Transporters on Demand: Intracellular Reservoirs and Cycling of Bile Canaliculal ABC Transporters*

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An important advance in hepatobiliary physiology was the discovery in 1986 that the secretion of major biliary components is ATP-dependent rather than, as had long been accepted, driven by the transeellular electrochemical gradient. Studies using canaliculal membrane vesicles revealed separate transport processes for bile acids, phospholipids, and non-bile acid organic anions (glutathione, glucuronide, and sulfate conjugates) and cations (mainly drugs). Specific transporters were subsequently identified as ABC transporters (3) and included BSEP (ABCB11, bile acids), MDR3 (ABCB4, phospholipid flippase, MDR1 (ABCB, organic cations) (1), MR2 (ABCC2, non-bile acid organic anions), and ABCG5/8 (sterols) (4). Genetic studies in man and transgenic animals revealed recessively inherited hepatobiliary phenotypes resulting from mutations in specific transporter genes; defects in ABCB11 and MDR3 produced familial intrahepatic cholestasis types 2 and 3, respectively; defects in MR2 produced the Dubin Johnson syndrome, and defects in ABCG5 or ABCG8 resulted in sitosterolemia.

Attention was then directed to understanding how the transporters traffic to the canaliculal plasma membrane. Initial trafficking studies with antibodies recognizing canaliculal membrane proteins revealed trafficking to the basolateral plasma membrane followed by transcytosis to the canaliculal domain. It was then proposed that all canaliculal membrane proteins utilized the transcytotic route and that hepatocytes differed from other polarized epithelial cells in lacking a direct vesicular pathway from Golgi to the apical membrane; however, none of these studies examined ABC transporters. Subsequent immunohistochemical analyses revealed restriction of ABC transporters to the bile canaliculus and their absence from the basolateral plasma membrane.

Intracellular Reservoirs of Canaliculal ABC Transporters

Bile acid secretion increases in response to the enterohepatic circulation of bile acids and postprandial secretion of peptide hormones that increase cAMP production in hepatocytes. Treatment of rats with taurocholate or cAMP selectively increased canaliculal ABC transporters. Because these effects occurred within minutes and required an intact microtubular system, intracellular reservoirs of canaliculal ABC transporters were postulated from which additional transporters could rapidly be recruited.

The intracellular distribution and trafficking routes of canaliculal ABC transporters were identified in pulse-chase studies performed in rats (7, 8) (Fig. 1A). In contrast to other canaliculal proteins, newly synthesized ABC transporters were not detected in the basolateral plasma membrane before they reached the bile canaliculus. MDR1 trafficked directly from Golgi to the canaliculal membrane; however, BSEP trafficking required an additional hour after passage through the Golgi before appearing in the canaliculal membrane. In the intermediate time, BSEP chased through a post-Golgi endosomal fraction suggesting that the transporter was sequestered before apical delivery. These observations were consistent with immunoelectron microscopic demonstration of BSEP in both a subapical compartment and the plasma membrane. Administration of cAMP or taurocholate increased the amounts of MDR1, MDR2, MR2, and BSEP in the canaliculal membrane by ~3-fold, which was unaffected by cycloheximide (6, 8).

These studies confirm that additional ABC transporters in the canaliculal membrane result from recruitment from intracellular pools rather than from enhanced transcription or translation. The half-life of MDR1, MDR2, and BSEP is 5 days in rat liver suggesting that ABC transporters cycle between an intracellular vesicular pathway from Golgi to the apical membrane, which co-localizes with BSEP, secrete MR2 substrates into intracellular structures (9).

ABC Transporter Reservoirs and Recruitment Mechanisms

To quantify the distribution of individual canaliculal ABC transporters and determine the mechanism and regulation for
MINIREVIEW: Mechanisms of Helicases

![Diagram showing intracellular trafficking in hepatocytes of newly synthesized BSEP and MDR1 and the endosomal recycling pathway based on studies in vivo.](Image)

A. MDR1 traffics directly from the Golgi to the apical membrane without participation of subcellular compartments. In contrast, BSEP traffics to a post-Golgi mixed endosomal compartment that is enriched in Rab11a and Rab5. Administration of cAMP or taurocholate increased the rate and amount of BSEP and MDR1 transferred from postulated intracellular pools that cycle to and from the apical membrane. Because the effects of cAMP and taurocholate are additive, two distinct mechanisms are postulated. ER, endoplasmic reticulum.

B. Rab5. Post-Golgi compartments containing BSEP and MDR1 and the endosomal recycling pathway based on studies in WIFB cells infected with adenoviral BSEP-YFP (18) and knockdown of HAX-1 RNAi increased BSEP apical membrane content because of apical membrane retention of BSEP without affecting translation, exocytosis, or the half-life of the transporter. HAX-1 interacts with cortactin, an actin-binding protein involved in clathrin-mediated endocytosis (25). Expression of dominant-negative cortactin doubled the steady-state level of BSEP in the apical membrane. Therefore, HAX-1 and cortactin participate in clathrin-mediated endocytosis of BSEP and possibly other ABC transporters from the apical membrane. In contrast, a non-phosphorylatable dominant-negative MLC2 significantly reduced apical delivery of labeled BSEP (26). Thus, MLC2a is required for apical delivery of newly synthesized BSEP and possibly other ABC transporters to the apical membrane and/or TGN release of ABC transporter vesicles (27).

**Rab Proteins**—Different Rab proteins have been associated with different organelles in MDCK and hepatic cell lines. In hepatocytes, Rab11a co-localizes with vesicles containing ABC transporters before their insertion in the canalicular membrane (27), and Rab4 and -5 facilitate endocytosis and vesicle fusion (28). Co-localization of Rab11a and apical proteins and the role of Rab11-interacting proteins in endosomal recycling were initially characterized in MDCK cells (17, 28, 29). The Rab GTPase family has been designated a “master regulator of membrane trafficking” (30). Rab11 acts as a molecular switch in which GTP loading and hydrolysis are coupled to recruitment of membrane tethering and docking factors (36) and activation of vesicle-linked motor activity. In the active GTP-bound form, Rab11a binds to downstream effector proteins that localize to recycling endosomes and influences vesicle sorting (30). Rab proteins selectively bind to cytoskeletal components and protein kinases, thereby facilitating discrete steps in membrane transport.

**cAMP and Phosphatidylinositol 3-Kinase**—Increased hepatocellular cAMP promotes apical surface-directed trafficking of vesicles containing proteins and lipids, suggesting that cAMP controls the relative abundance of canalicular membrane proteins in response to postprandial changes in the enterohepatic transmembrane conductance regulator in duodenal epithelium (22), proton K\(^+\)-ATPase in gastric parietal cells (23), and Na\(^+\)-dependent glucose transporter in Caco-2 cells (24).

**Sorting Pathways for Canalicular Membrane Proteins**

Because many canalicular proteins are delivered to the apical membrane by transcytosis (e.g. polymeric immunoglobulin A receptor, 5'-nucleotidase) whereas others traffic directly from Golgi through a recycling endosome system (e.g. canalicular ABC transporters), sorting of cargo is required. Based primarily on studies in MDCK cells, the trans-Golgi network is considered to be the major sorting site (16, 17); however, sorting site(s) for carriers containing BSEP and other ABC transporters have not been established.

**Protein Components of the Trafficking System**

**Cellular Proteins That Bind ABC Transporters**—Yeast two-hybrid screens identified HAX-1 and MLC2a as potential binding partners for BSEP, MDR1, and MDR2 (25, 26). RNAi-mediated knockdown of HAX-1 RNAi increased BSEP apical membrane content because of apical membrane retention of BSEP without affecting translation, exocytosis, or the half-life of the transporter. HAX-1 interacts with cortactin, an actin-binding protein involved in clathrin-mediated endocytosis (25). Expression of dominant-negative cortactin doubled the steady-state level of BSEP in the apical membrane. Therefore, HAX-1 and cortactin participate in clathrin-mediated endocytosis of BSEP and possibly other ABC transporters from the apical membrane. In contrast, a non-phosphorylatable dominant-negative MLC2 significantly reduced apical delivery of labeled BSEP (26). Thus, MLC2a is required for apical delivery of newly synthesized BSEP and possibly other ABC transporters to the apical membrane and/or TGN release of ABC transporter vesicles (Fig. 2).

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circulation and peptide hormone secretion (31, 32). In pulse-chase studies in rats, cAMP administration did not affect the rate of transfer of BSEP to the canalicular membrane consistent with exchange with a large intracellular transporter pool (8). In HepG2 cells, inhibition of cAMP-induced dihydroceramide synthesis resulted in intracellular sphingosine accumulation, altered recycling endosome dynamics, and impaired bile canalicular formation (33).

cAMP activates PI 3-kinase in hepatocytes and other cells by different pathways (32). PI 3-kinase inhibition reduced bile secretion in perfused liver and ATP-dependent bile acid transport in canalicular membrane vesicles but had no effect on cAMP-stimulated trafficking of ABC transporters (44, 45). The latter effect was reversed by 3-phosphoinositides but not PI 4,5-trisphosphate (35), suggesting that PI 3-kinase association with the canalicular membrane and 3-phosphoinositide formation are required for BSEP localization and activity. Neither the mechanism nor the specificity of this regulation are known. A decaphptide mimic of the gelsolin binding site on PI biphosphosphate activates PI 3-kinase activity and bile acid transport in rat cytomegalovirus and WIFB cells and increased bile secretion in perfused rat liver (11, 35). These observations suggest that 3-phosphoinositides regulate canalicular bile acid secretion. In addition, PI trisphosphate regulates endocytosis of apical resident proteins (36).

The taurocholate-mediated intracellular pool of ABC transporters is also large (6); however, neither its subcellular site nor regulation have been defined. In contrast to the effect of cAMP, taurocholate-stimulated bile acid secretion and recruitment of canalicular ABC transporters were unaffected by inhibition of PI 3-kinase activity (34). Whereas the cAMP-mediated cycling event is similar to that present for other ligands in other cells, the taurocholate pool appears unique to liver.

Cytoskeleton: Microtubules—In WIFB cells, live cell imaging of BSEP-YFP revealed microtubular-dependent oscillatory movement of cargo-containing tubular vesicles along microtubules from the MTOC throughout the cell; however, attachment and fusion occurred only in the canalicular domain (13). In WIFB and MDCK cells, apical trafficking of cargo by transcytotic and direct routes was inhibited by microtubular disruption (12, 19). The source of the tubular carriers is the TGN from which a steady stream of proteins and lipids bud because of the action of dynamin. Cargo proteins transported along these tubules to basolateral or apical plasma membranes have transmembrane domains that mainly partition into lipid regions enriched in sphingolipids and cholesterol (“rafts”) (37). Kinesins move the tubules along microtubules. Delivery of canalicular membrane proteins requires an intact microtubule structure that is affected by PAR-1, a kinase for microtubular-associated proteins (38). Inhibition of PAR-1 prevents canalicular formation in WIFB cells (38).

Cytoskeleton: Actin—In hepatocytes and WIFB cells, the actin cytoskeleton is distributed in a ring around the bile canaliculus at the adherens cell junction and also in microvilli (2, 7). How actin participates in BSEP apical trafficking, cycling, and endocytosis is unclear; however, several observations support a role for actin. (i) Rab GTPases interact with the actin cytoskeleton as determined by co-localization studies using GFP Rab11a constructs (19). (ii) Cytochalasin and latrunculin A inhibit bile acid- and cAMP-mediated increases in bile acid secretion in vivo (39) and in WIFB cells (11). (iii) An actin-based complex participates in clathrin-mediated endocytosis of BSEP from the canalicular domain in MDCK cells (25). (iv) In WIFB cells expressing BSEP-YFP, photobleaching of all but the canalicular region resulted in rapid decline of BSEP-YFP fluorescence; cytochalasin D or latrunculin A prevented endocytosis indicating that impaired actin polymerization inhibits cycling of canalicular BSEP-YFP (19). (v) In MDCK cells, blebbistatin A, which inhibits MLC kinase, reduced BSEP trafficking to the canalicular membrane (26). In other experiments, latrunculin A modulated basolateral endosomal cycling (40). (vi) The most direct studies implicating actin in targeting or maintaining apical ABC proteins involve mice in which radixin, the dominant ezrin-radixin-moesin protein in liver, was eliminated by targeted mutation (41). The ezrin-radixin-moesin family of proteins cross-links actin filaments and integral membrane proteins. Removal of radixin, which is concentrated in the bile canalicular domain, resulted in progressive dilation of the canaliculus, decreased microvilli, jaundice because of impaired apical trafficking of MRP2, and disappearance of other canalicular ABC transporters.

Convergence of Trafficking Pathways

Immunoelectron microscopy of rat liver organelles revealed that the plga receptor, a transcytosis marker, and ABC transporters occurred in the same vesicles. In experimental choleresis in rat liver and WIFB cells, transcytotic and ABC transporters partially co-localized in intracellular organelles (42). In the steady-state transcytosis, markers and ABC transporters localized to Rab11a-positive endosomes in WIFB cells (18). The plga receptor is a GPI-linked protein that is endocytosed at the basolateral membrane and moves to the early endosome. Before trafficking to the canalicular domain, plga resides in a morphologically and kinetically distinct subapical compartment (43,
cells revealed fusion of post-Golgi transport intermediates directly with the basolateral-Golgi transport intermediates; few molecules enter the degradation pathway at the apical membrane, diffusion is rapid and distribution is limited by tight junctions. Microtubules do not attach directly to the apical membrane but interact with actin microfilaments along which cargo is delivered to or from the apical membrane.

Apical Endocytosis of ABC Transporters

Inaccessibility of the bile canaliculus to micro-sampling restricts studies of apical retrieval mechanisms. The apical surface area would double every 15–20 min if not compensated (37, 47). Loss of canalicular membrane because of the detergent effect of bile acids contributes to apical membrane turnover; however, the major retrieval mechanism is endocytosis and delivery of membrane components to lysosomes and proteasomes. In support of this view, clathrin-coated profiles, SNAREs, Rab, and annexins are located in the apical domain (47). Treatment of hepatocytes with PI 3-kinase inhibitors resulted in accumulation of apical plasma membrane proteins in lysosomes (47). In wortmannin-treated cells, transcytosing apical proteins traversed the subapical compartment, whereas apically internalized proteins did not (47). Whether the endogenous recycling process in rat liver and WIFB cells for canalicular ABC transporters is entirely clathrin-dependent has not been tested; however, caveolae do not participate (43).

Role of ABC Transporter Trafficking in Polarization

Sorting and trafficking pathways may be reprogrammed during differentiation and polarization (48). In non-polarized WIFB cells, GPI-linked and single transmembrane domain proteins traffic from Golgi to the entire plasma membrane, whereas canalicular ABC transporters remain in a perinuclear region associated with the MTOC (19, 41). Upon polarization, the apical ABC transporters traffic only to the canalicular domain and transcytotic proteins traffic to the basolateral domain from which they cross to the canalicular membrane. These studies further indicate that trafficking patterns are multiple and relate to differentiation and polarization.

Bile canalicular formation (apical polarization) is essential for biliary secretion and is disrupted in cholestatic disorders. Many components participate in apical polarization and targeting of canalicular membrane components, including ABC transporters; however, their specific interactions are not well defined. In WIFB cells, Rab11a and myosin Vb participate in apical targeting of recycling endosomes. Stable knockdown of Rab11a expression by RNAi or overexpression of a Rab11a-GDP-locked mutant prevented polarization (19). Overexpression of the tail domain of myosin Vb, which competes with full-length myosin Vb for binding to Rab11a, also prevented bile canalicular formation. The absence of Rab11a or myosin

Fusion of Post-Golgi Transport Intermediates with Apical Membrane

Fusion of transport vesicles with the plasma membrane releases cargo, and membrane lipids and proteins redistribute laterally by diffusion within the plasma membrane. Live cell imaging of BSEP-YFP in WIFB cells revealed that intracellular carriers elongated and dissociated as tubular elements from a globular structure adjacent to the MTOC (19). The tubular elements displayed oscillatory movement toward the entire plasma membrane but only fused with the canalicular membrane. Specificity in fusion could be because of specific motor proteins that transport BSEP-containing vesicles to their appropriate membrane domain or to downstream selectivity in membrane fusion. It is likely that attachment to and insertion into the canalicular plasma membrane require downstream signals that are not present in the basolateral plasma membrane. Downstream site(s) for attachment and fusion of cargo-containing vesicles to the canalicular membrane have not been identified; however, specific SNAREs may participate (45). Different forms and subcellular localization of v- and t-SNAREs involved in different membrane traffic pathways confer specificity. For example, trafficking of cargo from the TGN to the apical membrane requires SNAP-23 and syntaxin-3, whereas basolateral to apical transcytosis requires SNAP-23 and NSF. In WIFB cells and hepatocytes, syntaxin-3 is restricted to the apical region.

Total internal reflection microscopy of polarized MDCK...
Vb caused ABC transporters to remain intracellularly co-localized with transcytotic membrane proteins that were also transported to the plasma membrane. These results suggest that myosin Vb, which binds Rab11a and actin, is required for trafficking endosomes that contain apical ABC transporters to sites that initiate polarization. Myosin Vb may serve as a motor for delivery of endosomal cargo through the actin network in WIFB cells.

Several mechanisms may account for the role of Rab11a and myosin Vb in canalicular biogenesis. In polarized mammalian cells, Rab11a participates in targeting apical proteins to the apical surface (28). In Drosophila, Rab11a participates in morphogenesis (48). Both Rab11a and myosin Vb may be required for directing critical junctional, cytoskeletal, targeting, and other components to the plasma membrane. In their absence, proteins that normally traffic to the canalicular membrane by the transcytosis route are primarily directed to the plasma membrane, whereas ABC transporters that normally traffic by the direct route remain intracellular, and normal turnover of canalicular membrane proteins and lipids results in loss of polarity and inability to polarize. Rab11a- and myosin Vb-positive endosomes constitute a reservoir for proteins that, in concert with unidentified polarization cues, are required for formation and trafficking of ABC transporters (and perhaps transcytotic membrane proteins) to the canalicular membrane. These endosomes may contain or be linked to specific targeting molecules, such as SNAREs, which facilitate membrane targeting and fusion.

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