ABC transporter trafficking in rat liver induced by cAMP or taurocholate and \([^{35}S]\)methionine metabolic labeling followed by subcellular fractionation were used to identify and characterize intrahepatic pools of ABC transporters. ABC transporter trafficking induced by cAMP or taurocholate is a physiologic response to a temporal demand for increased bile secretion. Administration of cAMP or taurocholate to rats increased amounts of SPGP, MDR1, and MDR2 in the bile canalicular membrane by 3-fold; these effects abated after 6 h and were insensitive to prior treatment of rats with cycloheximide. Half-lives of ABC transporters were 5 days, which suggests cycling of ABC transporters between canalicular membrane and intrahepatic sites before degradation. \textit{In vivo} \([^{35}S]\)methionine labeling of rats followed by immunoprecipitation of (sister of P-glycoprotein) (SPGP) from subcellular liver fractions revealed a steady state distribution after 20 h of SPGP between canalicular membrane and a combined endosomal fraction. After mobilization of transporters from intrahepatic sites with cAMP or taurocholate, a significant increase in the amount of ABC transporters in canalicular membrane vesicles was observed, whereas the decrease in the combined endosomal fraction remained below detection limits in Western blots. This observation is in accordance with relatively large intracellular ABC transporter pools compared with the amount present in the bile canalicular membrane. Furthermore, trafficking of newly synthesized SPGP through intrahepatic sites was accelerated by additional administration of cAMP but not by taurocholate, indicating two distinct intrahepatic pools. Our data indicate that ABC transporters cycle between the bile canalculus and at least two large intrahepatic ABC transporter pools, one of which is mobilized to the canalicular membrane by cAMP and the other by taurocholate. In parallel to regulation of other membrane transporters, we propose that the "cAMP-pool" in hepatocytes corresponds to a recycling endosome, whereas recruitment from the "taurocholate-pool" involves a hepatocyte-specific mechanism.

The bile canalicular membrane of the mammalian hepatocyte contains several primary active transporters that couple ATP hydrolysis to the transport of specific substrates into the bile canalculus (1–4). These transporters are members of the superfamily of ATP binding cassette (ABC) \(^1\) membrane transport proteins (5) and currently include P-glycoprotein (MDR1) for organic cations (6), MDR2 for phosphatidylcholine translocation (7, 8), sister of P-glycoprotein (SPGP), the canalicular bile salt export pump (BSEP) (9), and MRP2 for non-bile acid organic anions (10).

Recent studies indicate that the amount of each ABC transporter in the canalicular membrane is regulated by the physiological demand to secrete bile acids. Intravenous administration to rats of dibutyryl-cAMP (Bt2cAMP) or taurocholate (TC) rapidly and selectively increased the functional activity and amount of each ABC transporter in the canalicular membrane; these effects were 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, which is sensitive to active phosphatidylinositol 3-kinase (12), is required for trafficking of ABC transporters to the canalicular membrane. ATP-dependent transport activity of SPGP and MRP2 within the bile canalicular membrane requires active phosphatidylinositol 3-kinase and is regulated by 3'-phosphoinositide products of phosphatidylinositol 3-kinase (13).

ABC transporters are essential for biliary secretion in mammalian liver, and their amounts and activities in the canalicular membrane are tightly regulated. Inherited defects in SPGP and MRP2 proteins result in familial intrahepatic cholestasis (for review, see Ref. 14). Therefore, defects in trafficking and regulation of ABC transporters may result in insufficient amounts or activity of ABC transporters in the bile canalicular membrane, the consequences of which are impaired bile secretion and cholestasis (15).

The increase in the content of ABC transporters in the canalicular membrane after administration of Bt2cAMP or TC \textit{in vivo} (11) and in isolated perfused rat liver (12, 13) occurred within minutes after the administration of the effectors. These observations suggest that the increment in canalicular ABC transporters is likely to represent recruitment from pre-existing intrahepatic pools rather than from enhanced transcription or translation. Furthermore, the simultaneous administration of Bt2cAMP and TC resulted in additive rather than alternative effects (11). These observations suggest that Bt2cAMP and TC recruit ABC transporters to the canalicular membrane from...
Intrahepatic Pools of ABC Transporters

**Experimental Procedures**

**Materials**—Expre\(^{35}\)S\(^{35}\)S protein label was supplied by PerkinElmer Life Sciences. All other chemicals were of highest purity available and were purchased from Sigma. Monoclonal antibody C219 (anti-MDR1/MDR2) was from Centocor (Malvern, PA). Polyclonal anti-SPGP antibody is specific for rat MDR1 and MDR2 and that polyclonal LVT90 antibody is specific for SPGP (16). After treatment with Bt\(_2\)cAMP, an increase in MDR1, MDR2, and SPGP in CMVs was observed after 15 min; the effect peaked at 45 min to 4 h and disappeared after 6 h. TC treatment resulted in a significant increase in MDR1, MDR2, and SPGP after 15 min; the effect peaked at 45 to 90 min and then declined to the basal level after 4 h. From three sets of independent experiments, the maximal increase in the amount of ABC transporters in the canalicular membrane was 3.6 ± 0.9-fold after stimulation with Bt\(_2\)cAMP and 2.7 ± 0.7-fold after treatment with TC.

**RESULTS**

**Bt\(_2\)cAMP and TC Transiently Increase the Canalicular Amount of ABC Transporters**—Gatmaitan et al. (11) demonstrate that intravenous injection of rats with Bt\(_2\)cAMP or TC increased the amount of canalicular proteins in the bile canalicular membrane. To determine the duration and magnitude of the response to Bt\(_2\)cAMP and TC administration, we investigated the time courses of these effects. Groups of rats were injected with either Bt\(_2\)cAMP (20 \(\mu\)mol/kg) or TC (50 \(\mu\)mol/kg) for various time periods, and canalicular amounts of ABC transporters were determined in CMVs by Western blotting (Fig. 1). We demonstrated earlier that monoclonal C219 antibody is specific for rat MDR1 and MDR2 and that polyclonal LVT90 antibody is specific for SPGP (16). After treatment with Bt\(_2\)cAMP, an increase in MDR1, MDR2, and SPGP in CMVs was observed after 15 min; the effect peaked at 45 min to 4 h and disappeared after 6 h. TC treatment resulted in a significant increase in MDR1, MDR2, and SPGP after 15 min; the effect peaked at 45 to 90 min and then declined to the basal level after 4 h. From three sets of independent experiments, the maximal increase in the amount of ABC transporters in the canalicular membrane was 3.6 ± 0.9-fold after stimulation with Bt\(_2\)cAMP and 2.7 ± 0.7-fold after treatment with TC.

**TC and cAMP Effects Are Independent of de Novo Protein Synthesis**—Additional ABC transporters in the bile canalicular upon stimulation with Bt\(_2\)cAMP or TC could theoretically result from enhanced protein biosynthesis and/or recruitment of transporters from pre-existing intracellular pools. To discriminate between these possibilities, we determined whether the effect in ABC transporters in the bile canalculus remained when protein synthesis was inhibited. Protein biosynthesis was efficiently blocked by intraperitoneal injection of cycloheximide (5 mg/kg) for 30 min. Pretreatment with cycloheximide completely abolished metabolic labeling with \[^{35}\]Smethionine of proteins in CMVs. This was demonstrated by detection of \[^{35}\]S-labeled proteins in CMVs with a scintillation counter and of radiolabeled proteins using PhosphorImager after separation of CMV proteins by SDS-PAGE (Fig. 2).
**Protein Biosynthesis**

To establish the effectiveness of cycloheximide (CHX) in blocking protein biosynthesis in vivo, a rat was treated with an intraperitoneal injection of cycloheximide (5 mg/kg) for 30 min before metabolic labeling with $[^{35}S]$methionine for 2 h. CMVs were prepared, and radioactivity was determined from an aliquot by liquid scintillation counting (A). Furthermore, an aliquot of CMVs was separated by SDS-PAGE and stained with Coomassie (B), and radioactivity was then detected by phosphorimaging (C). The results were compared with CMVs from a rat without cycloheximide pretreatment. The results shown were established in a single experiment; error bars indicate S.D. from three replica.

**Rats were injected intraperitoneally with cycloheximide for 30 min followed by intravenous injection of either Bt$_2$cAMP or TC for 1 h. CMVs were then prepared, and proteins from rat liver homogenate and CMVs were separated by SDS-PAGE. The amount of ABC transporters in both preparations was quantified by Western blotting with C219 (MDR1, MDR2) and LVT90 (SPGP) antibodies and compared with control experiments (Fig. 3).**

Cycloheximide inhibits protein biosynthesis; however, in some instances cycloheximide can also cause superinduction of proteins (24, 25). In rat liver homogenate no significant change in the amount of ABC transporters was observed after pretreatment of rats with cycloheximide, Bt$_2$cAMP, and TC in various combinations (Figs. 3, A and B). These data indicate that superinduction of proteins by cycloheximide is not a concern in our study regarding ABC transporters. The total amount of SPGP, MDR1, and MDR2 in rat liver remained constant after administration of cycloheximide, Bt$_2$cAMP, and TC.

**Pretreatment of rats with cycloheximide for 30 min had no effect on the basal protein amount of MDR1, MDR2, and SPGP in CMVs. Cycloheximide pretreatment did not reduce the increase in ABC transporters in the bile canaliculus in response to Bt$_2$cAMP or TC.** Regardless of prior cycloheximide administration, Bt$_2$cAMP or TC significantly increased the canalicular content of MDR1, MDR2, and SPGP when compared with control animals (Figs. 3, C and D). These experiments demonstrate that additional ABC transporters in the bile canalicular membrane after stimulation with Bt$_2$cAMP or TC do not result from enhanced translation but from a redistribution between intrahepatic ABC transporter pools and the bile canalicular membrane.

**Half-lives of Canalicular ABC Transporters**—Where do the canalicular ABC transporters go after peak stimulation by Bt$_2$cAMP or TC and their amounts in the bile canalicular membrane return to basal levels? Two possible scenarios were considered as follows. ABC transporters either return to intrahepatic sites, or in parallel with other canalicular protein receptors, they are degraded. The latter presumably should be manifested by relatively short half-lives for canalicular ABC transporters. Therefore, we determined the half-lives of the canalicular ABC transporters and compared the results to previously established half-lives of other hepatocyte plasma membrane proteins.

A group of rats was labeled by intravenous injection with $[^{35}S]$methionine for 1, 3, 5, 8, and 10 days (without chase), and the content of newly synthesized MDR1, MDR2, and SPGP was determined by immunoprecipitation from CMVs and homogenate. The half-life of cCAM105 was also determined as a control. Protein half-lives were determined from semi-logarithmic plots of $^{35}$S intensity versus labeling time (Fig. 4). As observed earlier (16), MDR1 and MDR2 could not be immunoprecipitated with C219 antibody from liver homogenate, probably due to low abundance of the antigens.

Assuming first order decay, cCAM105 had an apparent half-life of 5 days as measured in homogenates and CMVs, which corresponds to an earlier published report (26). A 5-day half-life was also determined for MDR1 and MDR2 in CMVs. For SPGP, a half-life of 4 days was measured from CMVs and 6 days from homogenate. It is not clear why the apparent half-lives differ when determined from the two preparations. However, it has been observed before that the apparent half-lives of hepatic proteins are shorter when determined from plasma membranes as compared with results in homogenates (26).

The half-lives of several hepatocyte plasma membrane proteins are presented in Table I and were obtained from metabolic studies using radiolabeled amino acids. Except for nucleotide pyrophosphatase and the polymeric IgA receptor, which have short half-lives, all other hepatic plasma membrane proteins previously investigated have half-lives of 2–9 days. The short half-life of the polymeric IgA receptor is accounted for by extensive loss into the bile (26). Since it is difficult to prevent long term label reincorporation in vivo (26, 29), the data probably represent upper limits. Similar to other hepatic plasma membrane proteins (Table I), canalicular SPGP, MDR1, and MDR2 have relatively long half-lives ($t_{1/2} > 1$ day) and are likely to undergo lysosomal degradation (31).

**trafficking of ABC transporters from intrahepatic sites to the bile canalicular membrane is physiologic and results from increased need to secrete bile. Therefore, we postulate that canalicular ABC transporters cycle between the apical membrane and intrahepatic sites prior to undergoing degradation.**

**Large Amounts of SPGP Reside in Intracellular Pools**—A CEF from rat liver was prepared (20) to investigate the dynamics and steady state levels of SPGP in intrahepatic pools. CEFs, prepared by centrifugation of a microsomal fraction of rat liver on a discontinuous sucrose gradient, are highly enriched in endosomal markers Rab5 (early endosomes) and Rab11 (apical recycling endosomes) (32) as compared with homogenates and CMV and SMV plasma membrane subfractions (Fig. 5).

In a recent paper (16), we described trafficking of newly synthesized SPGP by investigating the kinetics of $[^{35}S]$methionine-labeled SPGP in liver homogenate, Golgi membranes, CMVs, and SMVs. SPGP was never observed in SMVs, indicating nontranscytotic targeting. Furthermore, in study of SPGP trafficking, there was a 1.5-h lag between passage through Golgi and arrival at the canalicular membrane, which suggests transient sequestration in intrahepatic sites. We expanded this experiment to include CEF. Trafficking of newly synthesized SPGP after pulse-chase labeling with $[^{35}S]$methionine through
Golgi membranes, CEF, and CMVs is shown in Fig. 6. Radiolabeled SPGP peaked in Golgi membranes after a chase time of 30 min and thereafter was virtually absent from the Golgi, indicating that processing and passage of SPGP through Golgi is complete after 30–60 min. SPGP peaked in CEF at 1 h and, after a chase time of 2 h, first appeared in CMVs. These experiments demonstrate that newly synthesized SPGP is targeted through an endosomal compartment before reaching the bile canalicular membrane. Furthermore, SPGP is not completely transferred from CEF, and a significant amount of SPGP remains in the endosomal fraction. These results suggest a distribution of SPGP between canalicular membrane and intracellular pools (2- and 3-h chase). This was also observed after a chase time of 20 h, which presumably represents steady state distribution of SPGP between the bile canaliculus and intracellular pools under basal conditions.

Immunoblots were used to determine the distribution of SPGP and MDR1/MDR2 between CMVs and CEF under steady

![Fig. 3.](image)

**FIG. 3.** TC and cAMP effects are independent of de novo protein synthesis. Rats were injected intraperitonally with cycloheximide (CHX) (5 mg/kg) for 30 min followed by intravenous injection of either Bt2cAMP (20 μmol/kg) or TC (50 μmol/kg) for 1 h. In control experiments, rats received an injection with PBS. Equal amounts of homogenate (HOM, A and B) (50 μg/lane) and CMVs (C and D) (20 μg/lane) were separated by SDS-PAGE, blotted onto nitrocellulose membrane, and probed with LVT90 (SPGP) and C219 (MDR1/MDR2) antibodies. Panels A and C show representative results observed in three independent sets of rats. Panels B and D show densitometric quantitations of Western blots from three independent sets of rats (mean values ± S.D., n = 3). The asterisk indicates values significantly different from control using Student’s t test (p < 0.05).

![Fig. 4.](image)

**FIG. 4.** Half-lives of canalicular proteins. Rats were metabolically labeled by intravenous injection with [35S]methionine (5 mCi) for 1, 3, 5, 8, and 10 days. cCAM105 (A) and MDR1/MDR2 and SPGP (C) were then immunoprecipitated from liver homogenate (HOM) and CMVs. Immunoprecipitates were separated by SDS-PAGE, and radiolabeled bands were detected and quantified by phosphorimaging. Half-lives of the proteins were estimated from semi-logarithmic plots (B and D) of the relative 35S intensity (highest values equal 100) versus labeling time. Assuming first order decay, data points were fitted linearly by the least square method. Determined half-lives are: cCAM105, 5 days; MDR1 and MDR2, 5 days; SPGP, 4 days (CMVs) and 6 days (HOM). The half-lives were established with one set of five rats.
state conditions and after induction of trafficking with Bt2cAMP or TC (Fig. 7). Administration to rats of Bt2cAMP or TC for 1 h significantly increased the amounts of SPGP and MDR1/MDR2 in the canalicular membrane, whereas the amounts in liver homogenate remained constant. These data suggest a redistribution of existing ABC transporters between intracellular pools and the canalicular membrane upon stimulation with Bt2cAMP and TC. Surprisingly, no decrease in the ABC transporter amount could be detected in CEF; the amount of SPGP, MDR1, and MDR2 remained constant after stimulation with Bt2cAMP and TC. These observations can be best explained by a large intrahepatic pool of ABC transporters, only a small portion of which is present in the bile canalicular membrane. In this scenario, a small portion of ABC transporters trafficking from a large endosomal pool to a small bile canalicular pool results in significantly increased amount of ABC transporters in CMVs, whereas any decrease in ABC transporter amount in CEF remained below the detection limit.

**Targeting of Newly Synthesized SPGP to the Bile Canaliculus Is Accelerated by Bt2cAMP But Not by TC**—Newly synthesized SPGP is targeted through intrahepatic sites before reaching the bile canalicular membrane. This was concluded from the 1.5-h lag time between passage through Golgi and arrival at the hepatocyte apical pole (16) and was confirmed by [35S]methionine metabolic pulse-chase labeling of a CEF from rat liver (Fig. 6). The pattern of ABC transporter trafficking after injection of rats with Bt2cAMP or TC also suggests the existence of intrahepatic pools. The two approaches were combined to determine whether intrahepatic SPGP pools, in which newly synthesized SPGP is sequestered before reaching the bile canalicular, is also mobilized by Bt2cAMP and/or TC. Typically [35S]SPGP is first detected in CMVs after 2 h in metabolic labeling studies. Therefore, we determined whether [35S]SPGP can be detected in CMVs after 1 h of labeling after additional administration of Bt2cAMP or TC (Fig. 8).

As demonstrated before, treatment of rats with Bt2cAMP or TC for 30 min significantly increased the steady state amount of SPGP in CMVs. However, accelerated trafficking of newly synthesized SPGP to the bile canalicular membrane after 1 h was observed only when rats were pretreated with Bt2cAMP. Pretreatment with TC did not accelerate Golgi-to-bile canalculus trafficking of newly synthesized SPGP. Altered schedules for TC administration 15 or 45 min after metabolic labeling also did not accelerate [35S]SPGP trafficking to the canalicular membrane (data not shown). Thus, trafficking of newly synthesized SPGP through intrahepatic pools to the bile canalicular membrane was accelerated by cAMP but not by TC.

### DISCUSSION

Previous morphological studies in rats rendered cholestatic by bile duct ligation (33), phalloidin (34), or lipopolysaccharide (35) suggest that MRP2 and SPGP may traffic from the bile canalculus to intracellular sites. In addition, MRP2 (35) and SPGP (9) were observed by immunogold staining and electron microscopy in undefined vesicular structures that were distinct from the bile canalculus.

The present study demonstrates that selective increase in ABC transporters in the canalicular membrane after stimulation with cAMP or TC is independent of protein de novo synthesis, indicating that additional transporters, upon stimulation, are recruited from pre-existing intrahepatic pools. Stimulation by the second messenger, cAMP, and the SPGP substrate, TC, probably mimic naturally occurring processes. The cAMP and TC effects are transient, and the half-lives of those other hepatic plasma membrane proteins, which suggest that ABC transporters cycle between intrahepatic pools and the canalicular membrane. The steady state distribution of
SPGP between intrahepatic pools and canalicular membrane is probably regulated by cAMP and TC depending on the physiological demand to secrete bile.

Previous studies (11) indicate that the increasing effects of Bt2cAMP and TC in bile canalicular ABC transporter amount are additive rather than alternative, which suggests the presence of at least two distinct intrahepatic pools of ABC transporters, one of which is mobilized to the canalicular membrane by Bt2cAMP (cAMP pool) and the other by TC (TC-pool). We here demonstrate that targeting of newly synthesized SPGP through intrahepatic sites to the bile canalicular membrane is accelerated by Bt2cAMP but not by TC. This observation supports the hypothesis of two distinct intrahepatic pools of SPGP. After passage through Golgi, SPGP accumulates in an intrahepatic cAMP pool and later equilibrates with the TC pool. Whether equilibration of newly synthesized SPGP with the TC pool occurs from the bile canalicular membrane or the cAMP pool remains unclear. A tentative model for intrahepatic pool targeting of newly synthesized SPGP is accelerated by cAMP but not by TC. Rats were pulse-labeled for 15 min with [35S]methionine (5 mCi) and then chased with unlabeled methionine for 1 h. At a chase time of 30 min, rats received an intravenous injection of PBS (control), Bt2cAMP (20 μmol/kg), or TC (50 μmol/kg). CMVs were prepared from the rat livers from which SPGP was immunoprecipitated. A, immunoprecipitates (IP) were separated by SDS-PAGE and [35S]SPGP detected by phosphorimaging (a typical result, observed in three independent sets of three rats is shown). B, equal protein amounts (20 μg) of the same CMV preparations were separated by SDS-PAGE, blotted onto nitrocellulose membrane, and probed with anti-SPGP antibody. WB, Western blot. C, Western blots from three independent sets were quantitated with a laser densitometer and compiled; mean values ± S.D., n = 3. The asterisk indicates values significantly different from control using Student's t test (p < 0.05).
most MDR1, MDR2, and SPGP appears to reside in intrahepatic pools rather than in the canalicular membrane. However, the increase in the amount of ABC transporter in CMVs after stimulation with Bt2cAMP or TC is ~3-fold for each effector. Taking into account that Bt2cAMP and TC recruit transporters from different intracellular sources, the intrahepatic pool of ABC transporters is at least 6-times more than the amount present in the bile canalicular membrane. Since this calculation presumes that all intracellular ABC proteins are translocated to the bile canalicular membrane upon stimulation, this number represents a lower limit. Thus, the intrahepatic/canalicular ratio of MDR1, MDR2, and SPGP probably exceeds 6:1.

Upon stimulation with cAMP, trafficking of membrane transporters from intracellular sites to the plasma membrane has been described in several systems, i.e. (i) cystic fibrosis transmembrane regulator (CFTR) channel into the apical surface of rat duodenal villous epithelia (36); (ii) sodium taurocholate cotransport protein (ntcp) in the basolateral membrane of parietal cells (39); (iii) aquaporin-2 water channel into the apical membrane of gastric parietal cells (39); (v) insulin-responsive glucose transporter 4 (40) in the plasma membrane of rat adipocytes (40). In each of these examples, recruitment of transporters to the plasma membrane from a recycling endosome has been suggested. In particular, trafficking of glucose transporter 4 in rat adipocytes has parallels to that of ABC transporter trafficking in rat hepatocytes. Glucose transporter 4 traffics from distinct intracellular sites to the plasma membrane in response to CAMP and insulin (40). In analogy to these other systems, we propose that CAMP recruits ABC transporters to the bile canalicular membrane from a recycling endosome, whereas the effect of TC appears to be hepatocyte-specific and involves a different mechanism.

The present study demonstrates that a substantial portion of canalicular ABC transporters resides in intracellular pools in hepatocytes and that the transporters can be transiently recruited from different intracellular pools to the bile canalicular membrane in response to cAMP and TC. Since ABC transporters are critical for bile formation, the present studies prompt revision of current concepts of bile secretion and raise a question with regard to the mechanism. Do intracellular pools of ABC transporters supply additional transporters to the bile canalicular only to cope with temporarily higher metabolic demand to secrete bile, or are these intracellular ABC transporter pools part of an unidentified more sophisticated bile secretion mechanism? The latter possibility is supported by the finding that intrahepatic pools of MRP2, which also colocalize with SPGP, secrete a MRP2 substrate into intracellular structures (33). A further challenge is the correlation of biochemically observed intrahepatic ABC transporter pools with morphological structures. In this regard, studies in Wif-B cells, a tissue culture model for functionally active, polarized hepatocytes (17, 41, 42), will be a useful tool. Wif-B cells infected with an adenoviral construct containing SPGP tagged with enhanced yellow fluorescent protein showed cycling of SPGP between the canalicular membrane and an intracellular compartment that colocalized with anti-Rab11 (recycling endosomes) antibody immunostaining (43). This project is presently being pursued in our laboratory.

REFERENCES