Baculovirus-mediated expression of recombinant rat phosphatidylcholine transfer protein

Li Feng and David E. Cohen[1]

Marion Bessin Liver Research Center, Department of Medicine, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461

Abstract **Phosphatidylcholine transfer protein catalyzes intermembrane transfer of phosphatidylcholines exclusive of all other phospholipid classes. Although postulated to participate in phosphatidylcholine biosynthesis and biliary trafficking in liver, the molecular basis underlying the substrate specificity of phosphatidylcholine transfer protein remains to be elucidated. Having demonstrated the inability of *Escherichia coli* to express recombinant phosphatidylcholine transfer protein, we infected *Spodoptera frugiperda* (Sf9) cells with recombinant baculovirus. When assayed in vitro, cytosol of recombinant but not control infected cells demonstrated high levels of intermembrane phosphatidylcholine transfer activity and no transfer activity for phosphatidylethanolamine. A two-step purification protocol in which 10 mg of cytosolic protein was subjected to anion exchange chromatography followed by hydroxylapatite chromatography yielded 0.1 mg active protein which was >92% pure. The identity of purified protein was confirmed by matrix-assisted laser desorption–ionization mass spectrometry and by amino acid sequencing. Based on the recovery of 30% of PC transfer activity after purification, we estimate that recombinant rat phosphatidylcholine transfer protein accounted for ~3–6% of cytosolic protein mass of infected cells. These results demonstrate the utility of baculovirus for expressing recombinant phosphatidylcholine transfer protein and should facilitate studies designed to elucidate the structural biology and physiological functions of this uniquely specific phospholipid transfer protein.—**Feng, L., and D. E. Cohen. **Baculovirus-mediated expression of recombinant rat phosphatidylcholine transfer protein. *J. Lipid Res.* 1998.** 39: **1862–1869.**

Supplementary key words phospholipid • membrane • bile • protein purification

In 1968, Wirtz and Zilversmit (1) reported that hepatic cytosol dramatically accelerated intermembrane transfer of phospholipids. This observation led to the purification of phosphatidylcholine transfer protein (PC-TP), which exhibits exquisite specificity for phosphatidylcholines (PCs) (reviewed in ref. 2). Early studies demonstrated that PC-TP binds PC in a tight 1:1 stoichiometric complex, and even minor chemical modifications of the phosphorylcholine head group of PC greatly reduce or abolish transfer by PC-TP (3). Subsequent biochemical and physical–chemical studies have suggested that a lipid-binding pocket is formed by hydrophobic segments within the primary structure of bovine PC-TP (4), which binds *sn*-1 palmitoyl- and *sn*-2 unsaturated-acyl chains preferentially (2). Recent cDNA cloning of PC-TP from rat and bovine liver (5–8) has demonstrated these homologues to be highly similar (80% and 76% nucleotide and amino acid identities, respectively), but to be unrelated to other cytosolic lipid transfer proteins (9). These collective observations notwithstanding, the molecular basis underlying the unique specificity of PC-TP for PC as well as its function(s) in vivo have remained largely unresolved.

Detailed studies of structure–function relationships of PC-TP necessitate a facile expression system for production and purification of recombinant protein. Whereas *E. coli* has been used to express recombinant sterol carrier protein 2 (10–12) and phosphatidylinositol transfer protein (13, 14), which are cytosolic proteins capable of promoting intermembrane transfer of PC in addition to other phospholipids, we have found this prokaryotic expression system to be inadequate for expression of recombinant PC-TP.[2] Here we describe expression and purification of

Abbreviations: PC, phosphatidylcholine; PC-TP, phosphatidylcholine transfer protein; PE, phosphatidylethanolamine; MLV, multilamellar vesicles; SUV, small unilamellar vesicles; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

[1]To whom correspondence and reprint requests should be addressed.

[2]In preliminary experiments, expression of recombinant PC-TP was first attempted using *E. coli*. At the time these experiments were undertaken, our laboratory had not yet completed cDNA cloning of rat PC-TP. Therefore, the coding region of a cDNA encoding bovine PC-TP (6) was cloned into an appropriate expression vector downstream from a T7 RNA polymerase promoter (15). Upon induction of a transformed host bacterial strain, no evidence for expression of recombinant PC-TP could be demonstrated either by detection of PC-TP activity (16) or by appearance of an appropriately sized (~25 kDa) band by SDS-PAGE. Moreover, there was no evidence for the formation of inclusion bodies in these experiments.

recombinant rat PC-TP using baculovirus-infected *Spodoptera frugiperda* (Sf9) cells.

MATERIALS AND METHODS

Materials

Restriction enzymes and other molecular reagents were purchased from Boehringer Mannheim (Indianapolis, IN). Grade I egg yolk PC, egg yolk phosphatidylethanolamine (PE), and bovine heart cardiolipin were from Avanti Polar Lipids (Alabaster, AL). Phospholipids were >99% pure by high performance liquid chromatography (17). l-α-1-Palmitoyl-2-[1-^{14}C]linoleoyl-PC (58 mCi/mmol), [1,2,6,7-^{3}H(N)]cholesteryl oleate (71 Ci/mmol), and l-α-1-palmitoyl-2-[1-^{14}C]arachidonyl-PE (56 mCi/mmol) were from NEN Life Science Products (Boston, MA). Pre-swollen diethylaminoethyl cellulose (DE-52) and carboxymethyl cellulose (CM-52) were obtained from Whatman (Maidstone, England), and Sephadex G50 was from Pharmacia Biotech (Piscataway, NJ). Hydroxylapatite chromatography medium (BIO-GEL® HTP) and protein assay reagents were from Bio-Rad Laboratories (Hercules, CA). All other chemicals were either ACS or reagent grade purity (Sigma, St. Louis, MO). Native rat PC-TP was purified according to the method of Teerlink, Poorthuis, and Wirtz (18).

Cloning of a cDNA encoding rat PC-TP

A cDNA containing a complete open reading frame (ORF) encoding rat PC-TP was constructed using genomic and complementary DNA molecules previously cloned in our laboratory (8). A partial, 1683 bp cDNA was isolated by screening a rat liver cDNA library (GenBank accession no. AF040261) and spanned from the polyA+ tail at its 3′-extent to include a 585 nt open reading frame at its 5′-extent. The deduced amino acid sequence, which lacked an amino terminus, was 78% identical to the homologous region of bovine PC-TP (6). The remaining nucleotide sequence encoding the amino terminus was derived from a cloned genomic DNA fragment that contained the first exon of the rat PC-TP structural gene (GenBank accession no. AF040262). A single 1979 bp clone containing a full-length (642 bp) coding sequence was constructed by utilizing a common *AocI* restriction site. The presence of an additional *AocI* restriction site in the cDNA necessitated its partial digestion with *AocI* followed by gel purification of the appropriate product (19) prior to ligation with the genomic *XbaI-AocI* fragment. Proper construction of the composite DNA clone in pBluescript SK (Stratagene Cloning Systems, La Jolla, CA) was confirmed by nucleotide sequencing.

Preparation of recombinant baculovirus

Recombinant PC-TP baculovirus was prepared using the Bac-to-Bac™ baculovirus expression system (GibcoBRL, Gaithersburg, MD). Briefly, the full-length rat PC-TP cDNA was cloned into the donor plasmid pFastBac1™, and transformed into component *E. coli* DH10Bac, which contained baculovirus genomic DNA (bacmid) as well as a helper plasmid. In ~8% of transformed cells, the helper plasmid facilitated a recombination event in which the PC-TP cDNA was translocated into the bacmid DNA. Because this recombination event also interrupted a *LacZ* gene which had been commercially introduced into the bacmid, clones containing recombinant bacmids were selected by their white color when grown on LB plates supplemented with X-Gal. Bacmid DNA was amplified and purified from selected recombinant clones. Introduction of the PC-TP cDNA into the bacmid was verified by PCR using oligonucleotide primers positioned both within the cDNA and in the adjacent bacmid DNA followed by nucleotide sequencing of the PCR products. Bacmid DNA was then lipofected into Sf9 cells and, after 48 h of incubation at 27°C in SF900II serum-free medium (SFM) (GibcoBRL), recombinant baculovirus was harvested from the medium. Viral stocks were amplified by re-infection into Sf9 cells at a multiplicity of infection (MOI) of 0.1 for 72 h at 27°C to achieve final viral titers of 7×10^7 pfu/mL as assessed by viral plaque assay. Recombinant baculovirus containing a cDNA encoding a 65 kDa fusion protein of murine IgG2a and *Helicobacter pylori* urease B for use as a control was a generous gift from Drs. Andreas Christ and Richard Blumberg (Brigham and Women's Hospital, Boston, MA).

Expression of recombinant rat PC-TP

Prior to infection with recombinant baculovirus, Sf9 cells were grown in suspension to log-phase (viability >98%) in 400 mL serum-free medium (density ~1–2 × 10^6 cells/mL). Cells were then concentrated ~10-fold and infected at an MOI of 5 by incubation in the dark at 27°C for 1 h prior to dilution to their original densities in SFM. Infected Sf9 cells were grown in suspension at 27°C in a rotary shaker (140 rpm) for 48–72 h until the proportion of non-viable cells reached 20–40% as assessed by trypan blue staining. Cells were then harvested by centrifugation (120 *g* at 4°C) and then resuspended in 20 mL buffer containing Tris-HCl (20 mm), EGTA (5 mm), EDTA (1 mm), β-mercaptoethanol (10 mm), NaN_3 (3 mm), and the protease inhibitor phenylmethylsulfonyl fluoride (PMSF, 1 mm) at pH 7.2. Cells were lysed by four brief (15 sec) periods of sonication on ice using a Branson Model W140 Sonifier Cell Disrupter (Heat Systems Inc., Plainview, NY). Supernatants containing cytosolic proteins were recovered after removal of membrane fragments by centrifugation at 100,000 *g* for 1 h at 4°C.

In vitro phospholipid transfer activity assay

Transfer activity of PC-TP was determined by measurement of intermembrane transfer of radiolabeled phospholipids between model membranes essentially as described by DiCorleto and Zilversmit (16). Briefly, donor small unilamellar vesicles (SUV) were prepared by bath sonication (Special Ultrasonic Cleaner, Laboratory Supply Corp., Hicksville, NY) of 1 mg of pure PC or of PC plus PE (1:1 molar ratio), [^{14}C]PC (0.1 × 10^6 dpm), [^{3}H]cholesteryl oleate (1 × 10^6 dpm) and the antioxidant butylated hydroxytoluene (2 μg) dispersed in 1.0 ml of buffer (5 mm EDTA and 50 mm Tris at pH 7.2). In some experiments, [^{14}C]PE (0.1 × 10^6 dpm) was substituted for [^{14}C]PC. Multilamellar vesicles (MLV) consisting of PC and cardiolipin (95:5 mol%) were prepared by rotary shaking (250 rev/min) of PC (104.6 mg), cardiolipin (10.4 mg), and butylated hydroxytoluene (10 μg) dispersed in the same buffer. In order to isolate a population of multilamellar vesicles (MLV) that could be separated readily from SUV by centrifugation, rotary shaken suspensions were centrifuged at 55,000 *g* for 30 min, the supernatant was discarded, and the pellet was gently resuspended in 6 ml of buffer. Solutions containing recombinant PC-TP (10–100 μl) were incubated together in a 37°C shaking water bath with donor SUV (50 μl), acceptor MLV (100 μl) adjusted to a final volume of 500 μl with buffer. Control samples contained no added protein. After 30 min of incubation, intervesicular phospholipid transfer was arrested by rapid cooling on ice. MLV were pelleted by centrifugation (55,000 *g*) at 4°C for 30 min, and the [^{14}C]PC or [^{14}C]PE and [^{3}H]cholesteryl oleate contents in a 350 μl aliquot of SUV, which remained in suspension, were determined by liquid scintillation counting (Liquid Scintillation System LS 6500TD, Beckman, Fullerton, CA). Because cholesteryl oleate is not transferred by PC-TP (20), ^{3}H concentrations were used to correct for small losses of SUV during centrifugation. In this manner, [^{14}C]PC or [^{14}C]PE transferred from SUV to MLV was reflected

by decreases in the $^{14}C/^{3}H$ ratio of SUV. Transfer activity (nmol/h) of PC or PE was calculated as described by Zilversmit and Hughes (21).

Purification of recombinant PC-TP

Purification of recombinant rat PC-TP was achieved by modification and abbreviation of a procedure used by Teerlink, Poorthuis, and Wirtz (18) to purify the native protein. All purification procedures were conducted at 4°C. Cytosolic preparations from Sf9 cells were dialyzed against 5 mm Tris-HCl buffer (pH 7.2) containing 10 mm β-mercaptoethanol, 1 mm PMSF, and 3 mm NaN_3 and then applied to a 12 × 1.5 cm DE-52 anion exchange column equilibrated with the same buffer. PC transfer activity, which eluted with the unbound protein fraction, was dialyzed against 10 mm potassium phosphate buffer (pH 6.8, 10 mm β-mercaptoethanol, 1 mm PMSF and 3 mm NaN_3) and applied to a 13 × 1 cm hydroxylapatite column equilibrated with this buffer. Bound transfer activity was eluted using 10 column volumes of a 10–300 mm potassium phosphate gradient buffer (pH 6.8) at a flow rate of 0.15 mL/min and fraction volume of 5 mL.

Analytical techniques

Protein concentrations were determined by using a Bio-Rad kit based on the method of Bradford (22) using bovine serum albumin as a standard. Protein purities were assessed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (23) followed by Coomassie brilliant blue staining. Relative intensities of protein bands were quantified by scanning laser densitometry and image analysis using ImageQuant™ software (Molecular Dynamics, Sunnyvale, CA). For estimation of pI value, 2-dimensional electrophoresis was performed on a Multiphor II flatbed electrophoresis system (Pharmacia Biotech, Piscataway, NJ). In preparation for mass spectrometry, reverse-phase high performance liquid chromatography (HPLC) was used to remove salts and minor protein impurities. Briefly, 4 μg of purified recombinant rat PC-TP was applied to a reverse-phase C4 column (30 × 2.1 mm, 5 μm particle size, Brownlee Labs, Inc., Santa Clara, CA) and eluted with water–acetonitrile gradient (containing 0.01% trifluoroacetic acid) at 0.2 mL/min (1%/min) using a Hewlett-Packard model 1090 HPLC apparatus. HPLC-purified protein was also subjected to tryptic digestion (24) and peptide fragments were separated by the same HPLC procedure. Molecular weights of purified recombinant PC-TP and tryptic digest fragments were determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (24) using a Voyager RP Biospectrometry Work Station (Perspective Biosystems, Framingham, MA).

Nucleotide sequences of both plasmid DNA and polymerase chain reaction (PCR) products were determined by fluorescence cycle sequencing with dye-labeled terminators using an Applied Biosystems model 377 automated sequencer (Perkin-Elmer, Foster City, CA). Amino acid sequencing was performed using a Precise™ 494 Protein Sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA).

RESULTS

Cloning of a cDNA for expression of rat PC-TP

Figure 1 presents the nucleotide and deduced amino acid sequences of the DNA clone used for expression of recombinant rat PC-TP. Within the nucleotide sequence, we have underlined the *AocI* restriction site used for construction of the full-length ORF. The 642 bp ORF encodes a 214aa protein with predicted molecular mass of 24,733 Da. The underlined amino acid sequence indicates the tryptic peptide fragment of the expressed recombinant protein that was subjected to detailed analysis (see below), and the experimentally determined sequence is italicized.

Expression and purification of recombinant rat PC-TP

Expression and purification of recombinant rat PC-TP was facilitated by in vitro determinations of intermembrane phospholipid transfer activity (16). **Figure 2A** shows the PC transfer activity of cytosolic preparations from Sf9 cells prior to and after infection by recombinant PC-TP or recombinant control baculovirus. Only baculovirus recombinant for rat PC-TP yielded high levels PC transfer activity. Activity increased linearly as functions of total cytosolic protein mass and then tended toward leveling-off with protein amounts in excess of 15 μg (corresponding to 30 μl of cytosolic protein solution). Figure 2B compares transfer rates of [^{14}C]PE and [^{14}C]PC from SUV composed of PC to MLV in the presence of cytosolic proteins from recombinant PC-TP baculovirus-infected Sf9 cells. Substitution of [^{14}C]PE for [^{14}C]PC completely abolished transfer of the radiolabeled phospholipid. To ensure that the absence of PE transfer activity reflected specificity of the recombinant protein and not sequestration of [^{14}C]PE within the inner hemileaflet where it would be inaccessible to the transfer protein (25), these results were verified using SUV composed of an equimolar mixture of PC and PE (26) (data not shown).

For purification of recombinant protein, cytosolic preparations (10 mg total protein) were first subjected to anion exchange chromatography at pH 7.2. PC transfer activity, which eluted with unbound protein fractions, was applied to a hydroxylapatite affinity column. As shown in **Fig. 3A**, PC transfer activity bound to the column at low salt concentration (10 mm potassium phosphate), and was selectively eluted using a linear salt gradient. Figure 3B displays SDS-PAGE analysis after each step in recombinant PC-TP expression and purification. When compared either with cytosolic proteins from Sf9 cells prior to or after infection with recombinant control baculovirus, the electrophoretic pattern of cytosol from recombinant PC-TP baculovirus-infected Sf9 cells did not clearly demonstrate a prominent band close to the anticipated molecular weight of recombinant PC-TP. However, a major band was visualized near 24 kDa after anion exchange chromatography. Based on preliminary experiments in which the molecular weight associated with PC transfer activity was demonstrated by Sephadex G50 gel filtration chromatography to elute near 24 kDa (not shown), the protein band that migrated near the 24 kDa standard in lane 5 of Fig. 3B was tentatively identified as recombinant rat PC-TP. Analysis by laser scanning densitometry revealed the purity of the putative recombinant protein to be ~30% after this initial step. Lanes 6 to 10 in Fig. 3B show SDS-PAGE analysis of concentrated fractions from the hydroxylapatite column which comprised the peak of PC transfer activity in Fig. 3A (i.e., fractions 17–21). These results show that the maximum PC transfer activity was closely matched with the intensity of a protein band with molecular weight of 24 kDa.

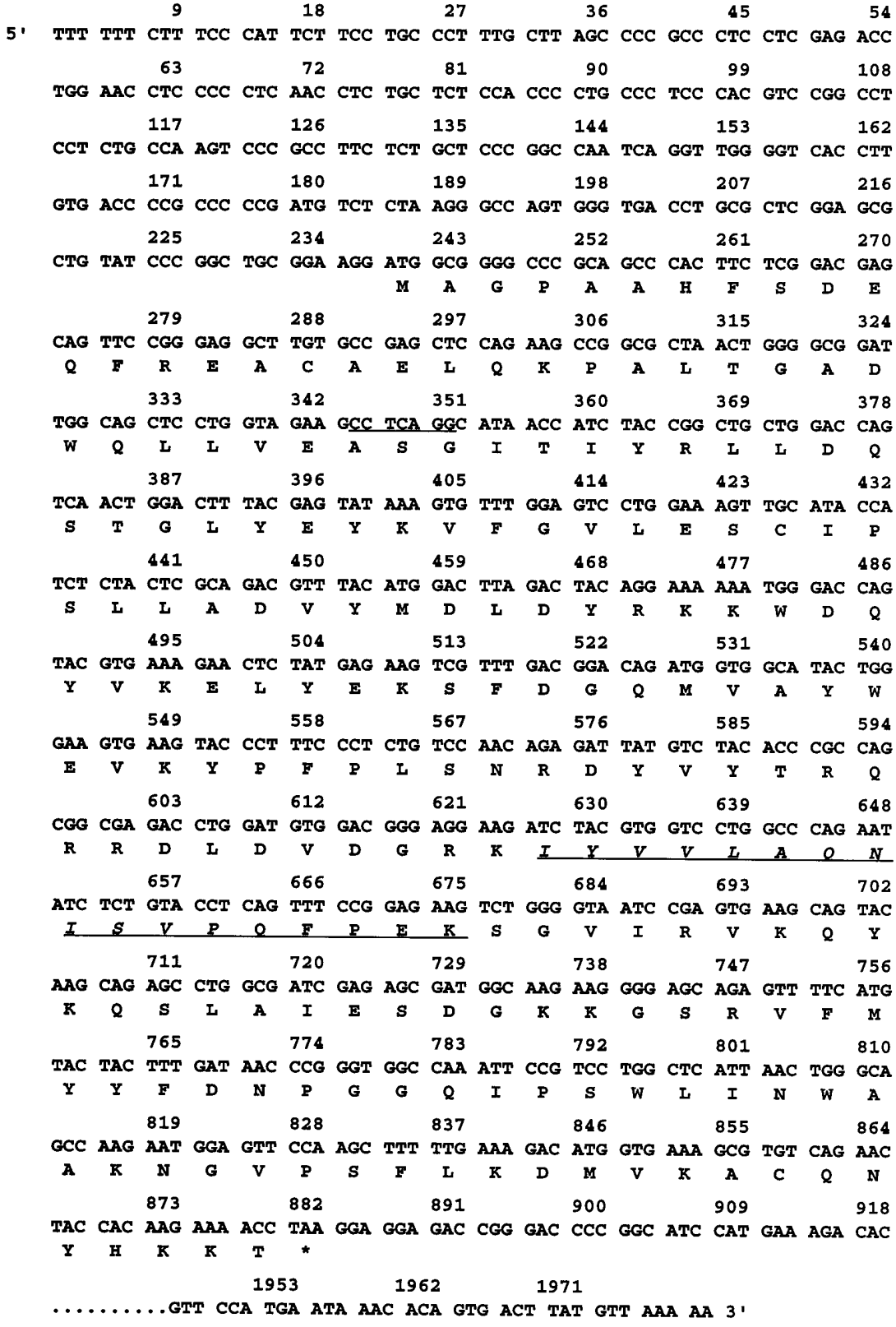

Fig. 1. Nucleotide sequence of rat PC-TP cDNA. The deduced 214aa sequence (one-letter amino acid symbols) ends with a stop codon indicated by an asterisk. Dots signify abbreviation of the 3′ untranslated region. The nucleotide sequence of the common *AocI* restriction site used for cloning is underlined. Also underlined is the amino acid sequence of the tryptic digest fragment that was identified by mass spectrometry and purified by HPLC. The experimentally determined amino acid sequence is italicized.

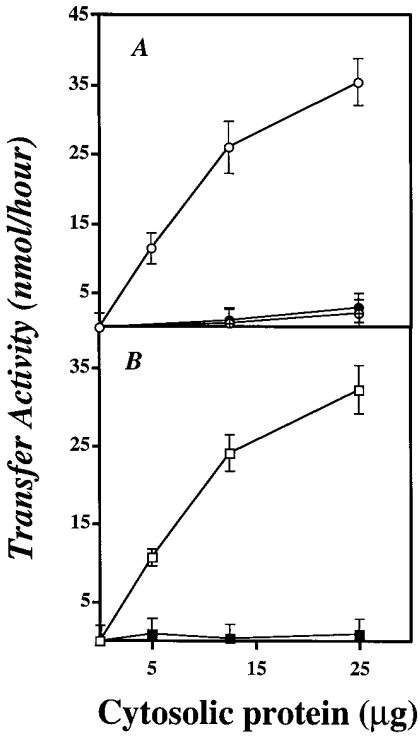

Fig. 2. Phospholipid transfer activity in Sf9 cell cytosol. (A) Intermembrane PC transfer activity was determined for cytosolic proteins prepared from Sf9 cells prior to (⊕) and after infection with recombinant PC-TP baculovirus (○) or recombinant control baculovirus (●). (B) Intermembrane transfer activity of PC (□) and PE (■) was measured for cytosolic proteins prepared from Sf9 cells after infection with recombinant rat PC-TP baculovirus. Data points represent mean ± SD of three experiments.

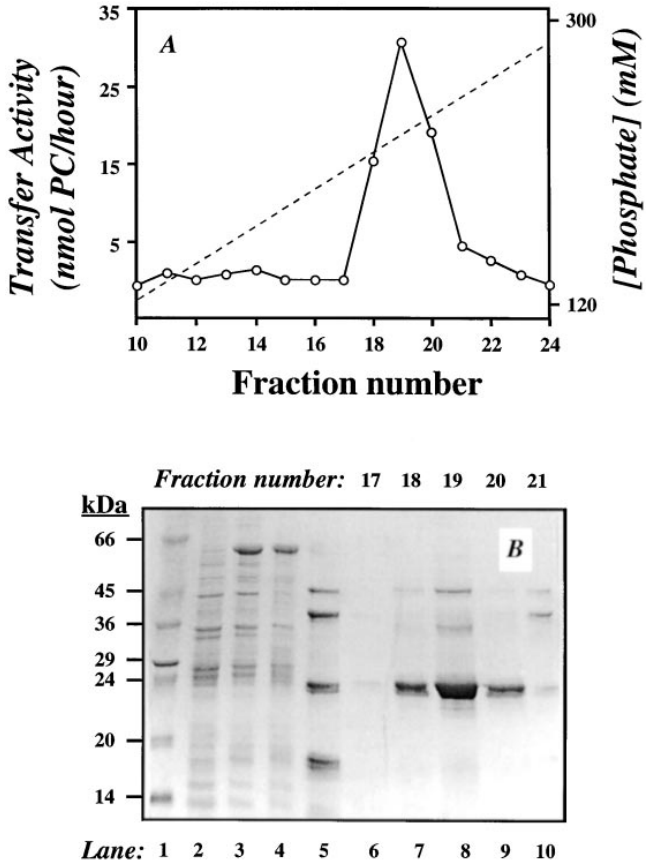

Fig. 3. Purification of recombinant rat PC-TP. (A) Cytosol harvested from Sf9 cells after infection with recombinant PC-TP baculovirus initially purified by DE-52 anion exchange chromatography was applied to a hydroxylapatite column. Using a linear potassium phosphate gradient, PC transfer activity eluted near 200 mm phosphate. (B) SDS-PAGE analysis of PC-TP purification. Lane 1: protein molecular weight standards; lane 2: Sf9 cell cytosol; lane 3: Sf9 cell cytosol after infection with recombinant control baculovirus; lane 4: Sf9 cell cytosol after infection with recombinant PC-TP baculovirus; lane 5: cytosolic protein preparation from lane 4 after partial purification by anion exchange chromatography; lanes 6–10: fractions 17 to 21 from the hydroxylapatite column (panel A). Lanes 2–5 were loaded with ~7 μg of protein whereas lanes 6–10 were each loaded with 4 μl of protein solutions after 50-fold concentration by centrifugation using a 10 kDa cut-off filter (Centricon-10, Amicon, Inc., Beverly, MA). Under these conditions, 4 μg of protein was applied to lane 8 which contained the peak of PC transfer activity in panel B.

By 2-dimensional electrophoresis, the pI of purified recombinant PC-TP (lane 8 in Fig. 3B) was found to lie between 8 and 9 (not shown), which is consistent with the pI of native rat PC-TP (8.4) (27). Laser scanning densitometry analysis of lane 8 demonstrated the purity of the putative recombinant PC-TP to be >92%. This purification procedure yielded 100 μg of purified protein (fraction 19, Fig. 3B) from 10 mg cytosolic protein. Compared with the cytosolic protein preparation, ~30% of PC transfer activity was recovered after hydroxylapatite chromatography (fractions 18–20, Fig. 3B). The specific activity of the purified recombinant protein was 30 nmol/μg per h and did not differ appreciably from the specific activity of the native rat PC-TP.

Molecular confirmation of recombinant PC-TP expression

To establish that purified protein in Fig. 3C was full-length recombinant rat PC-TP, we analyzed the peak hydroxylapatite fraction (fraction 19, Fig. 3A) by mass spectrometry and amino acid sequencing. Salt and minor impurities were removed by reverse-phase HPLC, and **Fig. 4A** demonstrates a representative chromatogram after elution using a water/acetonitrile gradient. A major peak eluted at 51 vol% acetonitrile with a much smaller peak at 53 vol% acetonitrile. Although PC transfer activity could not be detected in either of these two fractions after removal of organic solvent and reconstitution in buffer (5 mm EDTA and 50 mm Tris at pH 7.2), Fig. 4A also shows SDS-PAGE analysis of the two samples that revealed in each a single protein that migrated near 24 kDa. Mass spectrometry demonstrated the molecular mass of the major peak to be 24,760 Da (Fig. 4B) and the minor peak to be 24,793 Da (not shown). Because our inability to obtain N-terminal amino acid sequence suggested that the protein was blocked, tryptic digestion was performed. The sequence of the first twelve amino acids of an HPLC-purified fragment was determined and was found to

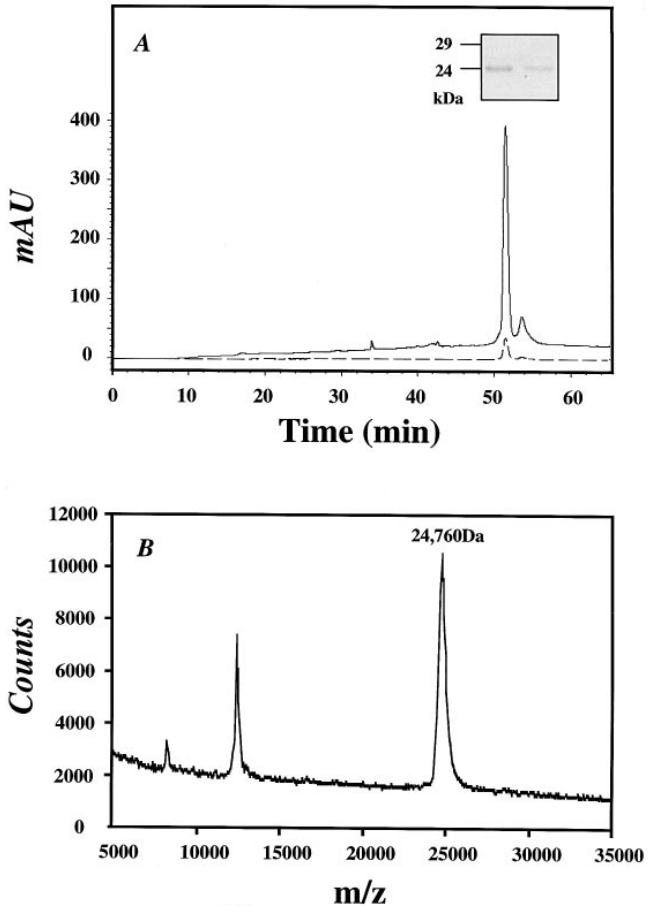

Fig. 4. Molecular mass confirmation for recombinant rat PC-TP. (A) The fraction containing peak PC transfer activity after hydroxylapatite chromatography (fraction 19, Fig. 3A) was further purified by application to a C4 reverse phase HPLC column followed by elution using a linear acetonitrile/H_2O gradient (A_{280}, dashed line; A_{214}, solid line). Peaks eluting at 51 and 53 min were concentrated by lyophilization and analyzed by SDS-PAGE (inset figure). (B) MALDI-TOF-MS analysis of peak eluting at 51 min (panel A) indicating a molecular weight (24,760 Da) consistent with recombinant rat PC-TP.

match exactly the deduced sequence of recombinant rat PC-TP from amino acids 130–141 (Fig. 1). Based on the deduced amino acid sequence, this tryptic fragment was predicted to span from amino acid 130 to 146 (underlined in Fig. 1) and to display a molecular mass of 1,944 Da. This was confirmed by mass spectrometry, which demonstrated that the tryptic digest mixture contained a 1,945 Da fragment (data not shown).

DISCUSSION

Absolute specificity for PC to the exclusion of all other phospholipid classes distinguishes PC-TP as the most selective of cytosolic lipid transfer proteins (2), yet the molecular basis responsible for this selectivity as well as its physiological function has remained elusive. In order to facilitate studies that will elucidate PC-TP structure and function, we have constructed a DNA clone encoding the rat homologue and have expressed and characterized the recombinant protein.

Under physiological conditions, PC is tightly bound to PC-TP non-covalently in a 1:1 stoichiometry (2). We therefore hypothesized that availability of PC might be required to facilitate PC-TP synthesis, and that the absence of PC in *E. coli* membranes (28) may have prevented PC-TP expression in our preliminary experiments. Baculovirus has been demonstrated to be useful for high level expression of a variety of heterologous genes (29). Moreover, membranes of *Spodoptera frugiperda* (Sf9) cells, insect ovarian cells commonly used for recombinant protein production after baculoviral infection, contain PC as a major phospholipid class (30). Therefore, we examined whether recombinant rat PC-TP could be expressed using a baculovirus/Sf9 cell system (29). Although production of recombinant rat PC-TP clearly does not prove a requirement of PC for heterologous expression, these experiments demonstrate the utility of this system for expression of this highly specific lipid transfer protein.

In addition to mediating intermembrane transfer activity for PC but not PE (Fig. 2), the physical characteristics of recombinant PC-TP exhibited during purification (Fig. 3) demonstrate strong similarities between the native and recombinant proteins. Anion exchange chromatography, hydroxylapatite affinity chromatography as well as cation exchange chromatography (data not shown) yielded the same elution profiles as described for native rat PC-TP (18, 27). The specific activities of the purified recombinant and native PC-TPs were similar, and the experimentally determined pI value for the recombinant protein agrees well with the reported value of 8.4 for native rat PC-TP (27).

In the current study, molecular identification of purified recombinant protein was accomplished by MALDI-TOF-MS analysis and by amino acid sequencing. During HPLC purification used for desalting and removal of minor impurities, loss of PC transfer activity was not unanticipated as a result of the relatively high concentration of organic solvent (51 vol% acetonitrile) required to elute recombinant PC-TP after binding to the reverse phase column. This finding is consistent with the observation by Kamp et al. (31) that the water-miscible solvent dioxan irreversibly inactivated PC-TP activity at concentrations in excess of 15 vol% apparently due to induction of conformational change(s) in the protein. Indeed, the finding of a variably sized minor peak of the same molecular weight (Fig. 4) suggests the likelihood that an alternative conformation may also be adopted by the protein under the HPLC chromatographic conditions.

Based on the deduced amino acid sequence, the predicted molecular mass of recombinant PC-TP is 24,733 Da. Our experimental finding of slightly larger values 24,760 Da and 24,793 Da (Fig. 4B) are consistent with a blocked N-terminus. Blocking by acetylation as is observed for native bovine PC-TP (32), would increase the predicted molecular mass to 27,775 Da, which agrees well with the experimentally determined values. Collectively, these analytical findings suggest that recombinant PC-TP

is expressed by baculovirus with good fidelity and should serve as an excellent surrogate for studies of structural features that confer absolute specificity of this protein for PC.

The technical utility of the baculovirus/Sf9 cell system for studies of PC-TP structure and function may be assessed by several criteria. As demonstrated in Fig. 2, an in vitro lipid transfer assay (16) was used for detection and purification of recombinant PC-TP. The same activity assay should facilitate rapid screening of the functional consequences of alterations in the primary structure of PC-TP introduced by site-directed or random mutagenesis (33). Moreover, recent advances in methods for production of recombinant baculovirus (e.g., the Bac-to-Bac™ system) now permit facile preparation of recombinant viral stocks after cloning of cDNA constructs (34). We further demonstrated that recombinant PC-TP can be readily purified from Sf9 cell cytoplasm (Fig. 3) without the need for modification of the target protein, such as by addition of a histidine tag. Under the current experimental conditions (MOI ~5, duration of infection ~48 h), our recovery from 10 mg of cytosolic protein of 0.1–0.2 mg of recombinant PC-TP and ~30% of PC transfer activity after hydroxylapatite chromatography (Figs. 3B and 4C) indicate that the recombinant protein comprised 3–6% of cytosolic protein. Compared with a value of ~0.02% similarly calculated for rat liver from the data of Teerlink et al. (18), baculovirus infected Sf9 cells represent a 150- to 300-fold richer source of starting material for PC-TP purification.

Based on our recent demonstration that submicellar bile salt concentrations greatly enhance in vitro transfer activity of PC-TP (5, 17) and the long-standing observation that biliary phospholipids are highly enriched in PC (35), we have hypothesized that a key physiological function of PC-TP in liver might be the selection and cytosolic trafficking of PC molecules destined for secretion into bile (17). The capability to express recombinant PC-TP will now permit studies designed to elucidate molecular mechanisms underlying specific intermembrane transfer of PC in liver cytosol.

The authors gratefully acknowledge technical assistance provided by Andrew N. Leonard as well as expert advice from the Laboratory for Macromolecular Analysis at Albert Einstein College of Medicine (Dr. Ruth Hogue Angeletti, director). This work was performed while D. E. Cohen was a Pfizer Scholar in Cardiovascular Medicine and an American Liver Foundation Liver Scholar. Additional support was from center grant DK41296 from the National Institutes of Health to the Marion Bessin Liver Research Center.

Manuscript received 6 March 1998 and in revised form 6 May 1998.

REFERENCES

1. Wirtz, K. W. A., and D. B. Zilversmit. 1968. Exchange of phospholipids between liver mitochondria and microsomes in vitro. *J. Biol. Chem.* **243:** 3596–3602.
2. Wirtz, K. W. A. 1991. Phospholipid transfer proteins. *Annu. Rev. Biochem.* **60:** 73–99.
3. Kamp, H. H., K. W. A. Wirtz, P. R. Baer, A. J. Slotboom, A. F. Rosenthal, F. Paltauf, and L. L. M. van Deenen. 1977. Specificity of the phosphatidylcholine exchange protein from bovine liver. *Biochemistry.* **16:** 1310–1316.
4. Akeroyd, R., A. Lenstra, J. Westerman, G. Vriend, K. W. A. Wirtz, and L. L. M. van Deenen. 1982. Prediction of secondary structural elements in the phosphatidylcholine-transfer protein from bovine liver. *Eur. J. Biochem.* **121:** 391–394.
5. Cohen, D. E., and R. M. Green. 1995. Cloning of cDNAs for specific phosphatidylcholine transfer proteins (PC-TP) from liver: evidence for species conservation of the phosphatidylcholine (PC) binding site (Abstract). *Gastroenterology.* **108:** A1051.
6. Cohen, D. E., and R. M. Green. 1995. Cloning and characterization of a cDNA encoding the specific phosphatidylcholine transfer protein from bovine liver. *Gene.* **163:** 327–328.
7. Geijtenbeek, T. B. H., A. J. Smith, P. Borst, and K. W. A. Wirtz. 1996. cDNA cloning and tissue specific expression of the phosphatidylcholine transfer protein gene. *Biochem. J.* **316:** 49–55.
8. Wu, M. K., M. O. Boylan, and D. E. Cohen. 1998. Cloning and gene structure of rat phosphatidylcholine transfer protein (Abstract). *FASEB J.* **12:** A1281.
9. Wirtz, K. W. A. 1997. Phospholipid transfer proteins revisited. *Biochem. J.* **324:** 353–360.
10. Ossendorp, B. C., T. B. H. Geijtenbeek, and K. W. A. Wirtz. 1992. The precursor form of rat liver non-specific lipid-transfer protein, expressed in *Escherichia coli*, has lipid transfer activity. *FEBS Lett.* **296:** 179–183.
11. Moncecchi, D., A. Pastuszyn, and T. J. Scallen. 1991. cDNA sequence and bacterial expression of mouse liver sterol carrier protein-2. *J. Biol. Chem.* **266:** 9885–9892.
12. Matsuura, J. E., H. J. George, N. Ramachandran, J. G. Alvarez, J. F. Strauss 3rd, and J. T. Billheimer. 1993. Expression of the mature and the pro-form of human sterol carrier protein 2 in *Escherichia coli* alters bacterial lipids. *Biochemistry.* **32:** 567–572.
13. Tremblay, J. M., G. M. Helmkamp, and L. R. Yarbrough. 1996. Limited proteolysis of rat phosphatidylinositol transfer protein by trypsin cleaves the C terminus, enhances binding to lipid vesicles, and reduces phospholipid transfer activity. *J. Biol. Chem.* **271:** 21075–21080.
14. Geijtenbeek, T. B., E. de Groot, J. van Baal, F. Brunink, J. Westerman, G. T. Snoek, and K. W. Wirtz. 1994. Characterization of mouse phosphatidylinositol transfer protein expressed in *Escherichia coli*. *Biochim. Biophys. Acta.* **1213:** 309–318.
15. Studier, F. W., and B. A. Moffatt. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189:** 113–130.
16. DiCorleto, P. E., and D. B. Zilversmit. 1977. Protein-catalyzed exchange of phosphatidylcholine between sonicated liposomes and multilamellar vesicles. *Biochemistry.* **16:** 2145–2150.
17. 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.
18. Teerlink, T., B. J. H. M. Poorthuis, and K. W. A. Wirtz. 1983. Phosphatidylcholine transfer protein from rat liver: purification and radioimmunoassay. *Methods Enzymol.* **98:** 586–592.
19. Bloch, K. D., and B. Bartos. 1994. Digestion of DNA with endonucleases. *In* Current Protocols in Molecular Biology. F. M. Abusbel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, editors. John Wiley & Sons, New York. 3.1.1–3.1.20.
20. Johnson, L. W., M. E. Hughes, and D. B. Zilversmit. 1975. Use of phosphatidylcholine exchange protein to measure inside-outside transposition in phosphatidylcholine liposomes. *Biochim. Biophys. Acta.* **375:** 176–185.
21. Zilversmit, D. B., and M. E. Hughes. 1976. Phospholipid exchange between membranes. *Methods Membr. Biol.* **16:** 211–259.
22. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
23. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
24. Henzel, W. J., and J. T. Stults. 1995. Matrix-assisted laser desorption/ionization time-of-flight mass analysis of peptides. *In* Current Protocols in Protein Science. J. E. Coligan, B. M. Dunn, H. L. Ploegh, D. W. Speicher, and P. T. Wingfield, editors. John Wiley & Sons, New York. 16.2.1–16.2.11.

25. Crain, R. C. 1990. Phospholipid transfer proteins as probes of membrane structure and function. *Subcell. Biochem.* **16:** 45–67.
26. Crain, R. C., and D. B. Zilversmit. 1980. Two nonspecific phospholipid exchange proteins from beef liver. 1. Purification and characterization. *Biochemistry.* **19:** 1433–1439.
27. Lumb, R. H., A. D. Dloosterman, K. W. A. Wirtz, and L. L. M. van Deenen. 1976. Some properties of phospholipid exchange proteins from rat liver. *Eur. J. Biochem.* **69:** 15–22.
28. Runquist, E. A., and G. M. Helmkamp, Jr. 1988. Effect of acceptor membrane phosphatidylcholine on the catalytic activity of bovine liver phosphatidylcholine transfer protein. *Biochim. Biophys. Acta.* **940:** 21–32.
29. O'Reilly, D. R., L. K. Miller, and V. A. Luckow. 1992. Baculovirus Expression Vectors. A Laboratory Manual. W. H. Freeman, New York.
30. Yeh, L. H., R. K. Bajpai, and G. Y. Sun. 1997. Membrane lipid metabolism and phospholipase activity in insect *Spodoptera frugiperda* 9 ovarian cells. *Lipids.* **32:** 481–487.
31. Kamp, H. H., E. D. Sprengers, J. Westerman, K. W. A. Wirtz, and L. L. M. van Deenen. 1975. Action of phospholipases on the phosphatidylcholine exchange protein from beef liver. *Biochim. Biophys. Acta.* **398:** 415–423.
32. Akeroyd, R., P. Moonen, J. Westerman, W. C. Puyk, and K. W. A. Wirtz. 1981. The complete primary structure of the phosphatidylcholine-transfer protein from bovine liver. *Eur. J. Biochem.* **114:** 385–391.
33. Sambrook, J., E. Frisch, and T. Maniatis. 1989. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Plainview, NY.
34. Luckow, V. A., S. C. Lee, G. F. Barry, and P. O. Olins. 1993. Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli. J. Virol.* **67:** 4566–4579.
35. Hay, D. W., and M. C. Carey. 1990. Chemical species of lipids in bile. *Hepatology.* **12:** 6S–16S.