Isolation and Characterization of Rat Cholangiocyte Vesicles Enriched in Apical or Basolateral Plasma Membrane Domains†

Pamela S. Tietz,‡ Ralph T. Holman,§ Laurence J. Miller,‡,|| and Nicholas F. LaRusso*,‡,||

Center for Basic Research in Digestive Diseases, Division of Gastroenterology and Internal Medicine, and Department of Biochemistry and Molecular Biology, Mayo Medical School, Clinic and Foundation, Rochester, Minnesota 55905, and Hormel Institute, University of Minnesota, Austin, Minnesota 55912

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ABSTRACT: Cholangiocytes, the epithelial cells that line intrahepatic bile ducts, are composed of plasma membranes with discrete apical (lumenal) and basolateral domains. While these domains are thought to contain different transporters, exchangers, channels, and receptors, no methodology currently exists for the isolation of these different membrane compartments. Thus, our aim was to develop a technique to isolate plasma membranes from cholangiocytes enriched in apical or basolateral domains. We isolated a cholangiocyte-enriched population of cells from rats 3 weeks after bile duct ligation (BDL), a maneuver which stimulates selective cholangiocyte proliferation. Using isopycnic centrifugation on linear sucrose gradients, we prepared a mixed cholangiocyte plasma membrane (MCPM) fraction from which we further generated separate apical and basolateral cholangiocyte plasma membranes (ACPM and BCPM, respectively). We characterized these fractions by specific marker enzyme assays, transmission electron microscopy (TEM), lipid analysis, anisotropy measurements, one- and two-dimensional gel electrophoresis, and quantitative immunoblots of the cystic fibrosis transmembrane conductance regulator (CFTR). Marker enzyme assays and TEM revealed that the MCPM fraction was essentially devoid of other organelles but was enriched approximately 70-fold in phosphodiesterase I, a general plasma membrane marker; the ACPM and BCPM were appropriately enriched in the respective apical and basolateral markers. TEM of ACPM and BCPM revealed homogeneous preparations of vesiculated membranes without contamination by other organelles. Lipid analysis, one- and two-dimensional gel electrophoresis, CFTR immunoblots, and anisotropy measurements showed unequivocal differences in lipid and protein composition and in fluidity between the ACPM and BCPM domains. This methodology provides an initial attempt to develop highly purified apical and basolateral membrane domains from cholangiocytes, which provides a starting point for further studies.

The liver is composed of a variety of resident cells, including hepatocytes and bile duct epithelial cells, or cholangiocytes (Jones, 1990). These two cell types are the only epithelial cells in the liver, are involved in both absorptive and secretory activities, and represent highly polarized units containing morphologically and, presumably, functionally distinct plasma membrane domains (Hubbard et al., 1983; Ishii et al., 1989; Meier et al., 1984; Tavoloni, 1987). We know a great deal about the transport activities of hepatocytes, in part because the physicochemical properties and functional topography of their sinusoidal (basolateral) and canalicular (apical) membranes have been extensively characterized by several groups (Blitzer & Donovan, 1984; Evers et al., 1989; Hubbard et al., 1983; Meier et al., 1984) utilizing methodology for isolating preparations highly enriched in each of these membrane domains. On the other hand, our knowledge about the transport processes in cholangiocytes is relatively embryonic.

Cholangiocytes are simple epithelia which line an extensive array of interconnecting conduits or ducts within the liver, and provide an enormous surface area for the exchange of materials with bile and blood. The importance of these cells to the normal function of the liver and in disease states is being increasingly recognized. From a physiologic perspective, cholangiocytes are actively involved in the bidirectional movement of both water and solutes (e.g., ions, sugars, probably amino acids, possibly bile acids) in response to an array of regulatory agents, including hormones. In addition, these cells can selectively proliferate in response to a variety of stimuli, may participate in the metabolism of selected drugs, and are likely important in host defense against microbes. From a pathological viewpoint, cholangiocytes are the targets of a variety of immune-mediated, genetic, developmental, drug-induced, and malignant diseases (i.e., the cholangiopathies). While an increasing number of transporters, exchangers, channels, and receptors are being identified in these cells, the precise location of these proteins within cholangiocytes remains relatively obscure, in part because no methodology is currently available to isolate subcellular fractions enriched in specific organelles.

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* Address correspondence to this author at the Center for Basic Research in Digestive Diseases, Mayo Clinic, 200 First St., S.W., Rochester, MN 55905. Phone: (507) 284-1006. Fax: (507) 284-0762. E-mail: larusso.nicholas@Mayo.edu.

‡ Center for Basic Research in Digestive Diseases, Mayo Medical School.

§ Hormel Institute, University of Minnesota.

|| Department of Biochemistry and Molecular Biology, Mayo Medical School.

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We have previously developed a technique for isolating cholangiocytes in high yield and excellent purity from rats in whom selective proliferation of cholangiocytes has been stimulated by bile duct ligation (BDL)[1] (Alpini et al., 1994b). In work described here, we extend this methodology to allow isolation and characterization of cholangiocyte plasma membrane fractions enriched in apical and basolateral domains.

MATERIALS AND METHODS

Animal Model of Selective Cholangiocyte Hyperplasia. In all experiments, we used male Fisher 344 rats weighing between 225 and 250 g (Harlan Sprague-Dawley, Indianapolis, IN). We fed the animals Purina laboratory chow (Ralston Purina, St. Louis, MO) and permitted them access to food and water ad libitum. To induce selective cholangiocyte hyperplasia in order to increase our cell yield, we performed BDL 3 weeks prior to cell isolation as previously described (Alpini et al., 1994b).

Isolation of Cholangiocytes. Parenchymal and nonparenchymal liver cells were separated from livers of BDL rats by collagenase perfusion, enzymatic digestion, and mechanical disruption as previously described (Alpini et al., 1994b). A second, higher activity digestion and several passes through a 22 gauge needle yielded a single-cell, cholangiocyte-enriched population.

Cells were counted and purity was assessed by staining for γ-glutamyl transpeptidase (γGT) (a cholangiocyte-specific marker) as previously described (Rutenberg et al., 1969).

Preparation of Mixed Cholangiocyte Plasma Membranes (MCPM). All solutions for plasma membrane isolation were prepared in the presence of 0.01% soybean trypsin inhibitor (STI) and 0.1mM phenylmethanesulfonyl fluoride (PMSF) (Sigma, St. Louis, MO). Densities of all sucrose solutions were adjusted by refractometry at room temperature.

The cholangiocyte-enriched cell population was pelleted for 10 min at 400*g*, the supernate discarded, and the pellet sonicated in 10 volumes by weight of 0.3 M sucrose with two bursts, 7 s each, using a Sonifier Cell Disrupter (Heat Systems-Ultrasonics, Inc., Plainview, NY). While vortexing, 1.43 volumes of 2.0 M sucrose was added to the suspension and the mixture transferred to a TI70 ultracentrifuge tube (Beckman Instruments Inc., Palo Alto, CA). The sucrose cushion was overlaid with 0.3 M sucrose and spun for 1 h at 240000*g*. The plasma membrane band was removed and diluted 1:3 with cold Nanopure water, and centrifuged at 240000*g* for 30 min. The resulting pellet was resuspended in 3 mL of Krebs−Ringers Hepes (KRH), pH 7.4, layered over a 9−60% linear sucrose gradient, and centrifuged at 90000*g* for 3 h in a Beckman SW 28 rotor (Beckman Instruments, Inc., Palo Alto, CA). For marker enzyme analysis, the bottom of the tube was punctured, and 35 1-mL fractions were collected. Routinely thereafter, the major plasma membrane-containing band was collected by aspiration from the top of the tube and represented the MCPM.

Biochemical Characterization of MCPM. To determine the content and degree of enrichment of the MCPM prior to separation into apical and basolateral domains, we performed organelle-specific enzyme assays on an aliquot of homogenate and on each of the 35 1-mL fractions from the 9−60% sucrose gradient. *N*-Acetyl-β-glucosaminidase (lysosomes) activity was assayed fluorometrically using a 4-methylumbelliferyl substrate (Koch-Light Laboratories, Ltd., Colnbrook, Buckinghamshire, U.K.) as previously described (Peters et al., 1972). Malate dehydrogenase (mitochondria) activity was determined from the rate of oxidation of NADH, measured at 340 nm in the presence of acetic acid, under conditions previously described (Dupourque & Kun, 1969). Microsomal esterase (endoplasmic reticulum) activity was measured by the method of Beaufay et al. (Beaufay & Berthet, 1974), and lactate dehydrogenase (cytosol) activity was assayed by the method of Reeves and Fimognari (Reeves & Fimognari, 1966). Alkaline phosphodiesterase I (APDI) (plasma membrane) activity was assayed with *p*-nitrophenylthymidine 5′-phosphate as a substrate as previously reported (Beaufay & Berthet, 1974). Activity of γGT (a cholangiocyte-specific marker) was assayed biochemically (Rutenberg et al., 1969). Enrichment (*i.e.*, relative specific activity) was expressed as (specific activity of membrane fraction)/(specific activity of homogenate). Total protein was quantitated by the fluorescamine method (Udenfriend et al., 1972).

Isolation of Apical and Basolateral Cholangiocyte Plasma Membrane Domains. The plasma membrane-enriched MCPM band was diluted with 4 volumes of 1 mM $NaHCO_3$, pH 7.5, and centrifuged at 7500*g* for 15 min, and the resulting pellet was washed with 10 volumes of bicarbonate buffer and centrifuged at 2700*g* for 15 min. This pellet was resuspended to a volume of 3 mL in 0.25 M sucrose and homogenized with a tight type B glass−glass Dounce homogenizer (Kontes, Vineland, NJ) by 50 up-and-down strokes. This suspension was layered on top of a three-step sucrose gradient consisting of 4 mL of 38% (w/w; density = 1.1663 g/mL), 2.4 mL of 34% (w/w; density = 1.1463 g/mL), and 2.4 mL of 31% (w/w; density = 1.1318 g/mL) sucrose. The tubes were centrifuged at 20000*g* for 3 h in an SW41 rotor (Beckman Instruments Inc., Palo Alto, CA). This procedure produced two distinct bands: one on top of the 31% sucrose layer which was characterized to be the ACPM, the other at the 34/38% interface which was characterized to be the BCPM. A less distinct band appeared at approximately 32% sucrose and was not further characterized. The ACPM and BCPM bands were diluted to 10 mL each in 0.125 M sucrose and pelleted at 105000*g* for 1 h in an SW41 rotor. The resulting pellets were resuspended in KRH buffer by passage 20 times through a 25 gauge needle and stored at −70 °C or in liquid nitrogen.

Biochemical Characterization of ACPM and BCPM. Domain-specific assays were performed on the ACPM and BCPM, and relative specific activity was calculated as in the characterization of MCPM. Alkaline phosphatase and leucine aminopeptidase, commonly used markers for the apical plasma membrane domain (Meier et al., 1984), were assayed biochemically using commercially available enzyme kits (Sigma). As an additional apical domain marker, we measured γGT activity using γ-glutamyl-*p*-nitroanilide as

[1] Abbreviations: ACPM, apical cholangiocyte plasma membrane; BCPM, basolateral cholangiocyte plasma membrane; BDL, bile duct ligation; CFTR, cystic fibrosis transmembrane conductance regulator; γGT, γ-glutamyl transpeptidase; KRH, Krebs−Ringers Hepes; MCPM, mixed cholangiocyte plasma membrane; PMSF, phenylmethanesulfonyl fluoride; STI, soybean trypsin inhibitor; TEM, transmission electron microscopy; DBI, double bond index; PUFA, polyunsaturated fatty acids; MMP, mean melting point.

substrate (Rutenberg et al., 1969). We measured membrane-associated Na^+,K^+-ATPase, previously validated in liver plasma membranes by Scharschmidt et al. (1979), as a basolateral membrane marker.

Transmission Electron Microscopy. Membranes were prepared for transmission electron microscopy by pelleting freshly isolated ACPM and BCPM in fixative: 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer with 0.25 M sucrose at 4 °C for 30 min. This mixture was spun for 6 min at 16000*g*, the supernate removed, and the pellet washed 3 times with 0.1 M sodium cacodylate buffer to remove excess fixative. After washing, we post-fixed the membranes for 1 h in 1% OsO_4 in 0.1 M sodium cacodylate buffer. Dehydration in alcohol, embedding, sectioning, and examination on a Phillips 201 electron microscope were done as previously described by Ishii et al. (1989).

Membrane Lipid Analysis. Total cholesterol was measured spectrophotometrically using the enzyme cholesterol analysis kit of Boehringer Mannheim (Indianapolis, IN), and total phospholipids were determined using the commercial kit of Wako Chemicals USA, Inc. (Richmond, VA).

Fatty acid analysis was performed with capillary gas chromatography (Holman, 1993; Holman et al., 1989). One sample each of apical and basolateral domains of cholangiocytes, each containing 300 μg of total protein, was extracted with chloroform/methanol (2:1). The lipids were transesterified with boron trifluoride in methanol (14% w/v) for 90 min at 85 °C to form the methyl esters of the fatty acids. The methyl esters were separated by capillary gas chromatography using a bonded FFAP2 silica column (Quadrex, New Haven, CT). Components were identified by comparison to authentic methyl ester standards. The double bond index (double bonds per fatty acid) and mean melting point were calculated from the fatty acid composition (Holman, 1993, Holman et al., 1989).

Membrane Anisotropy Measurements. Apical and basolateral membrane fluidity was estimated by measuring steady-state anisotropy by fluorescence polarization as previously described (Myers et al., 1991). Briefly, 100 μg of membrane protein was added to 2.0 mL of 250 mM sucrose buffer containing 5 μL of 1 mmol/L 1,6-diphenyl-1,3,5-hexatriene (DPH) (Molecular Probes, Eugene, OR) and allowed to equilibrate for 1 h. Steady-state anisotropy was measured in an SLM 4800 spectrofluorometer (SLM, Urbana, IL) at 25 °C. Excitation was at 363 nm and emission at 420 nm (Myers et al., 1991).

Polyacrylamide Gel Electrophoresis of Membrane Proteins. We first solubilized membrane proteins (300 μg each of ACPM and BCPM) in 10 volumes of cold HPLC-grade acetone overnight at −20 °C. The samples were then centrifuged for 5 min at 9000*g*, the supernate was discarded, and any remaining acetone was allowed to evaporate from the pellet.

One-dimensional SDS−polyacrylamide gel electrophoresis was performed using conditions described by Laemmli (1970). Optimum resolution was obtained running a 12% polyacrylamide gel under reducing and denaturing conditions.

Two-dimensional polyacrylamide gel electrophoresis was performed using the system of O'Farrell (Adams & Gallagher, 1992; O'Farrell, 1975). Isoelectric focusing was performed in 1.5 mm diameter tube gels with a pH gradient of ampholines (Serva, Westbury, NY) from 3 to 10. We used the Carbamlyte calibration kit (Pharmacia, Piscataway, NJ) to generate a standard curve of isoelectric point versus R_f, thereby confirming reproducible formation of the pH gradient. We ran second-dimension gels using 12% polyacrylamide and visualized the protein spots by Coomassie blue staining.

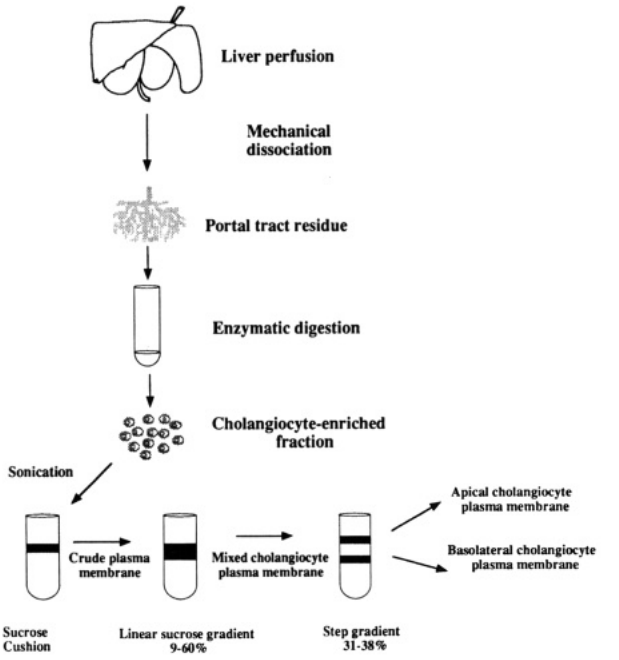

FIGURE 1: Isolation scheme for the separation of apical and basolateral plasma membrane domains from cholangiocytes isolated from bile duct ligated rats.

Western Blotting. Immunoblotting of CFTR was performed using solubilized plasma membranes from T84 cells, a human colon carcinoma cell line (ATCC, Rockville, MD) (positive control), rat hepatocytes (negative control), MCPM, ACPM, and BCPM. A 7.5% polyacrylamide gel was run containing 10 μg of T84 plasma membrane and 150 μg each of hepatocyte membranes, MCPM, ACPM, and BCPM.

We transferred the proteins to nitrocellulose and, following blocking, incubated the blot at 37 °C for 2 h with a 1:1000 dilution of primary whole antiserum. The CFTR antibody (amino acid residues 724−746, exon 13, R domain) was a generous gift from Dr. Pamela Zeitlin, Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, MD (Zeitlin et al., 1992). The blot was rinsed in wash buffer and exposed to secondary antibody (peroxidase conjugated goat anti-rabbit immunoglobulin; Tago, Inc., Burlingame, CA) at a 1:5000 dilution for 1 h at room temperature. We exposed the blot using an enhanced chemiluminescence detection system (ECL; Amersham, Arlington Heights, IL). We prepared the autoradiograms using Kodak XAR film and quantitated the bands by laser densitometry.

Statistical Analysis. Data were analyzed using an unpaired two-tailed Student's T test on the Clinfo data management and analysis system. Differences were considered to be significant at $p < 0.05$. All results are expressed as mean ± SE unless otherwise indicated.

RESULTS

Characterization of Plasma Membrane Preparations by Marker Enzyme Assays. An overview of the cell and membrane isolation procedure is shown in Figure 1. The

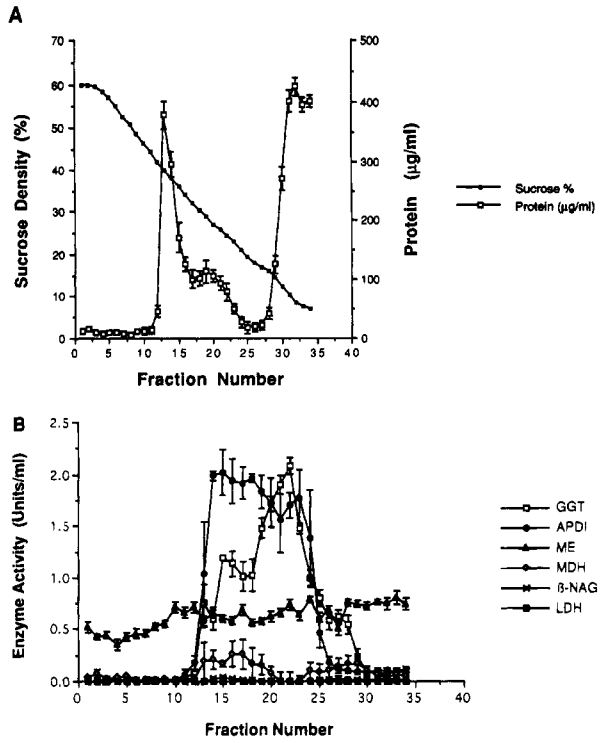

FIGURE 2: Isolation of a mixed cholangiocyte plasma membrane (MCPM) preparation was generated on a linear 9−60% sucrose gradient. One milliliter fractions were collected by puncturing the tube bottom, and protein (A) and marker enzymes (B) were measured on each fraction. The resulting MCPM was comprised of fractions 16−25 which were highly enriched in APDI (plasma membrane marker) compared to homogenate. Data are expressed as mean ± SEM. (n = 3 experiments in each group).

cholangiocyte isolation method described yielded a partially purified, single-cell suspension of 360 × 10^6 (±18.8) cells per 300 g rat which represents results (mean ± SE) for 20 membrane preparations. Cholangiocyte purity, as assessed by γGT positivity, was 65.7% (±1.2); previous work by us (Ishii et al., 1989) had shown that endothelial cells represent the major other cell type in this preparation. Crude membranes were prepared as described under Materials and Methods and further purified on a linear 9−60% gradient as demonstrated in Figure 2A. The use of a gradient maker ensured a reproducible linear sucrose gradient, and the distribution of membrane protein is as shown (Figure 2A). The amount of protein loaded was 9.3 (±0.46 mg). The distribution of organelle-specific enzymes is shown in Figure 2B. Lactate dehydrogenase was undetectable while malate dehydrogenase, microsomal esterase, and *N*-acetyl-β-glucosaminidase were present in minimal amounts. Alkaline phosphodiesterase 1 (plasma membrane marker) and γGT (cholangiocyte-specific marker) were detected in a broad band between fractions 13 and 24 of the gradient.

That portion of the gradient with the highest specific activity for the plasma membrane marker (i.e., highest APDI activity and lowest total protein) was found between fractions 16 and 25. These fractions were subsequently pooled and represented the fraction that was further separated into apical and basolateral membrane domains.

Table 1 shows the relative specific activity (i.e., the specific activity of the pooled fraction ratioed to the specific activity of the homogenate) for each of the organelle-specific marker enzyme assays. The plasma membrane marker APDI was enriched 68.7 (±8.34)-fold over the homogenate,

Table 1: Biochemical Analysis of MCPM, ACPM, and BCPM[a]

initial organelle preparation	rel sp act.[b]
N-acetyl-β-glucosaminidase	4.3 (±0.48)
microsomal esterase	0.11 (±0.07)
malate dehydrogenase	0.79 (±0.35)
lactate dehydrogenase	nondetectable
alkaline phosphodiesterase 1	68.7 (±8.34)
γ-glutamyl transpeptidase	2.1 (±0.004)

	rel sp act.	
apical markers	ACPM	BCPM
alkaline phosphatase	16.6	0.3
leucine aminopeptidase	4.9	1.1
γ-glutamyl transpeptidase	4.4	0.7

	rel sp act.	
basolateral marker	ACPM	BCPM
Na^+,K^+-ATPase	0.1	42.3

[a] Data are expressed as mean ± SEM with at least three experiments in each group. [b] Relative specific activity = specific activity of membrane fraction/specific activity of homogenate.

confirming a plasma membrane-enriched fraction with minimal contamination with other organelles. Following separation of the plasma membrane-enriched MCPM into ACPM and BCPM, respectively, the ACPM was found to be enriched in the domain-specific proteins alkaline phosphatase, leucine aminopeptidase, and γ-GT, while the BCPM was enriched in Na^+,K^+-ATPase when compared with the homogenate (Table 1). The total protein yield (milligram) generated from a membrane preparation using two BDL rats was 198.11 (±16.01) in homogenate, 2.71 (±0.16) in the MCPM, 0.55 (±0.04) in the ACPM, and 0.82 (±0.08) in the BCPM.

Transmission Electron Microscopy. Morphologic analysis by TEM revealed the ACPM and BCPM to be similar, homogeneous preparations of vesiculated membranes of varied shapes and sizes without apparent contamination by other organelles (Figure 3A,B).

Lipid Analysis. Table 2 shows the lipid analysis for the ACPM and BCPM. By our measurements, the ACPM contained approximately 2-fold the amount of cholesterol and phospholipid as the BCPM; the cholesterol and phospholipid ratios were approximately the same for both membrane domains. The amount of total fatty acids was found to be 7-fold higher in the ACPM compared to the BCPM. As shown in Table 2, over half of the fatty acids in both the ACPM and BCPM were comprised of three species—palmitic acid (16:0), stearic acid (18:0), and arachidonic acid (20:4ω6). While a number of differences between the two membrane compartments in fatty acid composition are apparent (Table 2), the most prominent difference between the two membrane domains was in the amount of arachidonic acid (20:4ω6) present; this biologically important eicosanoid precursor comprised ≈5% of the total fatty acid composition in the BCPM and ≈16% in the ACPM. Of the polyunsaturated fatty acids (PUFA), most were less abundant in the BCPM than in the ACPM. Total PUFA were 19.54% of the fatty acids in the BCPM and 34.43% of the fatty acids in the ACPM. These differences were counterbalanced by opposite changes of similar magnitude in the saturated fatty acids. Monoenoic acids were consistently less in BCPM than in ACPM phospholipids.

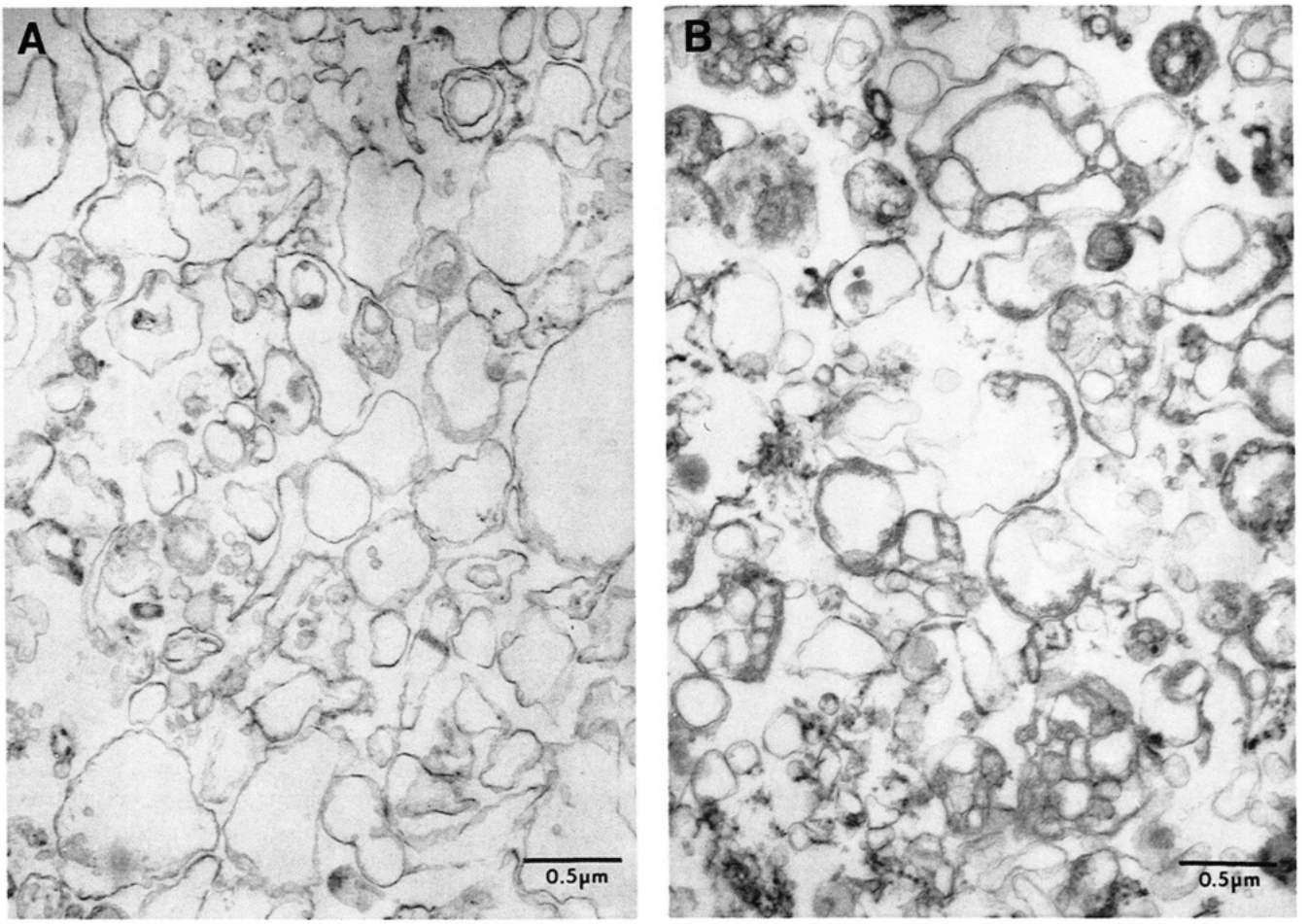

FIGURE 3: Transmission electron microscopy of apical (A) and basolateral (B) plasma membrane domains (22500×).

A composite index traditionally used to express the total fatty acid composition of a membrane is the double bond index (DBI) which calculates the number of double bonds per fatty acid chain. The DBI was calculated to be 0.72 for the BCPM and 1.32 for the ACPM phospholipid fatty acid, indicating that double bonds are nearly twice as many per mole of apical vs basolateral plasma membrane phospholipid. The mean melting point of the component fatty acids of the phospholipids was calculated for the ACPM and BCPM (Holman, 1993; Holman et al., 1989), and found to be 24.2 and 42.7 °C, also indicating the greater proportion of unsaturated fatty acids in the ACPM.

Membrane Fluidity. Steady-state anisotropy of ACPM and BCPM measured by fluorescence polarization of DPH showed a statistically significant ($p < 0.05$) difference between the two domains. Anisotropy measurement for the ACPM was 0.240 (±0.0028); the result for the BCPM was 0.210 (±0.0096).

Gel Electrophoresis Analysis of ACPM and BCPM. As demonstrated in Figure 4, approximately 18 distinct apical and 28−30 basolateral membrane protein bands could be resolved by one-dimensional SDS−PAGE and detected by Coomassie Blue staining. Approximately 11 bands demonstrate considerable enrichment in the BCPM domain and may represent specific cholangiocyte basolateral membrane proteins. We did not observe any bands present in the apical domain which were not also present in the basolateral domain lane.

Two-dimensional electrophoresis revealed a profile of approximately 10 major spots in the apical and 20 major spots in the basolateral domain which patterned in a slightly acidic range of isoelectric points between 5.0 and 6.8 (Figure 5A,B). As we observed by one-dimensional electrophoresis, there were distinct spots present in the basolateral domain which were not present in the apical domain.

Western Blotting. As shown in Figure 6, we detected a band at 165 kDa in the lanes containing membranes from T84, MCPM, and ACPM; this band was absent in hepatocytes and in BCPM. By quantitative laser densitometric scanning (a measurement of area units), the MCPM measured 150 122 (±888.86) and the ACPM 335 390 (±158.26), suggesting a 2.23-fold enrichment in the expression of CFTR in the apical domain compared to the mixed plasma membrane fraction.

DISCUSSION

The major findings reported here relate to the development of a technique to isolate cholangiocyte plasma membranes enriched in apical or basolateral domains. We describe a reproducible method based on principles of cell isolation and isopycnic centrifugation which yields fractions of cholangiocyte plasma membranes enriched in apical and basolateral domains. Moreover, we provide complete characterization of these fractions using morphologic, enzymatic, physicochemical, and electrophoretic techniques.

Several procedures have been published for the isolation from rat liver of hepatocyte membrane vesicles enriched in apical or basolateral domains (Blitzer & Donovan, 1984; Hubbard et al., 1983; Meier et al., 1984). In these studies,

Table 2: Lipid Analysis of ACPM and BCPM[a]

	ACPM	BCPM
cholesterol (μg/mg of protein)	343.33 (±3.52)	162.33 (±6.11)
phospholipids (μg/mg of protein)	333.3 (±7.21)	182.00 (±8.12)
cholesterol/phospholipid ratio	1.02 (±0.01)	0.89 (±0.04)
total fatty acids (mg/mg of protein)	78.54	11.66
anistropy (r)	0.240 (±0.0028)	0.210 (±0.0096)
individual fatty acids (mg %)		
12:0	0.02	0.09
14:0	0.56	1.03
14:1	0.53	0.61
16:0 br	2.48	1.54
16:0	21.59	28.54
16:1w7	1.50	1.17
17:0 br	0.55	1.69
18:0 br	1.78	1.14
18:0	18.33	27.69
18:1w9	9.95	6.67
18:1 isom	2.86	2.20
18:2w6	8.69	6.35
18:3w6	0.31	0.66
18:3w3	0.23	0.54
20:0	0.98	1.88
20:1w9	0.47	0.39
20:2w6	0.94	2.31
20:3w9	0.19	0.07
20:3w6	1.19	0.38
20:4w6	15.56	4.67
20:5w3	0.28	0.10
22:0	1.66	4.74
22:4w6	2.47	0.62
22:5w6	0.25	0.65
22:5w3	1.36	0.17
24:0	1.30	0.42
22:6w3	2.96	3.02
24:1	0.62	0.50

[a] Data are expressed as mean ± SE with at least two experiments in each group.

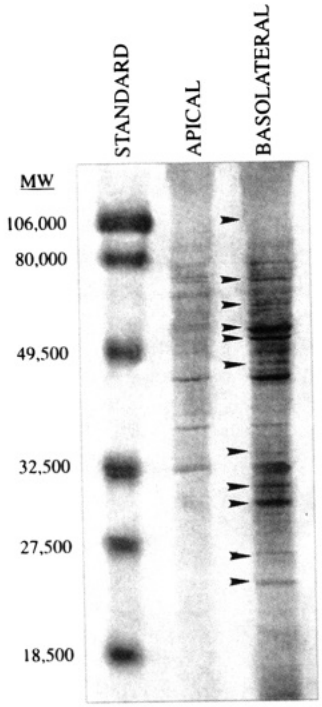

FIGURE 4: One-dimensional gel electrophoresis was run using a 12% polyacrylamide gel and 30 μg of protein per lane. (The arrowheads identify unique protein bands.)

investigators utilized a variety of preparative (e.g, isopycnic centrifugation, free-flow electrophoresis) and analytical (e.g., domain-specific marker proteins, TEM, protein electrophoresis, transport) approaches to prepare and characterize,

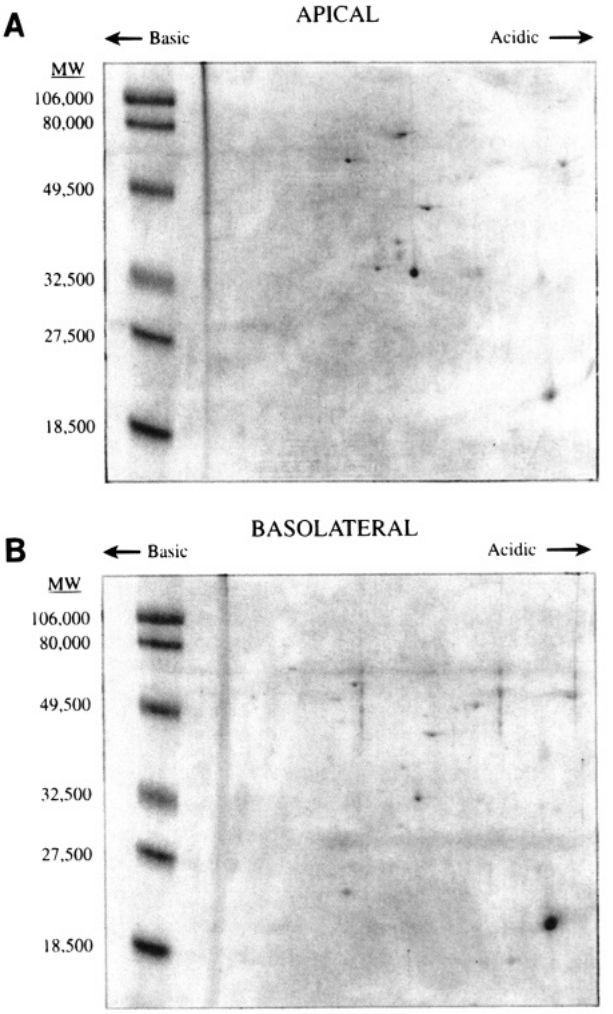

FIGURE 5: Two-dimensional gel electrophoresis of apical plasma membrane (A) and basolateral plasma membrane (B) was run by applying 300 μg of protein in the ISO dimension and running a 12% polyacrylamide gel in the DALT dimension.

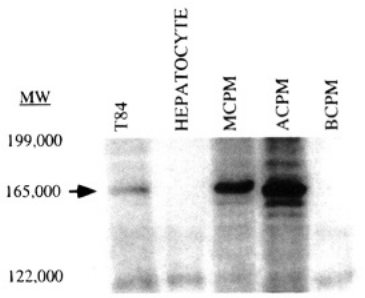

FIGURE 6: Quantitative immunoblot of CFTR was run using plasma membranes from T84 cell (10 μg) hepatocytes, MCPM, ACPM, and BCPM (150 μg each) on a 7.5% polyacrylamide gel. Bands were quantitated by laser densitometry.

respectively, their membrane fractions. These procedures served as useful models for developing our method, and their widespread application has generated important insights into hepatocyte biology (Blitzer & Donovan, 1984; Evers et al., 1989; Hubbard et al., 1983; Meier et al., 1984).

Given the fact that, under normal circumstances, cholangiocytes comprise only 3−5% of the total nuclear population of cells in rat liver, we employed BDL to stimulate selective proliferation of cholangiocytes in order to increase the number of cells available to yield plasma membrane vesicles. Thus, from a single rat liver after BDL, we could reliably generate approximately 350 million cells that were, on

average, 65% pure cholangiocytes. Our previous work showed that most of the noncholangiocyte cells in this preparation are endothelial cells (Ishii et al., 1989). Although we have reported alternative isolation procedures which result in an even purer cell preparation (i.e., over 90% cholangiocytes), the yield of cells (and, therefore, the membrane available for analysis) is markedly diminished. Thus, for our initial efforts at developing a reproducible fractionation scheme, we knowingly accepted a degree of contamination with noncholangiocyte cells in order to ensure sufficient membrane protein to yield a reasonable amount of apical and basolateral vesicles for characterization. The protein yield from a membrane preparation using two BDL rats and ≈700 million cells was consistently 0.5 mg of ACPM and 0.8 mg of BCPM. The isolation scheme we describe is patterned after similar schemes to generate apical and basolateral domains of hepatocyte membrane vesicles (Meier et al., 1984) from whole liver in which the hepatocyte comprises approximately 60% of the total number of liver cells (Alpini et al., 1994a). Thus, the purity of our cell preparation (65%) is actually comparable to the purity of hepatocytes in these methods (Meier et al., 1984).

There is a consensus among published plasma membrane vesicle isolation schemes as to which marker enzyme assays are indicative of each domain, and a certain degree of contamination of one domain by the other is anticipated. The most convincing evidence of a "pure" membrane preparation is the total absence of Na^+,K^+-ATPase in the apical vesicles (Keefe et al., 1979; Yeagle, 1983). While all of the marker enzyme assays described by us showed appropriate enrichment in their respective domains, Na^+,K^+-ATPase was essentially absent in the apical vesicles. TEM revealed the vesicles to be of varied sizes and shapes and devoid of other organelles. No obvious morphologic differences were apparent by TEM between ACPM and BCPM. We interpret these biochemical and morphologic data as indicating that (i) our method separates two plasma membrane fractions of cholangiocytes highly enriched in apical and basolateral domains and (ii) upon isolation the plasma membranes vesiculate. This latter point is particularly important (although not unexpected) because it indicates that these vesicles should be useful for direct transport studies in a fashion analogous to studies done on canalicular and basolateral vesicles derived from hepatocytes (Blitzer & Donovan, 1984; Meier, 1988). Ideally, determination of the sidedness (Blitzer & Donovan, 1984; Meier & Boyer, 1990) of our cholangiocyte membrane vesicles will be required for such transport studies.

The lipid composition of our cholangiocyte membrane domains was of great interest because it is markedly different from the membrane lipid composition reported by others for hepatocytes and for nonhepatic, epithelial membrane preparations (Brasitus & Schachter, 1984; Kapitulnik et al., 1979; Meier et al., 1984; Parola et al., 1988). The most notable differences included the very high cholesterol content, the high cholesterol/phospholipid ratio, and the unusual fatty acid composition. For example, the cholesterol/phospholipid ratio in cholangiocyte ACPM was approximately 8 times higher than that measured in hepatocyte membranes (Yano and LaRusso, unpublished work). We do not believe these differences can be accounted for by changes in the membrane lipid composition as a result of BDL, although others have reported that BDL alters the lipid composition of plasma membranes of renal tubule epithelial cells (Hise et al., 1984). Indeed, in other work, we have also observed very high cholesterol/phospholipid ratios in plasma membranes derived from essentially pure cholangiocytes isolated from normal rats (Yano and LaRusso, unpublished work). We also found that levels of cholesterol and phospholipid were higher in the ACPM compared to the BCPM of cholangiocytes. Cholesterol has a hydrolyzing effect on the acyl chain region of lipid bilayers and makes membranes more rigid and less permeable to water and small solutes such as glucose, K^+, and Ca^{2+} (Blok et al., 1977; Gennis, 1989; Meier et al., 1984; Storch et al., 1983). Membrane cholesterol also decreases Na^+,K^+-ATPase and glucagon and fluoride-stimulatable adenylate cyclase activity of erythrocytes and mixed liver plasma membranes, respectively (Gennis, 1989; Meier et al., 1984; Whetton et al., 1983). Therefore, the high cholesterol and phospholipid content of ACPM may serve special cytoprotective and regulatory functions for cholangiocytes since this cholangiocyte domain faces the lumen of bile ducts and, as such, is exposed to very high concentrations of bile acids, sugars, ions, xenobiotics, and, theoretically, a host of possible microbes originating from the intestine. Of interest also was our observation that the PUFA content of the ACPM was very high, a finding which suggests that these membranes may be particularly susceptible to oxidation to form free radicals and high-energy intermediates. We were unable to find published data on the lipid composition of plasma membranes derived from hepatic endothelial cells; however, data on the lipid composition of plasma membranes of nonhepatic endothelial cells (Gerritsen et al., 1991) would suggest that the unusual lipid composition we observed in our vesicles is not likely accounted for by contaminating membranes derived from endothelial cells.

In parallel with the studies presented here, we are evaluating polarized primary cultures of normal rat cholangiocytes which produce an uninterrupted monolayer of cells grown on collagen-coated filters of tissue culture inserts (Vroman et al., 1994); the plasma membranes of these cells can be separated into apical and basolateral domains using our isolation scheme. Potentially, the use of cultured cholangiocytes would allow us to generate higher yields of pure membrane vesicles. To date, we have found that the ACPM and BCPM differ in their cholesterol and phospholipid content and fluidity in a quantitatively similar fashion to membranes prepared from the freshly isolated, cell preparation described in this paper (Tietz and LaRusso, unpublished data). This provides additional support that the unusual lipid composition we observed in vesicles prepared from freshly isolated cholangiocytes is not due to contaminating membranes derived from endothelial cells.

There appear to be a large number of individual proteins present in the plasma membrane of cholangiocytes, based on our gel electrophoresis data. This is not surprising given the known involvement of cholangiocytes in a variety of transport processes (Alpini et al., 1994c; Roberts et al., 1993, 1994) and the responsiveness of these cells to hormones and peptides (Alpini et al., 1994c; Kato et al., 1992; Tietz et al., 1994; Ulrich et al., 1993). Our electrophoretic data represent an initial, novel effort to categorize these proteins based on their molecular weight and charge and to define their polar topography within the plasma membrane. The quantitative and qualitative differences observed between the ACPM and

BCPM by one- and two-dimensional gel electrophoresis suggest the presence of basolateral-specific plasma membrane proteins which remain to be identified.

Our ability to demonstrate an enrichment of CFTR on the apical membrane surface of cholangiocytes by immunoblotting supports the feasibility of using these vesicles for future studies on channels, transporters, and receptors. The detection of a band at 165 kDa is a size consistent with predictions for CFTR in studies of various epithelial cell lines (Zeitlin et al., 1992). In additional studies using Western blots of intestinal villus cell apical and basolateral membrane fractions, the immunodetectable CFTR was also associated with the apical fraction (Crawford et al., 1991).

In conclusion, the availability of a reproducible and reliable technique for isolating cholangiocyte apical and basolateral plasma membrane domains will permit systematic evaluation of key physicochemical and functional parameters of cholangiocytes, and the relative contributions of the apical and basolateral domains to cholangiocyte physiology. This methodology provides an initial attempt to generate highly purified apical and basolateral domains from cholangiocytes which provides a starting point for further studies. Future studies will allow direct measurements of the basal and hormone-induced permeability of apical and basolateral membrane vesicles to a variety of solutes, and will permit confident localization of various transporters, channels, and receptors. In addition, comparative studies can be done on the two populations of membrane vesicles in the presence and absence of physiologically relevant agonists (e.g., secretin and somatostatin) to determine if changes occur in the lipid and protein composition in response to these hormones. Such studies not only will provide novel data relevant to the plasma membrane topography of key proteins but also could yield important comparative information as to the similarities and differences between cholangiocytes and hepatocytes. The availability of the technology described here should make feasible direct experimental approaches to answer questions in all of these areas.

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