose (**Fig. 1A**). Forty percent of C_6-NBD-PC molecules (Fig. 1A, circles) co-sedimented with CMV. Between 12.8 and 23.3 nmol (n = 8) of fluorescent PC was transferred from donor liposomes to CMV which corresponds to 0.5–0.9% of the total amount of PC in the CMV fraction. In control experiments, sedimentation of CMV was identical in the presence (Fig. 1A, bars) or absence of donor liposomes (Fig. 1B). In contrast, donor liposomes in the absence of CMV did not sediment and remained almost entirely in the first four fractions (Fig. 1C). Similar results were obtained using NBD-labeled SMV (data not shown). For assessment of contamination of NBD-labeled CMV with donor liposomes, CMV were labeled with C_{12}-NBD-PC which has a slower transfer rate (33). Only 15% of C_{12}-NBD-PC molecules co-sedimented with CMV (Fig. 1D, triangles). Furthermore, in CMV that were incubated with "only inside-labeled donor liposomes", no C_6-NBD-PC-fluorescence was associated with CMV (Fig. 1D, open circles).

Functional integrity of NBD-CMV

Because assay of asymmetric PC distribution in the vesicular membrane depends on intact membrane vesicles, ATP-dependent TC transport was measured to determine the effect of incorporation of C_6-NBD-PC into CMV. ATP-dependent TC transport was not significantly different ($P > 0.05$) in CMV (22 ± 4.6 pmol TC/mg protein per 75 sec, n = 4), NBD-labeled CMV (23 ± 4.5 pmol TC/mg protein per 75 sec, n = 4) and NBD-labeled CMV treated with 40 mM dithionite (16 ± 4.5 pmol TC/mg protein per 75 sec, n = 4). These results indicate that neither incorporation of C_6-NBD-PC nor treatment of NBD-labeled CMV with dithionite causes leakiness or disruption of the bilayer.

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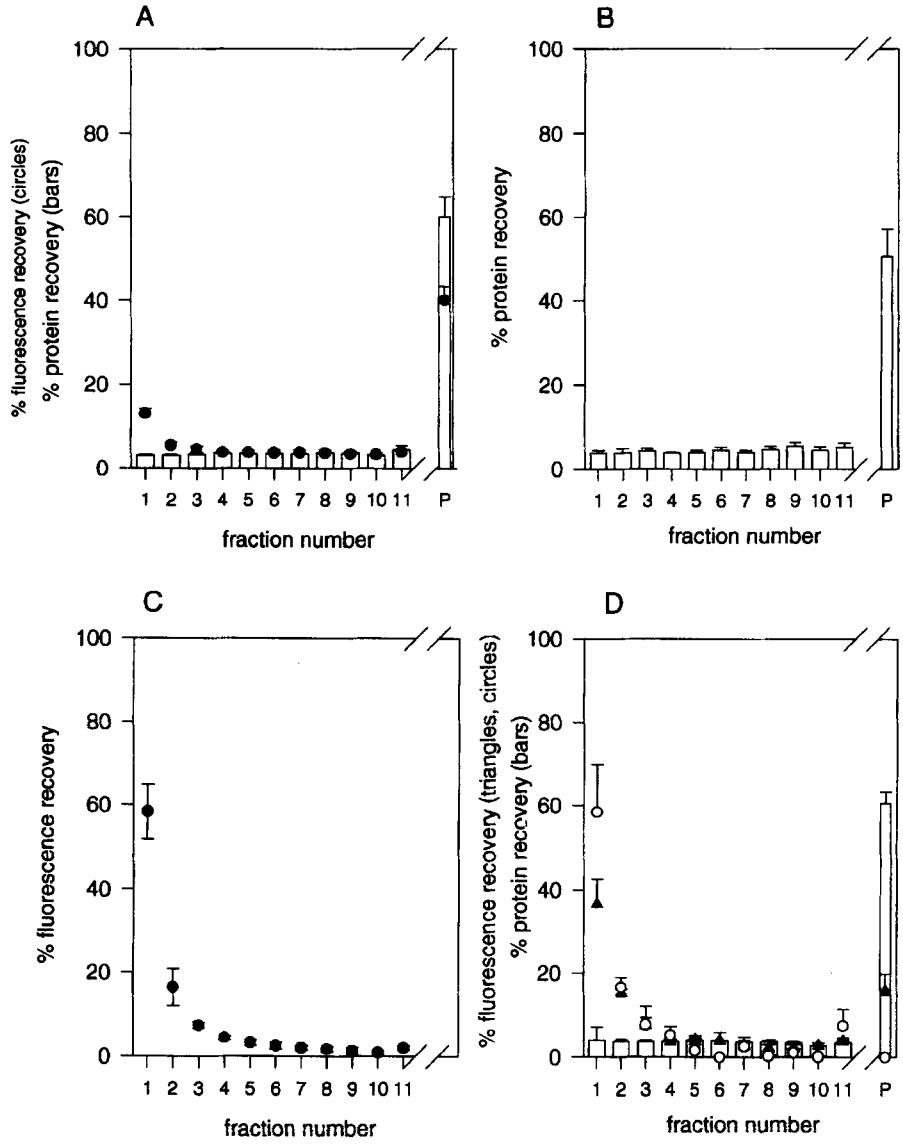

Fig. 1. Separation of free donor liposomes from NBD-PC-labeled CMV. CMV, free donor liposomes, and NBD-labeled CMV were centrifuged through 16% sucrose and 1-ml fractions were taken from top to bottom. Panel A shows recovery of C_6-NBD-PC-fluorescence (circles) and protein (bars) after centrifugation of NBD-labeled CMV through 16% sucrose. Panel B shows recovery of CMV-protein in the absence of liposomes. Panel C shows recovery of donor liposomes as measured by fluorescence emission in the absence of CMV. Panel D shows recovery of C_{12}-NBD-PC-fluorescence (triangles) and fluorescence due to "only inside-labeled donor liposomes" (open circles) and protein (bars) after centrifugation of NBD-labeled CMV through 16% sucrose. Data are given as a percentage of recovery from the initial amounts of C_6-NBD-PC (filled circles), C_{12}-NBD-PC (triangles), C_6-NBD-PC in "only inside-labeled donor liposomes" (open circles) and protein (bars). P, protein sediment. Data are means ± SE of 3 replicate experiments.

Assay of PC translocation

In **Fig. 2A,** a typical recording of a PC translocation assay is shown. Initial fluorescence (F_I) was measured until steady-state values were obtained. Upon addition of dithionite, a rapid decrease of the fluorescence signal was observed reaching a new steady-state level after 6–7 min (F_D) reflecting reduction of C_6-NBD-PC molecules associated with the outer leaflet of the vesicular membrane. The reaction was complete because increasing amounts of dithionite did not result in further decrease of fluorescence (data not shown). However, a very slow rate of dithionite reaction (0.08%/min of initial fluorescence) was observed when fluorescence was recorded for 30 min. Compared to the fast reaction of dithionite resulting in a loss of 95% of initial fluorescence within 2–3 min (Fig. 2A), this slow rate was negligible suggesting that NBD-labeled CMV are virtually impermeant to dithionite. Addition of Triton X-100 disrupts the vesicles permitting entry of dithionite. The observed further

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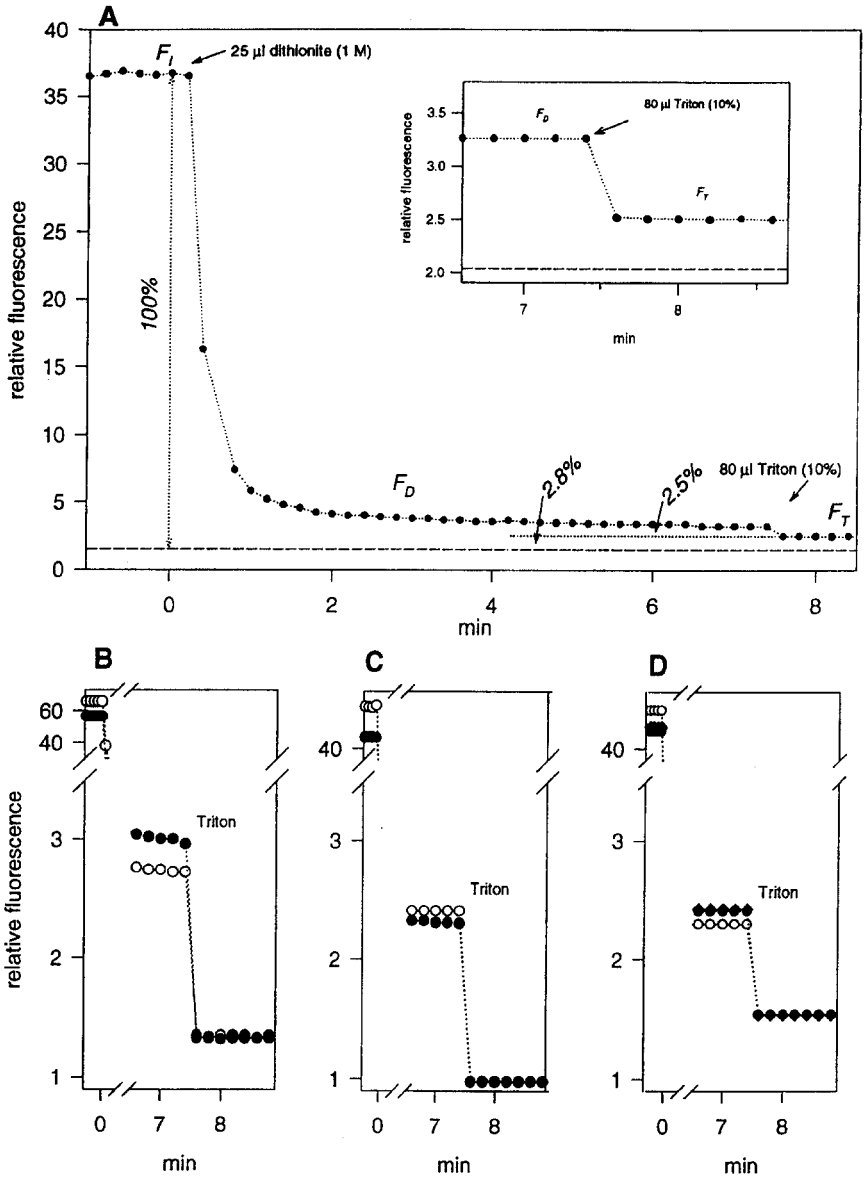

Fig. 2. Fluorescence emission recordings of C_6-NBD-PC-labeled CMV and C_6-NBD-PC-labeled SMV. Fluorescence emission was measured as described. Panel A shows a typical recording of labeled CMV directly recovered after centrifugation through 16% sucrose. The inset shows enlargement of the recording between 6.5 and 8.5 min. Panel B is a typical recording of C_6-NBD-PC-labeled CMV prior to (○) and after (●) incubation in ATP, Mg^{2+}, and an ATP-regenerating system at 37°C. Panels C and D are similar to panel B, but C_6-NBD-PC-labeled SMV and C_6-NBD-PC-labeled microsomes were used in the assay, respectively, and, in case of microsomes, a recording after incubation in buffer A at 37°C is shown (◆). Dithionite and Triton X-100 were added as indicated. All recordings represent characteristic results of duplicate profiles of 14 (CMV) and 3 (SMV, microsomes) replicate experiments (variation 5% in CMV- and 25% in SMV-recordings).

decrease in fluorescence signal (F_T, enlarged in inset) reflects the amount of C_6-NBD-PC associated with the inner leaflet and the intravesicular space. The reaction was complete because increasing amounts of Triton X-100 did not result in a further decrease of fluorescence (data not shown). However, decrease in the fluorescence signal in CMV to background emission levels in the absence of C_6-NBD-PC was not achieved (Fig. 2A, dashed line) suggesting that approximately 2.8% of C_6-NBD-PC molecules are inaccessible to dithionite and Triton X-100.

Properties of PC translocation in subcellular fractions: ATP-dependence

Fluorescence signal recordings of NBD-labeled CMV before and after 30 min incubation at 37°C in ATP,

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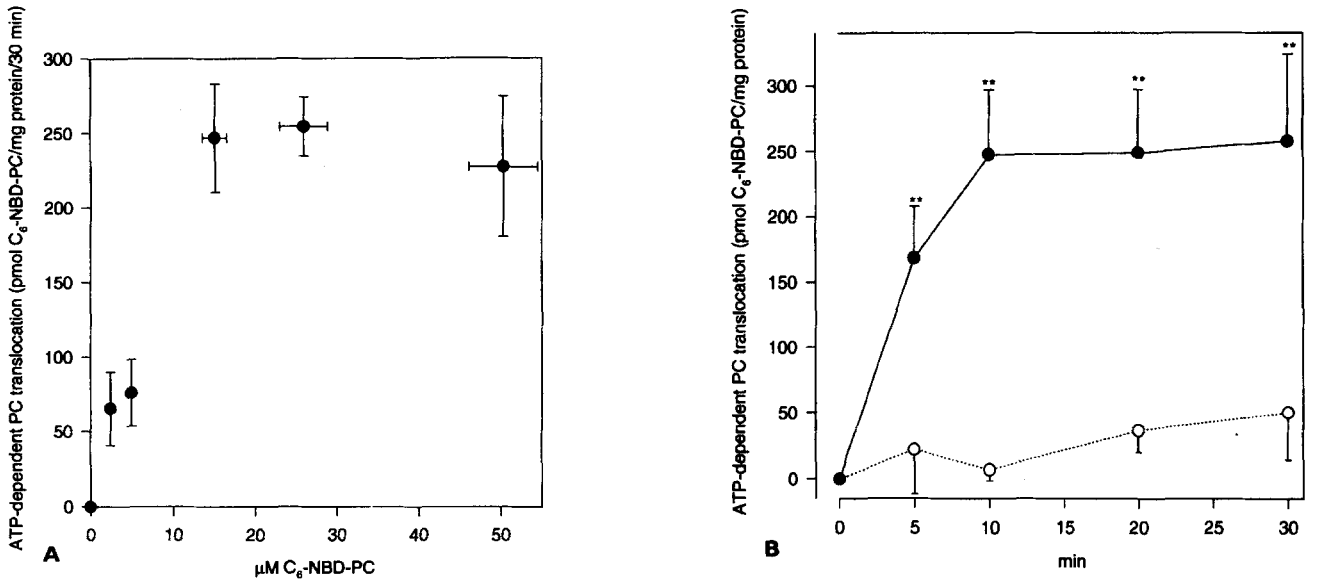

Fig. 3. Concentration-, time-, and temperature-dependence of ATP-dependent PC translocation. A: Concentration-dependence. C_6-NBD-PC-labeled CMV were prepared as described in Experimental Procedures by increasing the amount of donor liposomes added during the labeling procedure. Then, fluorescence emission of C_6-NBD-PC-labeled CMV in the presence of ATP was measured after 30 min as described in Experimental Procedures. B: Time- and temperature-dependence. Fluorescence emission of C_6-NBD-PC-labeled CMV in the presence of ATP was measured at indicated times at 37°C (●) or 4°C (○) at a concentration of 20 μM C_6-NBD-PC as described in Experimental Procedures. Data are means ± SE of duplicate measurements of 4 (A) and 3 (B) replicate experiments.

Mg^{2+}, and an ATP regenerating system are shown in Fig. 2B. Before incubation, 2.5 ± 0.1% (n = 14) of total NBD-labeled PC molecules were initially associated (i.e., background) with the inner leaflet (open circles) representing 249.6 ± 16.5 pmol C_6-NBD-PC/mg protein (n = 14). Incubation at 37°C significantly ($P < 0.05$) increased the amount of C_6-NBD-PC molecules associated with the inner leaflet of CMV to 297.4 ± 19.3 pmol C_6-NBD-PC/mg protein (n = 14, filled circles). The translocation activity resulted in 19.9 ± 2.8% increase over background fluorescence. As a control, PC translocation in SMV and total microsomes was measured. No PC-translocation was observed in SMV (Fig. 2C), and in total microsomes only ATP-independent PC translocation was measured (Fig. 2D).

Concentration-, time-, and temperature-dependence of PC translocation in CMV

ATP-dependent PC translocation is concentration-dependent and saturates beyond 15 μM NBD-C_6-PC at 250 pmol C_6-NBD-PC/mg protein/30 min (**Fig. 3A**). PC translocation activity is time-dependent, increasing in curvilinear fashion, and leveling off after 10 min to 250 pmol C_6-NBD-PC/mg protein (Fig. 3B, filled circles) after 30 min of incubation at 37°C. ATP-dependent translocation in vesicles incubated at 4°C was 50 pmol C_6-NBD-PC/mg protein per 30 min (Fig. 3B, open circles).

Separation of inside-out and right-side-out vesicles

Because the CMV preparation yields about 90% right-side-out and 10% inside-out oriented vesicles (22), it was of interest to determine which vesicle type accounted for ATP-dependent PC translocation. Therefore, after antibody-induced density perturbation two fractions were separated; a low density fraction mainly consisting of inside-out vesicles and a high density fraction mainly consisting of right-side-out vesicles (Table 1). ATP-dependent PC translocation was 12.9 ± 2.6-fold (n = 3) higher in NBD-labeled vesicles recovered from the low density fraction compared to results in NBD-labeled vesicles isolated from the high density fraction suggesting that PC translocation occurs primarily, if not exclusively, from inside-out oriented CMV (Table 1).

Substrate specificity

ATP-dependent translocation in CMV was specific for C_6-NBD-PC; C_6-NBD-PE was not translocated. C_6-NBD-PE (124.5 ± 26.2 pmol/mg protein) was initially associated with the inner leaflet and was unchanged after 30 min incubation at 37°C in ATP, Mg^{2+}, and an ATP-regenerating system (124.0 ± 31.9, n = 4).

No C_6-NBD-PC translocation was observed when NBD-labeled CMV were incubated for 30 min at 37°C without ATP, in AMP, or with the non-hydrolyzable ATP analogue, ATPγS (**Fig. 4**), suggesting that PC translocation requires ATP hydrolysis. PC translocation in CMV

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is ATP-dependent; however, after incubation of CMV with Pronase E for 30 min, all ATP-dependent PC translocation activity was lost (Fig. 4).

Effect of bile salts on PC translocation

Because PC translocation might be involved in hepatic bile formation by contributing PC for formation of mixed micelles with bile salts, the effect of bile salts on PC translocation was investigated. TC increased ATP-dependent PC translocation in a dose-dependent manner (**Fig. 5A**). TC did not significantly ($P > 0.05$) support PC translocation in the absence of ATP (Fig. 5B). The effect on ATP-dependent PC translocation was specific for TC; the non-micelle-forming bile salt, taurodehydrocholate, did not stimulate ATP-dependent PC translocation (Fig. 5B). These results suggest that the effect of TC may be due to its micelle-forming properties. This was further supported by the finding that in CMV, incubated with ATP, 0.05% Triton was sufficient to render C_6-NBD-PC molecules associated with the inner leaflet accessible to dithionite (Fig. 5C), whereas, in ATP and 10 μM TC (Fig. 5D), 0.1% of Triton was necessary to render the C_6-NBD-PC molecules accessible to dithionite reduction (n = 3). These results suggest that some C_6-NBD-PC molecules were present in micelles or aggregates, which required a higher detergent concentration for complete solubilization.

DISCUSSION

We have used a fluorescent phospholipid, acyl-(NBD)-caproyl-PC, to identify an ATP-dependent PC translocating activity in rat CMV. Because preparation of fluorescently labeled CMV and the phospholipid translocation assay were adapted from studies by Ruetz and Gros in yeast (21), it was first necessary to validate these methods.

Centrifugation of donor liposome/CMV mixtures through 16% sucrose proved optimal for quantitative separation of free donor liposomes from labeled CMV (Fig. 1), to ensure that no background fluorescence of free donor liposomes was present to interfere with fluorescence changes associated with labeled CMV. Because donor liposomes might be "sticking" to the vesicular membrane, two controls were performed: *1)* C_6- and C_{12}-NBD-PC both spontaneously transfer between liposomes with a rate of 94% of total fluorescence/min and 1.2% of total fluorescence/min, respectively (33). Thus, after 30 min of labeling, C_6-NBD-PC-label of CMV should be maximal, whereas C_{12}-NBD-PC-label should amount to 36% of total fluorescence. Assuming that C_6-NBD-PC associated with CMV (40% as shown in Fig. 1A) reflects the maximal transferable amount, only

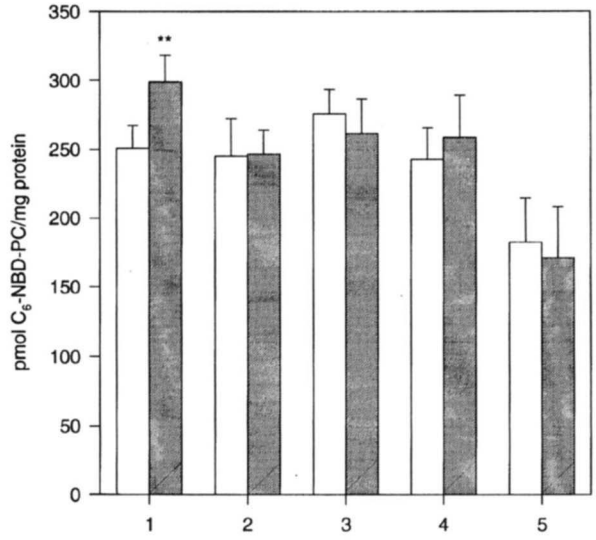

Fig. 4. Effect of nucleotides and Pronase E on PC translocation in NBD-labeled CMV. Translocation of C_6-NBD-PC was measured as described. Open bars, background; hatched bars, the amount of C_6-NBD-PC after incubation at 37°C for 30 min with (1) 5 mM ATP, Mg^{2+}, ATP-regenerating system, (2) buffer A, (3) 5 mM AMP, Mg^{2+}, ATP-regenerating system, (4) 5 mM ATPγS and (5) 5 mM ATP, Mg^{2+}, ATP-regenerating system and 10 mg/ml Pronase E. C_6-NBD-PC concentration was 5 μM. Data are means ± SE of duplicate measurements of 14 (column 1), 5 (columns 2–4), and 3 (column 5) replicate experiments. $^{**}P < 0.01$.

14.4% of C_{12}-NBD-PC should be associated with CMV. Because 15% of C_{12}-NBD-PC was associated with CMV, less than 1% of fluorescence is probably due to contamination by donor liposomes. *2)* Because "only inside-labeled donor liposomes" do not have any transferable fluorescence in their outer leaflet, any fluorescence observed in the CMV-sediment should be due to "sticking" of donor liposomes to the vesicular membrane. However, no C_6-NBD-PC fluorescence was associated with CMV so that contamination of NBD-labeled CMV with donor liposomes was negligible and most of the fluorescence was associated with the vesicular membrane. Neither labeling of CMV with C_6-NBD-PC nor treatment with dithionite altered the transport characteristics of TC, indicating that CMV retain functional integrity throughout the labeling procedure and phospholipid translocation assay. These results show that the described experimental system (21) is suitable for measurement of asymmetric PC distribution in rat CMV and that observed changes in the amount of C_6-NBD-PC are most likely due to a process residing in CMV.

The phospholipid labeling procedure resulted in CMV in which most of the label was accessible to dithionite, suggesting that C_6-NBD-PC incorporated preferentially into the outer leaflet. However, a Triton-dependent decrease in fluorescence was always observed in CMV immediately after their recovery from sucrose. This "background" varied between 200–300

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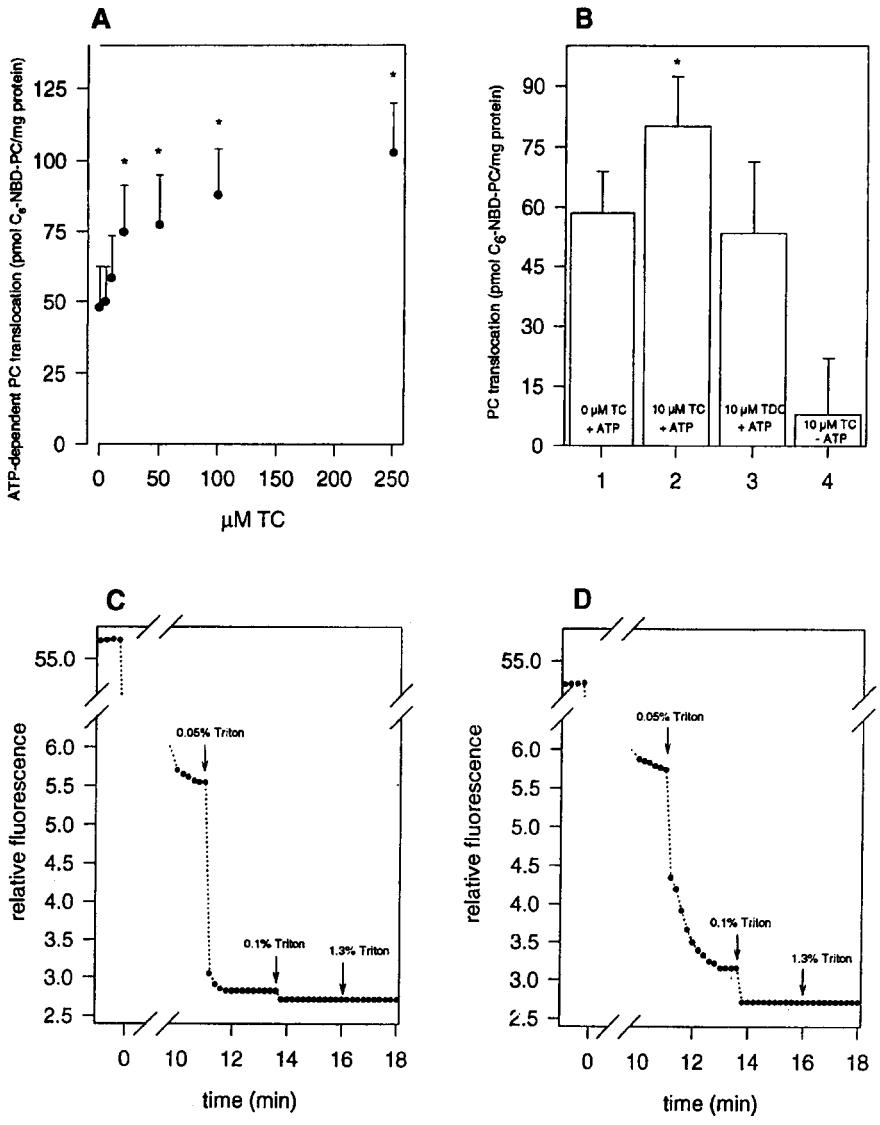

Fig. 5. Effect of bile salts on ATP-dependent PC translocation. Fluorescence emission of C_6-NBD-PC-labeled CMV in the presence of ATP was measured as described in Experimental Procedures in the presence of different TC concentrations (panel A, 4 replicate experiments; 0, 5, 10, 25, 50, 100, 250 μM TC). Panel B shows ATP-dependent PC translocation without bile salt (column 1; 7 replicate experiments), in 10 μM TC (column 2; 7 replicate experiments), and in 10 μM taurodehydrocholate (TDC, column 3; 4 replicate experiments) and PC translocation with 10 μM TC in the absence of ATP (column 4, 4 replicate experiments). ATP-dependent PC translocation is given in pmol C_6-NBD-PC/mg protein per 30 min at a concentration of 5 μM. Panels C and D show typical fluorescence emission recordings of C_6-NBD-PC-labeled CMV incubated for 30 min in the presence of ATP and in the absence (C) or presence (D) of 10 μM TC (3 replicate experiments). Arrows indicate the final concentration of Triton X-100 added into the translocation assay.

pmol C_6-NBD-PC/mg protein and may result from contamination by free donor liposomes or C_6-NBD-PC translocation during the labeling procedure. Because contamination by free donor liposomes is minimal (see above), background fluorescence is probably due to NBD-PC transferred during the phospholipid labeling procedure.

ATP-dependent PC translocating activity was 12.9-fold greater in inside-out CMV compared to results in right-side-out CMV (Table 1) indicating that PC movement in vivo is from the inner to the outer leaflet of the canalicular membrane. ATP-dependent PC translocating activity was only observed in CMV, but not in SMV and microsomes (Fig. 2B, C, D). ATP was necessary for translocation in CMV because the non-hydrolyzable ATP analogue, ATPγS, did not support translocation (Fig. 4) suggesting that ATP hydrolysis and not ATP binding is required for translocation. Time- and tem-

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perature-dependence of ATP-dependent translocation in CMV (Fig. 3) and its abolition by Pronase treatment (Fig. 4) provides further support that PC translocation is a protein-mediated process. Translocating activity was specific for PC, the aminophospholipid phosphatidylethanolamine was not translocated. These data provide evidence for a membrane-bound protein that has a cytoplasmic ATP-binding site and that translocates ("flips") PC from the inner to the outer leaflet of rat CMV.

Fluorescent phospholipids containing caproyl-NBD are identical to native phospholipids except for their solubility (34). Because they spontaneously transfer between liposomes at a much higher rate than their long-chain counterparts, dodecanoyl-NBD-phospholipids (33), they have been widely used for membrane labeling and as model substrates investigating phospholipid translocating processes in human erythrocytes (35), Chinese hamster fibroblasts (36), and yeast secretory vesicles (21, 37). Therefore, we used acyl-caproyl-NBD-PC in the present study as a model substrate for the ATP-dependent PC translocating process in CMV. Only PC, but not phosphatidylethanolamine, is a substrate, suggesting that other parts of the molecule, rather than the fluorescent moiety, are important for specificity. NBD-PC manifests self-quenching when inserted >1% into membranes (33). Because NBD-PC accounted for only 0.5–0.9% of total PC in CMV, considerable self-quenching probably does not exist in the described experimental setup.

Using also water-soluble phospholipids, two other phospholipid flippase proteins were recently described (14, 21). The protein described by Berr, Meier, and Stieger (14) passively transports short-chain PC (diC_4PC) across the canalicular membrane thus acting as an "equilibrative phospholipid pore" translocating 7 nmol diC_4PC/mg protein per 30 min. DiC_4PC uptake was also found in basolateral membrane vesicles (3 nmol diC_4PC/mg protein per 30 min) and microsomes (6 nmol diC_4PC/mg protein per 30 min); ATP had no stimulatory effect. In contrast, the system described in the present study specifically translocates acyl-C_6-PC with an absolute requirement for hydrolyzable ATP and is restricted to the canalicular membrane. During 30 min of incubation, only 250 pmol C_6-NBD-PC/mg protein were maximally translocated. In the present study, ATP-independent C_6-NBD-PC translocation may have occurred across the "phospholipid pore" during the phospholipid labeling procedure. This suggestion is supported by background fluorescence associated with the inner leaflet, amounting maximally to 0.8–1.2 nmol/mg protein.

The other recently described flippase activity is attributed to the product of murine mdr2, a member of the mdr gene family (21). The PC translocating activity described in the present study is similar to Mdr2-mediated flippase activity in many respects: flipping is temperature- and time-dependent and requires ATP hydrolysis (Fig. 3, Fig. 4). Additionally, ATP-dependent PC translocation was restricted to CMV as is the localization of Mdr2 as determined by isoform-specific antibodies (16). Therefore, it is likely that Mdr2 accounts for the observed ATP-dependent PC translocating activity in CMV. Unfortunately, however, homozygous mdr2-knockout mice are not available to us for study.

A further clue, as to whether the PC translocating protein is identical with Mdr2, was provided by the stimulatory effect of TC on ATP-dependent PC translocation (Fig. 5A), which is most likely not due to leakiness of CMV as ATP-dependent TC transport was detected up to 100 μM TC (1). As observed in ySV (37), in the presence of TC, a greater concentration of Triton was necessary to reveal C_6-NBD-PC compared to control conditions (Fig. 5C, D), indicating that part of the C_6-NBD-PC molecules may be in an environment that requires a higher detergent concentration. This environment is likely to be TC/C_6-NBD-PC micelles or aggregates in the intravesicular space, as was shown for ySV (37). Furthermore, taurodehydrocholate, a non-micelle-forming bile salt, did not stimulate ATP-dependent PC translocation (Fig. 5B). These results support the hypothesis that the PC-translocating protein is identical with Mdr2. In addition, other systems in rat liver canalicular membrane, such as ATP-dependent daunomycin transport by Mdr1 (38) or dinitrophenyl-*S*-glutathione transport by the non-bile-acid organic anion system (39), and the ectoATPase (data not shown) were unaffected by TC in vitro suggesting that these systems do not participate in ATP-dependent PC translocation.

Taken together, our results suggest that the observed ATP-dependent PC flippase activity in CMV is functionally similar to that mediated by yeast secretory vesicles transfected with murine mdr2 (21).

The involvement of this mechanism in bile formation in vivo requires further study, but may be speculated upon. Several models for biliary phospholipid secretion have been proposed: *1)* bilayer plasma membrane vesiculation ("fusion-budding", 6); *2)* release of preformed unilamellar lipid vesicles (7); and *3)* bile acid-induced release of phospholipids from the outer leaflet of the canalicular membrane. Recent work by Crawford et al. (15) demonstrated that bile salts induce formation of canalicular unilamellar phospholipid vesicles which were released from the outer leaflet of the canalicular membrane. These findings support the hypothesis that protein-mediated phospholipid translocation from the inner to the outer leaflet of the canalicular membrane, e.g., by Mdr2 (18, 21), exposes PC to the detergent

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action of bile salts. The present study shows that the rat bile canalicular membrane has the capability to translocate PC from the inner to the outer leaflet in an ATP-dependent manner.

Several lines of evidence showed that PC translocation by Mdr2 plays an important physiological role. *1)* In mice with a disrupted mdr2-gene, no PC is found in bile (18). *2)* When these mice are infused with TC, no bile acid-induced PC secretion is observed (13). The question arises whether ATP-dependent PC translocation in CMV is also physiologically significant. In the present study, maximal activity of PC translocation was 250 pmol C_6-NBD-PC/mg protein per 30 min. Only inside-out oriented vesicles translocated PC, so that the actual activity was 2.5 nmol C_6-NBD-PC/mg protein per 30 min. Because the average protein concentration of the CMV preparation was 1 mg/ml in a total volume of 1 ml, the total PC translocating activity recovered from 70 g liver, which was typically used, was 2.5 nmol C_6-NBD-PC/ml per 30 min. Assuming a recovery of canalicular proteins of approximately 1% (as measured for recovery of leucine aminopeptidase activity in CMV as compared to activity in homogenate), an in vivo activity of 250 nmol C_6-NBD-PC/70 g liver per 30 min or 1.7 nmol C_6-NBD-PC/14 g liver per min can be calculated. In isolated perfused rat liver (approximately 14 g fresh weight), a phospholipid output of 1.4 nmol/min was observed (40). Thus, the detected flippase activity, probably due to Mdr2, is sufficient for bile production and is important for translocating PC across the canalicular bilayer for formation of unilamellar vesicles that arise in the canalicular lumen under the detergent action of bile acids. Because CMV only tolerate a TC concentration of 0.25 mM before being disrupted (1), PC translocation increased only modestly by 2-fold in the presence of TC.

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REFERENCES

1. Nishida, T., Z. Gatmaitan, M. Che, and I. M. Arias. 1991. Rat liver canalicular membrane vesicles contain an ATP-dependent bile acid transport system. *Proc. Natl. Acad. Sci. USA.* **88:** 6590–6594.
2. Muller, M., R. Ishikawa, U. Berger, C. Klunemann, L. Lucka, A. Schreyer, and C. Kannich. 1991. ATP-dependent transport of taurocholate across the hepatocyte canalicular membrane mediated by a 110-kDa glycoprotein binding ATP and bile salt. *J. Biol. Chem.* **266:** 18920–18926.
3. Adachi, Y., H. Kobayashi, Y. Kurumi, M. Shouji, M. Kitano, and T. Yamamoto. 1991. ATP-dependent taurocholate transport by rat liver canalicular membrane vesicles. *Hepatology.* **14:** 655–659.
4. Inoue, M., T. P. Akerboom, H. Sies, R. K. H. Kinne, T. Tran, and I. M. Arias. 1984. Transport of glutathione-*S*-conjugate by rat liver canalicular membrane vesicles. *J. Biol. Chem.* **259:** 4998–5002.
5. Akerboom, T. P., M. Inoue, H. Sies, R. K. H. Kinne, T. Tran, and I. M. Arias. 1984. Biliary transport of glutathione disulfide studied with isolated rat liver canalicular membrane vesicles. *Eur. J. Biochem.* **141:** 211–215.
6. Coleman, R. 1987. Biochemistry of bile secretion. *Biochem. J.* **244:** 249–261.
7. Marzolo, M. P., A. Rigotti, and F. Nervi. 1990. Secretion of biliary lipids from the hepatocyte. *Hepatology.* **12:** 134S–142S.
8. Coleman, R., and K. Rahman. 1992. Lipid flow in bile formation. *Biochim. Biophys. Acta.* **1125:** 113–133.
9. Patton, G. M., J. M. Fasulo, and S. J. Robins. 1994. Hepatic phosphatidylcholines: evidence for synthesis in the rat by extensive reutilization of endogenous acylglycerides. *J. Lipid Res.* **35:** 1211–1221.
10. Cohen, D. E., M. R. Leonard, and M. C. Carey. 1994. In vitro evidence that phospholipid secretion into bile may be coordinated intracellularly by the combined actions of bile salts and the specific phosphatidylcholine transfer protein of liver. *Biochemistry.* **33:** 9975–9980.
11. McIntyre, J. C., and R. G. Sleight. 1994. Mechanisms of intracellular membrane lipid transport. *Curr. Top. Membr.* **40:** 453–481.
12. Higgins, C. F. 1994. Flip-flop: the transmembrane translocation of lipids. *Cell.* **79:** 393–395.
13. Oude Elferink, R. P. J., R. Ottenhoff, M. van Wijland, J. J. M. Smit, A. H. Schinkel, and A. K. Groen. 1995. Regulation of biliary lipid secretion by mdr2 p-glycoprotein in the mouse. *J. Clin. Invest.* **95:** 31–38.
14. Berr, F., P. J. Meier, and B. Stieger. 1993. Evidence for the presence of a phosphatidylcholine translocator in isolated rat liver canalicular plasma membrane vesicles. *J. Biol. Chem.* **268:** 3976–3979.
15. Crawford, J. M., G-M. Moeckel, A. R. Crawford, S. J. Hagen, V. C. Hatch, S. Barnes, J. J. Godleski, and M. C. Carey. 1995. Imaging biliary lipid secretion in the rat: ultrastructural evidence for vesiculation of the hepatocyte canalicular membrane. *J. Lipid Res.* **36:** 2147–2163.
16. Buschman, E., R. J. Arceci, J. M. Croop, M. Che, I. M. Arias, D. E. Housman, and P. Gros. 1992. Mdr2 encodes p-glycoprotein expressed in the bile canalicular membrane as determined by isoform-specific antibodies. *J. Biol. Chem.* **267:** 18093–18099.
17. Gottesman, M. M., and I. Pastan. 1993. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.* **62:** 385–427.
18. Smit, J. J. M., A. H. Schinkel, R. P. J. Oude Elferink, A. K. Groen, E. Wagenaar, L. van Deemter, C. A. A. M. Mol, R. Ottenhoff, N. M. T. van der Lugt, M. A. van Roon, M. A. van der Valk, G. J. A. Offerhaus, A. J. M. Berns, and P. Borst. 1993. Homozygous disruption of the murine *mdr2* p-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell.* **75:** 451–462.
19. Smith, A. J., J. L. P. M. Timmermans-Hereijgers, B. Roelofsen, K. W. A. Wirtz, W. J. van Blitterswijk, J. J. M. Smit, A. H. Schinkel, and P. Borst. 1994. The human MDR3 P-glycoprotein promotes translocation of phosphatidylcholine through the plasma membrane of fibroblasts from transgenic mice. *FEBS Lett.* **354:** 263–266.

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20. Gros, P., M. Raymond, J. Bell, and D. Housman. 1988. Cloning and characterization of a second member of the mouse *mdr* gene family. *Mol. Cell Biol.* **8:** 2770–2778.
21. Ruetz, S., and P. Gros. 1994. Phosphatidylcholine translocase: a physiological role for the mdr2 gene. *Cell.* **77:** 1071–1081.
22. Inoue, M., R. K. H. Kinne, T. Tran, L. Biempica, and I. M. Arias. 1983. Rat liver canalicular membrane vesicles. *J. Biol. Chem.* **258:** 5183–5188.
23. Inoue, M., R. K. H. Kinne, T. Tran, and I. M. Arias. 1982. Taurocholate transport by rat liver sinusoidal membrane vesicles: evidence of sodium cotransport. *Hepatology.* **2:** 572–579.
24. Saucan, L., and G. E. Palade. 1994. Membrane and secretory proteins are transported from the Golgi complex to the sinusoidal plasmalemma of hepatocytes by distinct vesicular carriers. *J. Cell Biol.* **125:** 733–741.
25. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
26. Schiefer, S., and H-O. Beutler. 1986. Phosphatidylcholine (Lecithin). *In* Methods in Enzymatic Analysis. H. U. Bergmeyer, editor. Verlag Chemie, Weinheim. 87–104.
27. Taussky, H. H., and E. Schorr. 1953. A microcolorimetric method for the determination of inorganic phosphorus. *J. Biol. Chem.* **202:** 675–685.
28. Kamimoto, Y., Z. Gatmaitan, J. Hsu, and I. M. Arias. 1989. The function of GP170, the multidrug resistance gene product, in rat liver canalicular membrane vesicles. *J. Biol. Chem.* **264:** 11693–11698.
29. Evers, J., H. Murer, and R. K. H. Kinne. 1976. Phenylalanine uptake in isolated renal brush border vesicles. *Biochim. Biophys. Acta.* **426:** 598–615.
30. McIntyre, J. C., and R. G. Sleight. 1991. Fluorescence assay for phospholipid membrane asymmetry. *Biochemistry.* **30:** 11819–11827.
31. Meyuhas, D., S. Yedgar, M. Rotenberg, N. Reisfeld, and N. Lichtenberg. 1992. The use of C_6-NBD-PC for assaying phospholipase A_2-activity: scope and limitations. *Biochim. Biophys. Acta.* **1124:** 223–232.
32. Kremer, J. M. H., v. d. Esker, C. Pathmamanoharan, and P. H. Wiersema. 1977. Vesicles of variable diameter prepared by a modified injection method. *Biochemistry.* **16:** 3932–3935.
33. Nichols, J. W., and R. E. Pagano. 1981. Kinetics of soluble lipid monomer diffusion between vesicles. *Biochemistry.* **20:** 2783–2789.
34. Pagano, R. E., and R. G. Sleight. 1985. Defining lipid transport pathways in animal cells. *Science.* **229:** 1051–1057.
35. Connor, J., and A. J. Schroit. 1988. Transbilayer movement of phosphatidylserine in erythrocytes: inhibition of transport and preferential labeling of a 31000-Dalton protein by sulfhydryl reactive reagents. *Biochemistry.* **27:** 848–851.
36. Sleight, R. G., and R. E. Pagano. 1985. Transbilayer movement of a fluorescent phosphatidylethanolamine analogue across the plasma membranes of cultured mammalian cells. *J. Biol. Chem.* **260:** 1146–1154.
37. Ruetz, S., and P. Gros. 1995. Enhancement of Mdr2-mediated phosphatidylcholine translocation by the bile salt taurocholate. *J. Biol. Chem.* **270:** 25388–25395.
38. Mazzanti, R., O. Fantappie, Y. Kamimoto, Z. Gatmaitan, P. Gentilini, and I. M. Arias. 1994. Bile acid inhibition of P-glycoprotein-mediated transport in multidrug-resistant cells and rat liver canalicular membrane vesicles. *Hepatology.* **20:** 170–176.
39. Nishida, T., C. Hardenbrook, Z. Gatmaitan, and I. M. Arias. 1992. ATP-dependent organic anion transport system in normal and TR$^-$ rat liver canalicular membranes. *Am. J. Physiol.* **262:** G629–G635.
40. Monte, M. J., R. A. Parslow, and R. Coleman. 1990. Inhibitory action of cyclobutyrol on the secretion of biliary cholesterol and phospholipid. *Biochem. J.* **266:** 165–171.

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