Newly synthesized canalicular ABC transporters are directly targeted from the Golgi to the Hepatocyte Apical Domain in Rat Liver

Helmut Kipp and Irwin M. Arias

From the Department of Physiology, Tufts University School of Medicine, Boston, Massachusetts 02111

Newly synthesized canalicular ectoenzymes and a cell adhesion molecule (cCAM105) have been shown to traffic from the Golgi to the basolateral plasma membrane, from where they transcytose to the apical bile canalicular domain. It has been proposed that all canalicular proteins are targeted via this indirect route in hepatocytes. We studied the membrane targeting of rat canalicular proteins by in vivo [35S]methionine metabolic labeling followed by preparation of highly purified Golgi membranes and canalicular (CMVs) and sinusoidal/basolateral (SMVs) membrane vesicles and subsequent immunoprecipitation. In particular, we compared membrane targeting of newly synthesized canalicular ABC (ATP-binding cassette) transporters MDR1, MDR2, and SPGP (sister of P-glycoprotein) with that of cCAM105. Significant differences were observed in metabolic pulse-chase labeling experiments with regard to membrane targeting of these apical proteins. After a chase time of 15 min, cCAM105 appeared exclusively in SMVs, peaked at 1 h, and progressively declined thereafter. In CMVs, cCAM105 was first detected after 1 h and subsequently increased for 3 h. These findings confirm the transcytotic targeting of cCAM105 reported in earlier studies. In contrast, at no time point investigated were MDR1, MDR2, and SPGP detected in SMVs. In CMVs, MDR1 and MDR2 appeared after 30 min, whereas SPGP appeared after 2 h of labeling. In Golgi membranes, each of the ABC transporters peaked at 30 min and was virtually absent thereafter. These data suggest rapid, direct targeting of newly synthesized MDR1 and MDR2 from the Golgi to the bile canaliculus and transient sequestering of SPGP in an intracellular pool en route from the Golgi to the apical plasma membrane. This study provides biochemical evidence for direct targeting of newly synthesized apical ABC transporters from the Golgi to the bile canaliculus in vivo.

The bile canaliculus of the mammalian hepatocyte contains several primary active transporters that couple ATP hydrolysis to the transport of specific substrates into the bile canaliculus (1–4). These transporters are members of the superfamily of ABC (ATP-binding cassette) membrane transport proteins (5) and currently include P-glycoprotein or MDR1 (multidrug resistance protein; organic cations) (6), MDR2 (phosphatidylcholine) (7, 8), SPGP (sister of P-glycoprotein; bile acids) (9), and MRP2 (multidrug resistance-associated protein; non-bile acid organic anions) (10).

The amount of each ABC transporter in the canalicular membrane is regulated by the physiological demand to excrete bile acids. Intravenous administration of rats with taurocholate or dibutylryl cAMP rapidly and selectively increased the functional activity and amount of ABC transporters in the canalicular membrane. This increase was inhibited by prior administration of colchicine, which disrupts microtubules (11), and wortmannin, which inhibits phosphatidylinositol 3-kinase (12). These observations indicate that an intracellular microtubule-dependent transport mechanism that is sensitive to active phosphatidylinositol 3-kinase is required to traffic ABC transporters to the canalicular membrane. In addition, lipid products of phosphatidylinositol 3-kinase directly regulate the ATP-dependent substrate transport activity of SPGP and MRP2 in the canalicular membrane (13). These studies indicate that bile secretion requires intrahepatic trafficking and regulation of the canalicular ABC transporters.

Membrane targeting of the newly synthesized canalicular ectoenzymes dipeptidyl peptidase IV, aminopeptidase N, and 5’-nucleotidase and the canalicular cell adhesion molecule cCAM105 (also known as HA4) has been studied in rat liver by in vivo metabolic pulse-chase labeling. After biosynthesis, these canalicular proteins are transferred from the Golgi to the basolateral membrane and subsequently reach the bile canaliculus only by transcytosis (14, 15). Based on these results, it was proposed that all newly synthesized canalicular proteins, including canalicular ABC-type transporters, are targeted via this indirect route (16, 17). Although the membrane targeting of newly synthesized canalicular ectoenzymes and cell adhesion molecule cCAM105 has been thoroughly studied, comparable investigations of canalicular ABC transporters have not been performed.

In this study, we used metabolic pulse-chase labeling followed by subcellular fractionation of rat liver and immunoprecipitation to investigate the intracellular trafficking of newly synthesized canalicular proteins. In particular, we focused on membrane targeting of newly synthesized transporters of the MDR family, including SPGP, and compared their trafficking with that of cCAM105 and with the basolateral membrane resident asialoglycoprotein receptor (ASGP-R).1

1 The abbreviations used are: ASGP-R, asialoglycoprotein receptor; CMV, canalicular membrane vesicle; SMV, sinusoidal membrane vesicle; PAGE, polyacrylamide gel electrophoresis.
FIG. 1. Schematic presentation of the protocol used to isolate CMVs, SMVs, and Golgi membranes from rat liver. HOM, homogenate.

EXPERIMENTAL PROCEDURES

Materials

Radiochemicals were supplied by NEN Life Science Products. All other chemicals were of the highest purity available and were purchased from Sigma. Monoclonal antibody C219 (anti-MDR1/MDR2) was from Centocor (Malvern, PA), and polyclonal anti-β-COP antibody was from Sigma. Other antibodies were kind donations: EAG15 (polyclonal, anti-MRP2), D. Kropper (10); Ab669 (polyclonal, anti-eCAM105), S. H. Lin, (18); HA301 (monoclonal, anti-dipeptidyl peptidase IV), A. L. Hubbard (19); and anti-ASGP-R, R. J. Stockert (20).

Generation of Anti-SPGP Antibody LVT90

A glutathione S-transferase fusion protein containing a 90-amino acid fragment of the SPGP linker region (amino acids 653–742, starting with LVT) was used as antigen to raise antibody LVT90. The corresponding coding DNA fragment was amplified from full-length SPGP cDNA (provided by P. Meier, (9) by polymerase chain reaction using the oligonucleotides 5′-AAT GAA TCC TGC TTG TGA CCC TGC AAA G-3′ (containing a BamHI site) and 5′-ATT GAC TAC CTA ACT GGG GCA GGT TC-3′ (containing a SalI site). The polymerase chain reaction product was digested by BamHI and SalI and ligated into the BamHI/SalI sites of the pGEX-SX-3 vector (Amersham Pharmacia Biotech). In-frame cloning was confirmed by DNA sequencing. Expression of the glutathione S-transferase fusion protein in Escherichia coli BL21 cells and purification using glutathione-Sepharose beads were performed according to protocols provided by Amersham Pharmacia Biotech.

A commercial service was employed to raise antibodies in rabbits (Covance, Denver, PA) using a standard protocol for immunization and bleeding.

Metabolic Labeling

Groups of five male Sprague-Dawley rats (300–350 g) kept on a standard diet were anesthetized with sodium pentobarbital (50 mg/kg, injected intraperitoneally) and were injected in the tail vein with 3.5 mCi of [35S]methionine/cysteine (1175 Ci/mmol; Exper 35S35S protein label, NEN Life Science Products) in 1 ml of phosphate-buffered saline. 15 min later, 50 mg of unlabeled methionine and 5 mg of unlabeled cysteine in 2.5 ml of phosphate-buffered saline were injected intraperitoneally. For investigation of membrane targeting, livers were removed after 15 min, 30 min, 1 h, 2 h, and 3 h and used for subcellular fractionation. Data presented are typical results observed in at least three sets of five rats.

Subcellular Fractionation of Rat Liver

Previously published methods were combined, modified, and optimized for a high yield of canalicular, sinusoidal/basolateral, and Golgi membranes from a single rat liver (Fig. 1). After gentle homogenization of rat liver, bile canaliculi remain attached to tight junctions and sediment with the low speed nuclear pellet. Canalicular membrane vesicles were prepared from the low speed pellet by nitrogen cavitation followed by calcium precipitation (21). The low speed supernatant was split and used for purification of basolateral membranes on a sucrose/Ficoll gradient (22) and for preparation of Golgi membranes by floating a microsomal fraction on a discontinuous sucrose gradient (23). Details are as follows.

Excised rat liver (10–15 g, wet weight) was rapidly perfused with ice-cold SHCa buffer (0.25 M sucrose, 10 mM HEPES/Tris, pH 7.4, and 0.2 mM CaCl2) supplemented with protease inhibitors (2 μg/ml leupeptin, 2 μg/ml pepstatin A, 20 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml benzamidine, and 2 μg/ml aprotonin) and homogenized in 50 ml of SHCa buffer with four strokes in a loose-fitting Dounce homogenizer. The suspension was filtered through a double layer of cheesecloth; homogenized again with 15 strokes; diluted with SHCa buffer, and centrifuged for 10 min at 1800 × g (Beckman JA-14, 3500 rpm). The pellet and fluffy layer were collected and used for preparation of CMVs. The supernatant (~130 ml) was split 1:1 and used for preparation of SMVs and Golgi membranes, respectively.

CMVs—The pellet was resuspended in 50 ml of SHCa buffer and centrifuged for 10 min at 3000 × g (Beckman JA-14, 4500 rpm). The resulting pellet was suspended in 50 ml of SHCa buffer, placed in a high pressure chamber (Parr Instrument Model 4635), and equilibrated with nitrogen at 550 p.s.i. for 15 min with shaking at 4 °C. Pressure was released within 3 min; and the contents of the chamber were homogenized with six strokes in a tight-fitting Dounce homogenizer, diluted with SHCa buffer to 120 ml, and supplemented with 1 mM CaCl2 stock solution to a final concentration of 1 mM; and centrifuged for 10 min at 130,000 × g (Beckman Ti-45, 27,000 rpm). The pellet was homogenized in 30 ml of SHCa buffer with six strokes in a tight-fitting Dounce homogenizer and centrifuged for 10 min at 3000 × g (Beckman JA-17, 4500 rpm). The supernatant was collected and centrifuged for 30 min at 47,000 × g (Beckman Ti-45, 27,000 rpm). The resulting pellet was homogenized in SHCa buffer to 3–4 mg/ml protein with a syringe and 24-gauge needle and stored at −80 °C until used.

SMVs—65 ml of the first supernatant was centrifuged for 10 min at 5500 × g (Beckman JA-14, 7500 rpm). The resulting supernatant and fluffy layer were collected and centrifuged for 30 min at 22,000 × g (Beckman JA-14, 12,000 rpm). The pellet was resuspended in SH buffer containing 1 mM EGTA (total volume of 12 ml) and layered on two discontinuous gradients consisting of 1 ml of 60% sucrose, 23 ml of 23% sucrose and 4% Ficoll 400, and 7 ml of 20% sucrose. After centrifugation for 90 min at 130,000 × g in a swinging bucket rotor (Beckman SW 27, 27,000 rpm), the interface between 20% sucrose and 23% sucrose and 4% Ficoll was collected, diluted six times with SHCa buffer, and centrifuged for 30 min at 47,000 × g (Beckman Ti-45, 27,000 rpm). The resulting pellet was homogenized in SHCa buffer at ~10 mg/ml protein with a syringe and 24-gauge needle and stored at −80 °C until used.
Direct Apical Targeting in Rat Hepatocytes

TABLE I
Specific activities of marker enzymes

| Marker enzyme                        | HOM   | CMV   | SMV   | Golgi  |
|-------------------------------------|-------|-------|-------|--------|
| γ-Glutamyl transpeptidase (μmol/h/mg) | 0.3 ± 0.1 | 14.1 ± 1.9 | 0.7 ± 0.4 | 0.6 ± 0.2 |
| pH 7.4, containing 0.02% NaN₃ and protease inhibitors (2 μg/ml) and aprotinin | (100%) | (47% [5.7%]) | (2% [11.1%]) | (2% [0.2%]) |
| Alkaline phosphatase (μmol/h/mg)    | 0.4 ± 0.1 | 17.6 ± 3.1 | 1.0 ± 0.3 | 1.2 ± 0.4 |
| Na⁺/K⁺-ATPase (μmol/h/mg)           | 0.3 ± 0.2 | ND    | 4.7 ± 14  | 0.7 ± 0.4 |
| Galactosyltransferase (nmol/h/mg)   | 0.8 ± 0.3 | ND    | 1.3 ± 0.3 | 19.3 ± 1.0 |
| (100%)                                |       | (16% [7.2%]) | (2% [0.3%]) |        |

...system (NEN Life Science Products). The method of Lowry et al. (26) was used for protein measurements with bovine serum albumin as the standard. The activities of marker enzymes were determined according to the following protocols: γ-glutamyl transpeptidase (27), alkaline phosphatase (28), Na⁺/K⁺-ATPase (29), and galactosyltransferase (30).

RESULTS

Properties of Subcellular Fractions—The quality of the membrane preparations was determined by measuring the enrichment of marker enzymes (Table I). Marker enzymes for the canalicular membrane γ-glutamyl transpeptidase and alkaline phosphatase were highly enriched only in CMVs (~45-fold) as compared with the homogenate. A marker for the basolateral membrane Na⁺/K⁺-ATPase was slightly increased in Golgi fractions, below the detection limit in CMVs, and enriched 16-fold in SMVs. The enrichment of marker enzymes was comparable to that reported previously (21, 22). Galactosyltransferase, a marker for Golgi membranes, was slightly enriched in SMVs, below the detection limit in CMVs, and 24-fold enriched in Golgi preparations. The yields of specific marker enzyme activities in CMVs and SMVs are consistent with data reported earlier (21, 22).

The quality of membrane preparations was further established by immunochemical methods. Monoclonal anti-β-COP antibody recognizes an epitope shared by the Golgi β-COP protein (110 kDa). Immunoblots probed with anti-β-COP antibody showed significant enrichment in Golgi membranes as compared with the homogenate, whereas β-COP was absent from canalicular and basolateral membranes (Fig. 2). CMV and SMV preparations were further characterized by probing with antibodies against apical and basolateral membrane resident proteins in immunoblots. As shown in Fig. 4, basolateral ASGP-R was detected only in SMVs and was absent from CMVs, whereas antibodies against the canalicular transporters MDR1 and MDR2 and SPGP and MRP2 reacted exclusively with antigens in CMVs.

Monoclonal antibody C219 (anti-MDR1/MDR2) met this property. Therefore, we generated a polyclonal antibody against the recently cloned canalicular bile acid transporter SPGP. Alignment of amino acid sequences revealed high similarity/homology among rat MDR1 (31), MDR2 (32), and SPGP (9): MDR1 versus SPGP, 48/68%; MDR1 versus MRP2, 69/82%; and MDR2 versus SPGP, 46/66%. A 90-amino acid peptide from the SPGP “linker” region that showed the lowest similarity/homology to the other MDR transporters was chosen as immunogen for the generation of anti-SPGP antibody LVT90.

Monoclonal antibody C219 was raised against MDR1 constitutively overexpressed in Chinese hamster ovary cells and is directed against two hexapeptides located close to the ATP-binding sites in CHO-MDR1, VQAALD and VQEALD (33).
Both recognition motifs are present in rat MDR1 and MDR2 at the same position. In rat SPGP, the corresponding sites are altered to VQEALN and VQTALD. In the first recognition sequence, aspartic acid, which is critical for antigen recognition (33), was replaced by asparagine. In the second recognition sequence, glutamic acid was replaced by threonine.

In immunoblots, the polyclonal anti-SPGP antibody LVT90 showed strong reaction with a 170-kDa protein in CMVs that was absent from SMVs (see Fig. 4). Preimmune serum did not show reactions with CMVs or SMVs. Reaction of LVT90 with CMVs was competed by addition of the glutathione \(-\text{transferase fusion protein (data not shown). To test for possible cross-reactivity between C219 and LVT90, we performed immunoprecipitations followed by immunoblotting (Fig. 3). A lysate of rat liver CMVs (500 µg) was immunoprecipitated first with anti-SPGP antibody LVT90 (lanes A) followed by anti-MDR1/MDR2 antibody C219 (lanes B). In a similar experiment, CMV lysate was first immunoprecipitated with C219 (lanes C) followed by LVT90 (lanes D). Immunoprecipitates were separated by SDS-PAGE, transferred onto nitrocellulose membrane, and probed with either LVT90 or C219. Reaction of LVT90 in immunoblots was observed only when LVT90 was used for immunoprecipitation. Whether LVT90 immunoprecipitation was before or after precipitation with C219 was uncritical. Furthermore, C219 immunoprecipitates showed no reaction with LVT90 in immunoblots. The same applied for C219 immunoblots. Positive reactions were observed only when C219 was used for the initial precipitation, and no reaction was observed with LVT90 precipitates. The sequence in which antibodies were employed for precipitation was not critical. These immunoprecipitation/blotting experiments indicate that there is no cross-reactivity between the C219 and LVT90 antibodies with regard to immunoprecipitation and immunoblotting. Thus, C219 is specific for rat MDR1 and MDR2, and LVT90 is specific for rat SPGP.

Diffusion of Canalicular Proteins in Immunoblots—Western blots of purified CMVs and SMVs that were probed with antibodies against canalicular proteins are shown in Fig. 4. Anti-dipeptidyl peptidase IV and anti-cCAM105 antibodies predominantly reacted with CMVs, and a small amount was regularly observed in SMVs. We explain the presence of these “canalicular” proteins in SMVs by the fact that these membrane proteins are initially transferred to the basolateral membrane after biosynthesis and subsequently reach the apical pole by transcytosis (14). This scenario is in good accordance with detectable steady-state levels in SMVs. Under the same conditions, antibodies against the canalicular ABC-type transporters, C219 (anti-MDR1/MDR2), LVT90 (anti-SPGP), and EAG15 (anti-MRP2), exclusively recognized antigens in CMVs, which suggests that newly synthesized canalicular ABC transporters may not be initially trafficked to the basolateral plasma membrane.

Metabolic Pulse-Chase Labeling—To test the hypothesis of direct apical targeting of canalicular ABC transporters in rat hepatocytes, we performed metabolic pulse-chase labeling experiments. Rats were injected with \[^{35}S\]methionine, and labeling of newly synthesized proteins was terminated 15 min later.
of antigens.

MRP2, the ectoenzyme dipeptidyl peptidase IV (DPP IV), the canalicular resident proteins MDR1 and MDR2 (detected with the C219 antibody (anti-MDR1/MDR2)), SPGP, the organic anion transporter (OAT1), and cCAM105 were prepared. Each fraction was immunoprecipitated with anti-ASGP-R, anti-cCAM105, C219 (anti-MDR1/MDR2), and LVT90 (anti-SPGP) antibodies; immunoprecipitates were separated by SDS-PAGE; and radioactive bands were detected and quantified with a PhosphorImager. The results are presented in Fig. 5.

Membrane targeting of ASGP-R was investigated as a control experiment to validate our experimental procedures using a sinusoidal membrane resident protein. ASGP-R consists of three subunits (43, 54, and 64 kDa) and is a well-established sinusoidal membrane protein that is involved in receptormediated endocytosis (35). ASGP-R in the basolateral membrane binds to ligands with terminal N-acetylgalactosamine residues, and the ligand-receptor complex is internalized into endosomes, where the complex is split; ASGP-R is recycled to the basolateral membrane, and the ligand is targeted to lysosomes. After immunoprecipitation and a chase time of 15 min, the three subunits of ASGP-R were detected exclusively in SMVs, peaked at 1 h, and declined thereafter. The decline after 1 h results from internalization of receptor from the sinusoidal membrane into endosomes. More important, this experiment shows that in immunoblots and by immunoprecipitation from CMVs and SMVs, ASGP-R is exclusively detected in SMVs, which further validates the quality of the plasma membrane preparations.

Radiolabeled MDR1, MDR2, and SPGP peaked in Golgi membranes after a chase time of 30 min and were virtually absent from the Golgi at later time points, indicating that processing and passage through the Golgi of these ABC transporters are complete after 30–60 min. This is also supported by the fact that immature forms of SPGP disappeared from homogenates at chase times later than 30 min. Under the same conditions, MDR1 and MDR2 could not be immunoprecipitated from the homogenate with C219 antibody, probably due to a much lower abundance of MDR1 and MDR2 compared with SPGP. Compared with ABC transporters, the passage of cCAM105 through the Golgi was slower. In Golgi membranes, cCAM105 was detected up to 2 h, which was paralleled by the presence of immature proteins in the homogenate for the same time period. The slower passage of cCAM105 through the Golgi may result from its greater extent of glycosylation compared with that of ABC transporters.

Beside passage through the Golgi, major differences were also observed among the canalicular membrane proteins with regard to membrane targeting. After a chase time of 15 min, newly synthesized cCAM105 appeared exclusively in SMVs, peaked at 1 h, and progressively declined thereafter. In CMVs, cCAM105 was first detected after 1 h and subsequently increased for 3 h. These observations are in accordance with transcytotic targeting of cCAM105 and confirm results from earlier studies (14). This experiment also demonstrates that our experimental procedure efficiently detects transcytotic targeting. After a chase time of 30 min, MDR1 and MDR2 appeared exclusively in CMVs and increased thereafter for the remaining time investigated. At no time was MDR1 or MDR2 detected in SMVs. The absence of MDR1 and MDR2 from CMVs at all investigated time points and the time course of their appearance in CMVs strongly suggest direct targeting from the Golgi to the canalicular membrane. A transcytotic pathway can also be excluded for the plasma membrane targeting of SPGP. At no time was SPGP detected in SMVs, but compared with MDR1 and MDR2, appeared exclusively in CMVs only after 2 h. Particular interesting are results after 1 h of labeling: the homogenate contained only mature SPGP, indicating that SPGP had already trafficked through the Golgi, but had not reached the cell surface. A likely explanation is that SPGP is transiently sequestered in an intracellular pool before it is delivered to the canalicular membrane.

Efficiency of Immunoprecipitation with C219 and LVT90 Antibodies—Critical to our hypothesis (that in contrast to other canalicular proteins, ABC transporters do not undergo transcytosis after biosynthesis, but are directly delivered from the Golgi to the apical membrane) is the absence of ABC transporters from SMVs in the metabolic labeling studies. Therefore, we investigated the efficiency of antibodies against canalicular ABC transporters for immunoprecipitation and quantified the detection limit of ABC transporters in SMVs.

To estimate the efficiency of immunoprecipitation, lysates of 1 mg of CMVs that had been immunoprecipitated with C219 or LVT90 under the same conditions used for metabolic labeling studies were probed with the same antibodies in immunoblots to detect remaining antigen. These results were compared with control experiments in which lysates of 1 mg of CMVs were “immunoprecipitated” under the same conditions with an equal volume of phosphate-buffered saline (control for monoclonal antibody C219) or an equal volume of preimmune serum (control for LVT90) (Fig. 6). Quantitation by a laser densitometer revealed that after immunoprecipitation with C219, 3% of MDR1/MDR2 remained in the lysate and 2% of SPGP remained after immunoprecipitation with LVT90. To determine whether any SPGP or MDR1/MDR2 remained after immunoprecipit-
tion, we used a sufficiently high amount of lysate that saturated the gray scale of the bands in the control experiments (Fig. 6). Therefore, the amount of remaining antigen after immunoprecipitation with C219 and LVT90 was even 2–3%. It is therefore reasonable to conclude that the amount of MDR1, MDR2, and SPGP remaining after immunoprecipitation is not significant and that the efficiency of antibodies C219 and LVT90 for immunoprecipitation is 100%.

The lower detection limit for putative ABC transporters in SMVs was determined using SMV/CMV mixing experiments. Rats were pulse-chase-labeled with [35S]methionine for 2 h since only at this chase time is SPGP present in CMVs (Fig. 5G). From the metabolically labeled rats, samples of SMVs (1 mg each) were supplemented with increasing amounts of CMVs (0, 25, 50, 100, 250, and 500 μg). The mixtures were immunoprecipitated with the C219 and LVT90 antibodies; immunoprecipitates were separated by SDS-PAGE; and radioactive bands were detected in a PhosphorImager (Fig. 7). Immunoprecipitation with C219 detected MDR1/MDR2 in a supplement of 50 μg of CMVs in 1 mg of SMVs, and immunoprecipitation with LVT90 detected SPGP in a supplement of 25 μg of CMVs in 1 mg of SMVs. For the metabolic labeling studies (Fig. 5), 1 mg of SMVs and CMVs, respectively, was used for immunoprecipitation. Therefore, in SMVs, <5% of C219 antigen and <2.5% of LVT90 antigen could be detected. Furthermore, this experiment demonstrates that there are no components in SMVs that prevent immunoprecipitation of ABC transporters with either C219 or LVT90.

**DISCUSSION**

Hepatocytes and other epithelial cells exhibit two morphologically distinguishable plasma membrane domains (apical and basolateral) that contain different membrane resident proteins that fulfill the physiological task of each pole of the cell. Maintenance of polarity requires mechanisms for targeting newly synthesized membrane proteins to their appropriate plasma membrane domains. After biosynthesis, basolateral membrane resident proteins are targeted directly from the Golgi to the basolateral plasma membrane. In contrast, alternative routes exist for newly synthesized proteins of the apical membrane. In various cell lines, they are either directly delivered from the Golgi to the apical pole or initially transferred to the basolateral domain and subsequently access the apical pole by transcytosis (for reviews, see Refs. 36 and 37). In polarized Madin-Darby canine kidney cells, newly synthesized endogenous apical proteins are predominantly delivered by a direct route (38–40); however, the transcytotic route can also participate as demonstrated for the polymeric immunoglobulin A
FIG. 6. Antibodies C219 and LVT90 efficiently precipitate their antigens.
CMVs were immunoprecipitated with C219 (control (contr) immunoprecipitation (IP) with phosphate-buffered saline) and LVT90 (control immunoprecipitation with preimmune serum) as described under “Experimental Procedures.” Aliquots of the remaining lysates were precipitated with trichloroacetic acid. The pellets were dissolved in sample buffer and subjected to SDS-PAGE and electroblotting. The blots were then reprobed with C219 and LVT90, respectively. Arrowsheads indicate the positions of the antigens. The amount of antigen was quantified by laser densitometry (lower panels), arb. units, arbitrary units.

FIG. 7. Low levels of [%sup]S-labeled SPGP, MDR1, and MDR2 can be detected in SMVs when supplemented with CMVs. Rats were pulse-chase-labeled with [%sup]S-methionine for 2 h, and CMVs and SMVs were prepared from the livers. SMVs were supplemented with various amounts of CMVs and immunoprecipitated (IP) with C219 (anti-MDR1/MDR2) and LVT90 (anti-SPGP) antibodies as described under “Experimental Procedures.” The immunoprecipitates were separated by SDS-PAGE, and [%sup]S-labeled antigens were detected in a PhosphorImager.

receptor (41, 42) and for endogenous apical glycoproteins (43). Both mechanisms for apical targeting are operative in Caco-2 cells, an epithelial cell line derived from native intestine (44, 45). In contrast, hepatocytes are assumed to lack the direct pathway for delivery of apical proteins (16) based on the investigations of dipeptidyl peptidase IV, aminopeptidase N, 5’-nucleotidase, and the canalicular cell adhesion molecule cCAM105 (14, 15). These canalicular proteins are selectively targeted by transcytosis in metabolic labeling studies in vivo.

Stable cell lines in culture are invaluable for investigating targeting of membrane proteins in polarized cells. However, similar targeting studies of endogenous membrane proteins in vivo are infrequent due to the difficulty in separating apical and basolateral membranes from native tissue for detailed investigation. Bartles and Hubbard (46) overcame these obstacles and developed a method to study targeting of newly synthesized membrane proteins in polarized rat hepatocytes in vivo. Livers from rats metabolically labeled with [%sup]S-methionine were used to prepare sheets of total plasma membranes, which were vesiculated by ultrasonification and resolved into apical and basolateral domains by centrifugation on a continuous sucrose gradient. Fractions of this gradient were immunoprecipitated with antibodies against apical proteins to document the distribution of newly synthesized proteins in either apical or basolateral membrane domains at a defined time after biosynthesis. In the present study, we used a similar approach to study membrane targeting of newly synthesized canalicular ABC-type transporters in vivo. We resolved apical and basolateral membrane domains by preparing highly purified CMVs and SMVs according to well established, reproducible methods (21, 22), which we have combined and optimized for high yield from a single rat liver. In addition, we prepared Golgi membranes from the same metabolically labeled rat to simultaneously study the trafficking of canalicular proteins through this intracellular compartment.

The metabolic pulse-chase labeling approach depends on availability of precipitating antibodies against canalicular ABC transporters. Since only the commercial C219 antibody met this requirement, a precipitating antibody (LVT90) was raised against a fusion protein containing a 90-amino acid sequence from the rat SPGP linker region. In sequence alignments, this region showed few matches with the homolog sequences of rat MDR1 and MDR2. As demonstrated in immunoprecipitation/blotting experiments, the C219 and LVT90 antibodies do not cross-react. Therefore, LVT90 is specific for SPGP, and C219 is specific for MDR1 and MDR2.

This study confirms the transcytotic pathway for apical targeting of newly synthesized canalicular cell adhesion molecule cCAM105 (HA4), which was described in an earlier study (14). In contrast, at no time between passage through the Golgi and arrival at the bile canaliculus were apical ABC transporters MDR1, MDR2, and SPGP detected in SMVs, indicating a direct Golgi-to-bile canaliculus pathway for their membrane targeting. These results are supported by the steady-state levels of apical proteins in isolated CMVs and SMVs as detected in immunoblots. Most of cCAM105 and dipeptidyl peptidase IV are present in CMVs, but a considerable amount is also present in SMVs. Their presence in SMVs is due to the fact that after biosynthesis, these molecules are targeted by transcytosis and are initially transferred to the basolateral membrane. Consistent with a direct Golgi-to-bile canaliculus targeting, the ABC transporters MDR1, MDR2, and SPGP were not detected in SMV immunoblots, but were exclusively found in CMVs, as was the canalicular ABC transporter MRP2. Therefore, it is reason-
able to assume that all apical ABC transporters known so far are targeted directly from the Golgi to the bile canalicus after biosynthesis.

Although newly synthesized MDR1, MDR2, and SPGP were not initially transferred to the basolateral membrane, their post-Golgi trafficking differed. After passage through the Golgi, MDR1 and MDR2 were rapidly delivered directly to the bile canalicus, whereas Golgi-to-bile canalicus trafficking of SPGP involved additional intermediate steps. At 1 h after metabolic labeling, only the mature form of SPGP was detected in the homogenate, indicating that processing and passage through the Golgi were complete at this point. This is also supported by decreased radioactivity to background levels in immunoprecipitates from Golgi membranes after 1 h. At this time point, SPGP was not detected in SMVs and CMVs and therefore had not reached the cell surface. The most likely explanation is that SPGP is sequestered in an intracellular pool prior to delivery to the canalicular membrane (Fig. 8). Interestingly, immunogold electron microscopic detection of SPGP in rat hepatocytes revealed that the distribution of SPGP in the rat hepatocyte is not restricted only to the bile canalicus, but labeling of SPGP was also detected in electron translucent vacuoles close to the apical membrane (9). Pericanalicular distribution of SPGP was also demonstrated by immunofluorescent staining of isolated rat hepatocyte couplets (47). These subapical structures containing SPGP may represent the transient intracellular pool in which newly synthesized SPGP is transiently sequestered prior to its targeting to the bile canalicus.

In metabolic pulse-chase experiments, ABC transporters peaked in Golgi membranes only 30 min after labeling; thus, it is unlikely that the labeled cohort of ABC transporters is not detected in SMVs due to very rapid transcytosis. But we cannot completely exclude the possibility that a very small portion of ABC transporters, which is below the detection limit, may be sequestering. The basolateral membrane and undergo transcytosis. SMV/CMV mixing experiments demonstrated that <5% of the labeled cohort of MDR1 and MDR2 (C219 antibody) and <2.5% of the labeled SPGP cohort (LVT90 antibody) can be theoretically detected in SMVs with our procedure. If a transcytotic pathway for the ABC transporters exists in rat hepatocytes, it is utilized by <2.5–5% of the canalicular ABC transporters that were studied. It is therefore reasonable to assume that transcytosis is not a significant pathway for membrane targeting of newly synthesized ABC transporters. In contrast, newly synthesized canalicular ABC transporters are predominantly if not exclusively targeted directly from the Golgi to the bile canalicus with or without intermediate intracellular sequestering.

There is additional evidence from earlier studies for a direct Golgi-to-bile canalicus pathway after biosynthesis of ABC transporters. Disturbance of transepithelial trafficking by either colchicine, which disrupts microtubules, or wortmannin, which inhibits phosphatidylinositol 3-kinase, results in accumulation of the transcytosing molecule cCAM105 in the basolateral membrane, but does not result in detectable levels of ABC transporters in the basolateral membrane (12). These results indicate non-transcytotic trafficking for canalicular ABC transporters.

WIF-B cells are a hybrid of rat hepatoma cells and human fibroblasts, have functional bile canaliculi, and serve as a useful model for hepatocytes (48, 49). Membrane targeting of an MDR1-green fluorescent protein chimera stably transfected into WIF-B cells was recently studied by Sai et al. (50). Fluorescence of the MDR1-green fluorescent protein chimera was exclusively detected in Golgi and bile canalicus membranes; no labeling of basolateral plasma membranes was observed. Time-lapse video imaging revealed that MDR1-green fluorescent protein traveled directly from the Golgi to the bile canalicus. Trafficking from the Golgi to the apical membrane occurred in tubular vesicular structures at 0.02–0.6 μm/s. These observations strongly support the rapid and direct pathway detected with the C219 antibody (anti-MDR1/MDR2) in the present in vivo study.

Direct Golgi-to-apical membrane targeting of newly synthesized ABC transporters is in contrast to earlier work in which transcytotic targeting was proposed for these canalicular transporters (17, 47). The assumption was based on immunofluorescence detection of canalicular ABC transporters in the basolateral membrane of isolated rat hepatocyte couplets. However, the “basolateral membrane” of rat hepatocyte couplets also originates from parts of other hepatocytes that are ripped from the bile canaliculi of adjacent hepatocytes. Consequently, the basolateral membrane of rat hepatocyte couplets represents a mixture of basolateral membrane and the remainder of former bile canaliculi, which likely explains detectable ABC transporter levels in the rat hepatocyte couplet periphery.

Soroka et al. (47) demonstrated, by immunogold electron microscopy, colocalization of the transcytosis marker polymeric immunoglobulin A receptor and SPGP (there called bile salt export pump) in the same vesicles from a population of tubulin-bound vesicles in rat liver and proposed transcytotic targeting of both newly synthesized proteins. In contrast, our study does not reveal evidence for newly synthesized SPGP to be initially targeted to the basolateral membrane. SPGP was transiently sequestered in an intracellular pool. Probably, this pool is a subapical compartment involved in sorting and/or apical recycling similar to that observed in other epithelial cells (i.e. WIF-B cells (51), Madin-Darby canine kidney cells (52), and Caco-2 cells (53)). Furthermore, in Madin-Darby canine kidney cells, a subapical compartment is an intermediate station for the polymeric immunoglobulin A receptor transcytotic basolateral-to-apical trafficking (54, 55). The observed intracellular SPGP pool is therefore most likely the site at which directly targeted biosynthesized canalicular ABC transporters and proteins undergoing transcytosis merge.

This study prompts revision of current views regarding the trafficking of newly synthesized canalicular proteins in hepatocytes. Direct Golgi-to-apical membrane trafficking of ABC transporters with or without subapical accumulation probably provides specific physiological regulation of transporter re-

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**Fig. 8.** Membrane targeting of newly synthesized canalicular proteins in rat hepatocytes.
Currently investigating these mechanisms.

**Acknowledgment**—We are grateful to Nipaporn Pichetshote for skillful technical assistance.

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