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TOPIC HIGHLIGHT

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Dynamic localization of hepatocellular transporters in health and disease

Marcelo G Roma, Fernando A Crocenzi, Aldo D Mottino

Marcelo G Roma, Fernando A Crocenzi, Aldo D Mottino, Instituto de Fisiología Experimental (IFISE)-Facultad de Ciencias Bioquímicasy Farmacéuticas (CONICET-U.N.R.), S2002LRL, Rosario, Argentina
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Correspondence to: Dr. Marcelo G Roma, Instituto de Fisiología Experimental (IFISE), Facultad de Ciencias Bioquímicas y Farmacéuticas, Suipacha 570, S2002LRL, Rosario, Argentina. mroma@fbioyf.unr.edu.ar
Telephone: +54-341-4305799 Fax: +54-341-4399473
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Abstract

Vesicle-based trafficking of hepatocellular transporters involves delivery of the newly-synthesized carriers from the rough endoplasmic reticulum to either the plasma membrane domain or to an endosomal, submembrane compartment, followed by exocytic targeting to the plasma membrane. Once delivered to the plasma membrane, the transporters usually undergo recycling between the plasma membrane and the endosomal compartment, which usually serves as a reservoir of pre-existing transporters available on demand. The balance between exocytic targeting and endocytic internalization from/to this recycling compartment is therefore a chief determinant of the overall capability of the liver epithelium to secrete bile and to detoxify endo and xenobiotics. Hence, it is a highly regulated process. Impaired regulation of this balance may lead to abnormal localization of these transporters, which results in bile secretory failure due to endocytic internalization of key transporters involved in bile formation. This occurs in several experimental models of hepatocellular cholestasis, and in most human cholestatic liver diseases. This review describes the molecular bases involved in the biology of the dynamic localization of hepatocellular transporters and its regulation, with a focus on the involvement of signaling pathways in this process. Their alterations in different experimental models of cholestasis and in human cholestatic liver disease are reviewed. In addition, the causes explaining the pathological condition (e.g. disorganization of actin or actin-transporter linkers) and the mediators involved (e.g. activation of cholestatic signaling transduction pathways) are also discussed. Finally, several experimental therapeutic approaches based upon the administration of compounds known to stimulate exocytic insertion of canalicular transporters (e.g. cAMP, tauroursodeoxycholate) are described.

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Key words: Hepatocellular transporters; Cholestasis; cAMP; Bile salts; Vesicular trafficking; Endocytosis; Signaling pathways

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INTRODUCTION

Bile secretion is a highly-regulated process. Such regulation is aimed at coping with the physiological demand for hepatocellular transport of endo- and xenobiotics. This is achieved by modulation of the constitutive expression, dynamic localization or intrinsic activity of relevant transport systems located at the sinusoidal (basolateral) and canalicular (apical) membranes of the hepatocyte.

Modulation of carrier transport activity may occur at different time scales. Long-term regulations occur by changes in carrier turnover, which leads to modification of the synthesis-degradation balance. Altered synthesis rate involves transcriptional or translational changes in carrier expression. On the other hand, modification of the carrier degradation rate is a post-translational

process. This latter event may involve, as an initiating step, sustained internalization of the carrier protein from its plasma membrane domain, followed by lysosomal breakdown.

In contrast to this irreversible fate, transitory, reversible changes in transporter localization by vesicle-mediated insertion/internalization from/to an endosomal recycling compartment may occur as part of a short-term, physiological mechanism aimed at quickly modulating carrier density at the plasma membrane. This is a tightly regulated process, and the signaling mediators involved are being actively characterized.

Apart from its role in biliary physiology, changes in the proper localization of hepatocellular carriers also occur in a number of pathological conditions, and they may partly explain the cholestatic manifestations in these liver diseases. This has encouraged investigators to better understand the mechanisms involved in this particular pathomechanism at a molecular level, and to envisage and test in experimental models of cholestasis new therapeutic approaches based upon its prevention.

This article aims to give an overview of this subject, by summarizing the current information available in the literature on physiological regulation and cholestatic changes in hepatocellular carrier dynamic localization, as well as its beneficial modulation by therapeutic agents.

HEPATOCELLULAR TRANSPORT SYSTEMS

The hepatocyte is a polarized cell that expresses differential transport systems in its plasma membrane domains. These transporters play a key role in the vectorial transfer of solutes and water from sinusoidal blood into bile, thus contributing to bile formation and the biliary excretion of many xenobiotics. Most of these transport proteins have been identified by molecular cloning, and their transport properties characterized by functional studies. Their localization and transport function are shown in Figure 1.

Sinusoidal solute uptake transporters

Liver sinusoids possess a specific architecture that allows passage of organic compounds bound to albumin through endothelial fenestrae into the space of Disse, from where they can be taken up by the sinusoidal transport systems of the hepatocytes[1].

Basolateral uptake transporters can be divided into Na^+-dependent and Na^+-independent systems. Na^+-dependent uptake involves co-transport of solutes with Na^+, and is driven by the electrochemical Na^+ gradient generated and maintained by the Na^+/K^+-ATPase, which is strategically localized at the sinusoidal membrane. The Na^+-independent transport of organic anions is driven primarily by anion exchange.

Bile salts are the predominant organic solutes in bile, and the main determinants of bile flow[2]. Bile salts are mainly taken up by the Na^+/taurocholate co-transporting polypeptide (NTCP/Ntcp for humans and rodents, respectively; also known as SLC10A1/Slc10a1)[3]. A remaining fraction is taken up by a Na^+-independent transport system mediated by the organic anion-transporting polypeptide (OATP/Oatp) family of transporters[4,5]. In addition to conjugated and unconjugated bile salts, Oatps/OATPs accept other cholephilic compounds, including glucuronidated (and maybe unconjugated) bilirubin, exogenous organic anions (e.g. sulphobromophthalein), leukotrienes, estrogen-conjugates (e.g. estrone-3-sulfate or estradiol-17-β-d-glucuronide), thyroid hormones, mycotoxins, and numerous xenobiotics[3,6-8]. Four OATPs have been cloned and characterized from human liver, namely: OATP1A2 (SLCO1A2/SLC21A3; formerly, OATP-A), OATP1B1 (SLC21A6; formerly, OATP-C or LST-1), OATP1B3 (SLC21A8; formerly, OATP-8) and OATP2B1 (SLC21A9; formerly, OATP-B). There are three Oatps identified in rats, namely: Oatp1a1 (*Slc21a1*; formerly, Oatp1), Oatp1a4 (*Slc21a5*; formerly, Oatp2) and Oatp1b2 (*Slc21a10*; formerly, Oatp4 or Lst-1). Oatp1b2 is the rodent ortholog of both OATP1B1 and OATP1B3[9].

Hepatocellular uptake of organic cations is mediated by two separate transport systems, which depends on the substrate molecular size[10]. Thus, small (type Ⅰ) organic cations are taken up by the organic cation transporter, OCT1/Oct1 (SLC22A1/Slc22a1), which is electrogenic in nature. On the other hand, human OATP-A (but not the remaining members of the OATP family) and rat Oatp2 mediate the uptake of bulky (type Ⅱ) organic cations.

Canalicular solute export transporters

After traversing the cell by Fick's diffusion, mostly bound to high-affinity cytosolic proteins, cholephilic compounds are excreted into bile mainly by ATP-dependent pumps of the superfamily of ATP-binding cassette (ABC) transporters, in particular those belonging to the family of multidrug-resistance proteins, MDR/Mdr, or to the family of multidrug-resistance-associated proteins, MRP/Mrp.

MDRs/Mdrs are members of the ABC superfamily that were originally described in cancer cell lines, where they confer resistance to therapeutic agents. Three gene products were identified in rodents, Mdr1a (Abcb1a), Mdr1b (Abcb1b) and Mdr2 (Abcb4), and two in humans, MDR1 (ABCB1) and MDR 3 (ABCB4). MDR1/Mdr1 functions as an efflux pump for a wide range of amphiphilic, bulky type Ⅱ cationic drugs, together with other hydrophobic compounds, such as endogenous and exogenous metabolites or toxins, steroid hormones, hydrophobic peptides and even glycolipids[8]. Two closely related but functionally distinct Mdr1 isoforms, mdr1a and mdr1b are present in the murine but not in the human phenotype[11]. MDR3/Mdr2 functions as a flippase, which translocates phosphatidylcholine (PC) from the inner to the outer leaflet of the canalicular membrane, followed by release of PC-containing vesicles from the outer leaflet into bile, a process facilitated by the detergent properties of luminal bile salts[12].

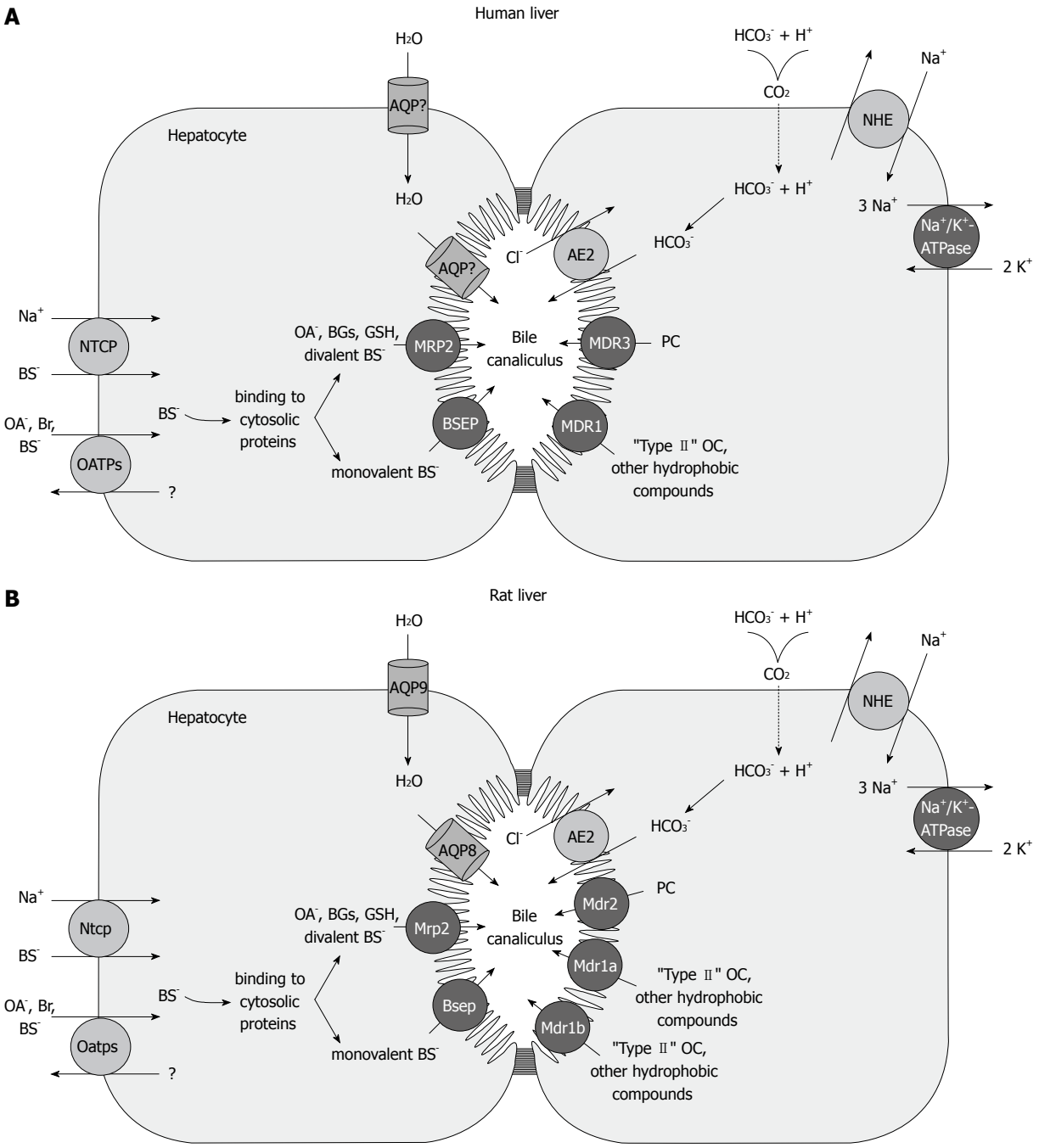

Figure 1 Localization and function of sinusoidal and canalicular hepatocellular transporters. A: humans; B: rodents. The Na^+-dependent sinusoidal uptake of bile salts is mediated by NTCP (human)/Ntcp (rat). The Na^+-independent hepatic uptake of organic anions (OA^-), Bile salts and type Ⅱ organic cations (OC^+) is mediated by members of the OATP/Oatp family. Sinusoidal uptake of type Ⅰ OC^+ is mediated by OCT1/Oct1. Transport across the canalicular membrane is driven mainly by ATP-dependent export pumps (black circles). MDR1/Mdr1a, Mdr1b mediates canalicular excretion of amphiphilic type Ⅱ OC^+ and other hydrophobic compounds. MDR3/Mdr2 functions as a phosphatidylcholine (PC) flippase. BSEP/Bsep mediates apical excretion of BSs. MRP2/Mrp2 transports non-bile-salt organic anions, such as bilirubin glucuronides, GSH, and sulfated/glucuronidated bile salts. Canalicular transport of HCO_3^- is mediated by the Cl^-/HCO_3^- exchanger AE2/Ae2. Aquaporins AQP9 and AQP8 are involved in the transport of water across the rat sinusoidal and the canalicular membrane, respectively. The nature of the water channels in human liver has yet to be characterized.

Monoanionic bile salts are excreted in the canalicular pole by the *bile salt export pump* (BSEP/Bsep; ABCB11/abcb11), another member of the MDR family[13]. In contrast, canalicular efflux of divalent, bipolar sulfated or glucuronidated bile salts is mediated by the multidrug-resistance-associated protein 2 (MRP2/Mrp2; ABCC2/Abcc2)[4,14]. This carrier is also engaged in the biliary excretion of many other organic anions, including glutathione S-conjugates (e.g. of leukotriene C4 or sulphobromophthalein, among others), glucuronides (e.g. of bilirubin and estrogens), and reduced (GSH) and oxidized glutathione (GSSG), the former with low affinity[15,16]. Both GSSG and GSH are major determinants of the so-called "canalicular bile-salt-independent bile flow"[17].

The canalicular membrane domain also contains the electroneutral anion exchanger 2 (AE2/Ae2; SLC4A2/slc4a2), which extrudes HCO_3^- by exchanging the anion

for biliary Cl^-[18]. It functions to regulate intracellular pH when hepatocytes are exposed to an alkaline load[18]. In addition, AE2/Ae2 plays a role in bile flow generation, since HCO_3^- excretion is thought to be an additional primary driving force of the canalicular bile-salt-independent bile flow[18,19]. Both in humans and rats, three transcript variants of AE2/Ae2 have been described, namely the full-length transcript AE2a/Ae2a, expressed from the upstream promoter in most tissues, and the alternative transcripts AE2b1/Ae2b1 and AE2b2/Ae2b2, expressed in a more tissue-restricted fashion (mainly in liver and kidney). AE2b1/2/Ae2b1/2 transcription is driven from overlapping promoter sequences within intron 2, which result in AE2/Ae2 protein isoforms with short N-terminal differences[20,21].

Water transporters

For a solute to drive blood-to-bile vectorial water transport primarily, resultant osmotic forces need to be associated with aquaporin (AQP)-mediated transcellular movement of water molecules from plasma to the bile canaliculus. Both immunochemical and functional studies have demonstrated the constitutive expression of the water channel AQP9 at the basolateral membrane of rat hepatocytes, and the regulated expression of the water channel AQP8 at the hepatocellular canalicular membrane domain[22-24]. As a result of it being inserted in the canalicular membrane on demand, AQP8 is suggested to play a role in bile formation, facilitating the osmotic movement of water under a choleretic stimulus[23,24]. AQP isoforms that mediate polarized water transport in human hepatocytes, if any, remain to be identified.

MECHANISMS OF NORMAL TRAFFICKING OF HEPATOCELLULAR TRANSPORTERS AND ITS REGULATION BY SIGNALING PATHWAYS

Basolateral transporters

NTCP/Ntcp: Basolateral targeting of NTCP is mediated by a sorting pathway that involves translocation of the protein from the endoplasmic reticulum (ER) to the Golgi apparatus, and from there to the plasma membrane, by a trans-Golgi-network-independent pathway[25]. The process may also involve microtubular and microfilamental motor proteins. A role for the cytoskeleton in NTCP translocation has been studied in detail using green fluorescent protein (GFP)-tagged NTCP expressed in the HepG2 cell line[26]. This study showed that targeting of NTCP to the plasma membrane consists of two steps: (1) delivery of NTCP to the region of the plasma membrane *via* microtubules, and (2) insertion of NTCP into the plasma membrane, by a microfilament-mediated mechanism; this actin requirement was also observed in isolated rat hepatocytes[27]. The latter step more likely involves targeting of NTCP from an early (recycling) endosomal compartment[28]. These NTCP/Ntcp-containing vesicles also express the microtubule-based motor proteins dynein and kinesin, and the actin-based motor myosin Ⅱa[28]. This compartment may serve as a reservoir of transporters for their rapid insertion into the sinusoidal membrane under a physiological stimulus that requires their function. It is therefore not surprising that recycling of NTCP/Ntcp from this compartment is a highly regulated process.

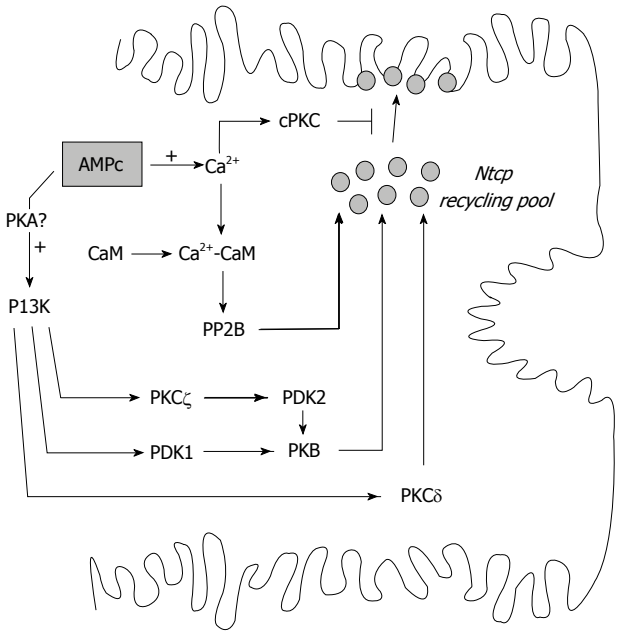

Figure 2 Signaling pathways that regulate the cAMP-induced exocytic insertion of Ntcp into the basolateral membrane. cAMP stimulatory effect involves elevations in cytosolic Ca^{2+} and activation of PI3K-dependent pathway, probably *via* protein kinase A (PKA). CaM complex activates phosphatase 2B (PP2B), which promotes insertion of Ntcp by dephosphorylation. This pathway is counter-regulated by cPKC. cAMP also stimulates Ntcp targeting by PI3K-dependent activation of PDK1 and subsequent PKB activation. Alternatively, PKB is activated by the concerted action of the atypical PKCζ and PDK2. Finally, cAMP/PI3K signaling stimulatory pathway may involve PKCδ.

The cAMP-elevating hormone glucagon and the permeant cAMP analog dibutyryl cAMP stimulate hepatocyte Ntcp maximal transport in rats by insertional exocytosis from intracellular vesicles that contain the transporter[29]. The signaling pathways evoked by cAMP that account for this stimulatory effect are depicted in Figure 2. Protein kinase A (PKA) activation[30], phosphatidylinositol 3-kinase (PI3K) activation[27,31] and elevations of cytosolic Ca^{2+}[30] all mediate the cAMP effect. Although the mechanism of PI3K activation by cAMP has not been elucidated as yet, there is evidence in other cell lines that the cAMP-dependent PKA can activate PI3K by phosphorylation of the PI3K regulatory subunit, p85[32]; if this applies to hepatocytes, this would explain the dual mediation of PKA and PI3K in the cAMP-stimulatory effect. The downstream mediators of the cAMP-PI3K signaling pathway are under debate, and may be multifactorial. The PI3K downstream enzyme, protein kinase B (PKB, also known as Akt), has been implicated[27,31]. Coincidently, hepatocellular swelling, which also evokes the PI3K/

PKB signaling pathway, favors Ntcp translocation to the plasma membrane as well[31,33]. The effect of PI3K/PKB on Ntcp translocation seems to be mediated by the PI3K-dependent activation of atypical protein kinase C zeta (PKCζ)[34]. PKCζ is downstream of PI3K, since PI3K products activate this PKC isoform[35,36]. The requirement of PKCζ for the PKB effect can be explained by PKCζ modulation of activators upstream of PKB. Activation of PKB requires phosphorylation by 3-phosphoinositide phosphate-dependent kinase 1 (PDK1), followed by phosphorylation by a second kinase, PDK2; this latter kinase phosphorylates and activates PKB fully only when associated with PKCζ[36,37]. In addition, a direct, non-PKB-mediated stimulatory role for PKCζ on Ntcp translocation has been suggested[34]. Apart from PKCζ, cAMP-stimulated PI3K phosphorylates the novel protein kinase C delta (PKCδ) at Thr-505, and the resulting activation seems to be involved in Ntcp membrane translocation as well[38]. The molecular target/s phosphorylated by PKB, PKCζ and PKCδ that ultimately account for the translocation of Ntcp are unknown. Ntcp itself seems not to be a target, since cAMP may promote dephosphorylation rather than phosphorylation of the carrier[39-41]. However, studies in transfected COS-7 and Madin-Darby canine kidney (MDCK) cells using GFP-fused Ntcp constructs that lack the cytoplasmic Ntcp tail, which serves as a signal for basolateral sorting, have demonstrated that this moiety has regulatory phosphorylation sites that are essential for cAMP-induced stimulation of Ntcp translocation[42]. The relevance of this finding needs to be tested in a more physiological context. Other possible phosphorylation targets, at least of PKCζ, are the microtubule motors that drive movement of Ntcp-containing vesicles. A majority (75%) of intracellular vesicles containing Ntcp were found to co-localize with PKCζ in rat hepatocytes, and the motility of these vesicles on microtubules, when assessed using an *in vitro* motility assay, was impaired by both PI3K and PKCζ inhibitors, and stimulated by PI3K products[28].

Apart from activating PKA and PI3K, cAMP induces elevations of cytosolic Ca^{2+} in hepatocytes[43,44]. The subsequent formation of the Ca^{2+}-calmodulin (CaM) complex influences Ntcp localization by activating the Ca^{2+}/CaM-dependent serine–threonine phosphatase PP2B (also known as calcineurin)[39]. cAMP promotes both serine and threonine dephosphorylation of Ntcp *via* PP2B[39-41], and dephosphorylated Ntcp is located preferentially in the plasma membrane[45]. Phosphorylated Ser-226 in the third cytoplasmic loop of Ntcp may be the target for cAMP-stimulated dephosphorylation[45]. This cAMP-dependent, Ca^{2+}-mediated pathway may be counter-regulated by activation of "classical" (Ca^{2+}-dependent) PKC (cPKC), since pan-specific activation of PKC with phorbol esters counteracts the cAMP-stimulatory effect[30].

OATP/Oatp: Unlike Ntcp, this family of transporters is not stored in intracellular vesicular compartments, and therefore regulation by trafficking is limited to modulation of its transfer from synthesis sites. Sorting of human OATP-C to the basolateral membrane is mediated by both the Golgi complex- and the vacuolar H^+-ATPase vesicle-mediated membrane sorting pathways, and cAMP positively regulates the first sorting mechanism *via* activation of PKA[46].

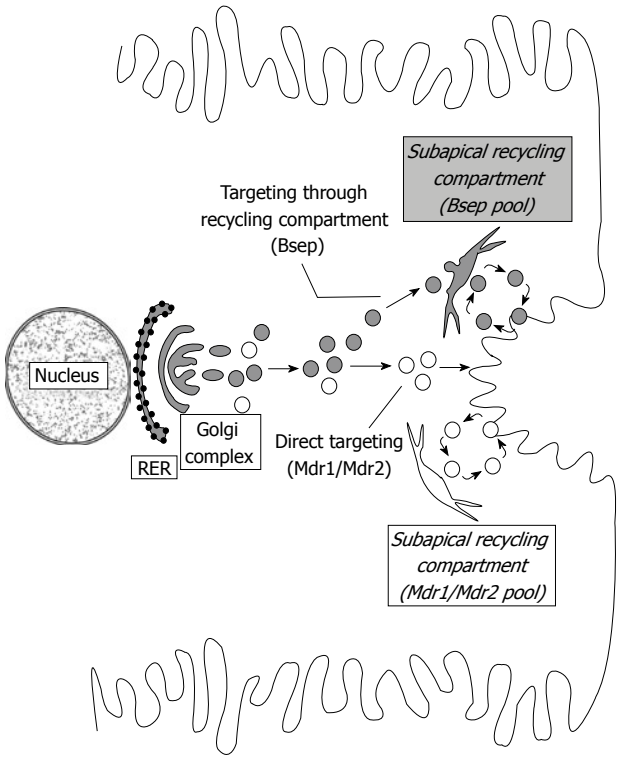

Figure 3 Routes involved in trafficking of canalicular transporters. The trafficking of vesicles delivering Bsep (gray vesicles) or Mdr1/Mdr2 (white vesicles) from the site of synthesis to the canalicular domain is distinct. Mdr1 and Mdr2 are directly targeted to the canalicular membrane, whereas Bsep is indirectly targeted *via* a subapical, endosomal compartment, which allows the recycling of transporters (exocytic insertion/endocytic internalization). Once targeted, Mdr1 and Mdr2 are also able to recycle between the subapical compartment and the canalicular membrane.

Canalicular transporters

ABC canalicular transporters: Vesicle-based trafficking steps of canalicular export pumps are depicted in Figure 3. Once synthesized by the rough ER, *de novo* ABC canalicular transporters belonging to either the MRP or the MDR family traffic *via* the Golgi complex directly to the apical membrane[47-49]. Pulse-chase studies using ^{35}S-methionine followed by immunoprecipitation of the ABC transporters from subcellular fractions have revealed that these transporters are targeted directly to the canalicular membrane, as at no time between passage through Golgi and arrival at the canalicular membrane are the ABC transporters localized at the sinusoidal membrane[49]. However, the post-Golgi trafficking differs among the ABC transporters studied. Mdr1 and Mdr2 are fully delivered to the canalicular membrane 30 min after ^{35}S-methionine administration[49]. This finding was confirmed for Mdr1 in WIF-B cells, a hybrid of rat hepatoma cells and human fibroblasts that has functional bile canaliculi[50]. Contrarily, Bsep only reaches

the canalicular membrane after 2 h, which suggests that, unlike Mdr1/2, Bsep is retained in an intracellular endosomal pool prior to delivery to the canalicular membrane[48]. This intrahepatic, large vesicular pool also serves as a reservoir of ABC transporters, which can be quickly recruited to the canalicular membrane on physiological demand that requires their function (e.g. increased biliary excretion of bile salts for lipid digestion/absorption during the post-prandial period). The recycling process involves exocytic insertion, followed by endocytic internalization once demand is satisfied[47,48].

Compelling evidence in the literature further supports the existence of this recycling compartment for canalicular hepatocellular transporters. Immunogold electron microscopy studies of rat hepatocytes have revealed that distribution of Bsep is not restricted to the canalicular membrane, but is also detected in electron-translucent vacuolar structures close to the apical, but not the basolateral membrane[51]. Pericanalicular localization of Mrp2, Bsep and Mdr1 has also been demonstrated by immunofluorescent staining in isolated rat hepatocyte couplets[52]. Finally, direct visualization of the recycling between the canalicular membrane and subapical endosomes has been observed for Bsep-GDP chimeras in WIF-B cells stably transfected with adenoviral Bsep-GFP constructs[53]. Chimeric Bsep co-localizes with the marker of recycling endosomes Rab11, and its recycling was microtubule- and microfilament-dependent in both ways[53]. On the contrary, and unlike the *de novo* transporter pathway, this recycling does not involve the Golgi complex, since it is unaffected by brefeldin A. This suggests that recycling represents an independent step in the whole trafficking of *de novo* ABC transporters to the canalicular membrane, and that only replenishment of this recycling compartment with newly-synthesized transporters is Golgi-dependent.

This large-range, Golgi-dependent vesicular trafficking of ABC transporters has been characterized by our group and others using the couplet model. Sorting of Mrp2 to the apical membrane has been analyzed by studying the spontaneous retargeting of the transporter after Mrp2 internalization that occurs during the isolation process[54,55]; this vesicle-based trafficking shares the route of newly-synthesized, apically-directed proteins, since it is sensitive to disruption of the Golgi complex function with brefeldin A[55]. Inhibitors of microtubule polymerization diminish, but do not completely block, the restoration of Mrp2 localization[54,55]. Re-establishment of hepatocyte couplet secretory polarity is instead strikingly dependent on microfilament organization[55]. A similar differential cytoskeletal dependency has been suggested to occur for Bsep, as inferred by functional studies upon restoration of the hepatocyte couplet capability to secrete apically the Bsep substrate, cholyl-lysylfluorescein (CLF), and also for Ca^{2+}/Mg^{2+}-ATPase, another canalicular transporter[56]. The vesicle motor protein myosin-Ⅱ may be crucially involved in the actin-dependent targeting of Bsep. Co-immunoprecipitation studies have identified myosin-Ⅱ regulatory light chain as a binding partner of BSEP, and reduced expression of this protein in dominant negative mutant MDCK cells reduces apical membrane BSEP levels[57]. Furthermore, pharmacological inhibition of myosin Ⅱ impedes delivery of newly synthesized transporter to the apical membrane in these cells[57]. These findings suggest that myosin-Ⅱ is required for BSEP trafficking to the apical membrane in polarized epithelial cells.

Trafficking of ABC transporters from their place of synthesis to the canalicular membrane is under signaling modulation. Studies using the re-polarization approach in hepatocyte couplets described above have shown that the spontaneous canalicular targeting of Mrp2 after isolation and culture is Ca^{2+}- but not PKA-dependent[55]. The Ca^{2+}-elevating compound thapsigargin (an inhibitor of the ER Ca^{2+}-ATPase) accelerates, whereas the intracellular Ca^{2+} chelator BAPTA/AM and the CaM inhibitor W7 greatly inhibit this process, which suggests Ca^{2+}-CaM dependency. On the other hand, the PKC-dependent signaling pathway is inhibitory in nature, since the PKC activator phorbol 12,13-dibutyrate inhibits this process, whereas both the pan-specific PKC inhibitor staurosporine and the specific inhibitor cPKC Gö6976 accelerate this process. This indicates that, under basal conditions, cPKC exerts an inhibitory effect on long-range trafficking of ABC transporters to the canalicular pole and that the stimulation induced by Ca^{2+} elevations may generate its own counter-regulatory mechanism, by activating cPKC. In this connection, selective activation of cPKC by administration of thymeleatoxin is associated with retrieval of Bsep and loss of bile salt secretory function in isolated rat perfused liver[58].

Both Roelofsen *et al*[54] and our group[55] have analyzed the influence of cAMP on the time-dependent re-targeting of Mrp2 after isolation-induced Mrp2 internalization. cAMP stimulates this process. This phenomenon is partially inhibited by inhibitors of microtubule polymerization. We have further examined this phenomenon by analyzing the involvement of signaling molecules downstream of cAMP, the cross talk with other signaling pathways, and the dependency of cAMP stimulus on cytoskeleton organization[55] (Figure 4). The cAMP-sensitive stimulatory pathway shares most downstream signaling constituents with the basal, spontaneous pathway described above, i.e. it is not PKA-dependent, but Ca^{2+}-dependent, *via* Ca^{2+}-CaM complex formation. This cAMP-dependent pathway is also counter-regulated by activation of cPKC[55]. Interestingly, a similar counter-regulatory cross-talk between cAMP- and PKC-dependent signaling pathways applies to the trafficking of other transporters, including Ae2[59] and Ntcp[30]. Another candidate to mediate cAMP-stimulatory effects is PI3K. Studies *in vivo* have revealed that cAMP-mediated stimulation of ABC transporter insertion is inhibited by the PI3K inhibitor wortmannin, and restored by phosphoinositide PI3K products[60]. PKCδ has been identified recently

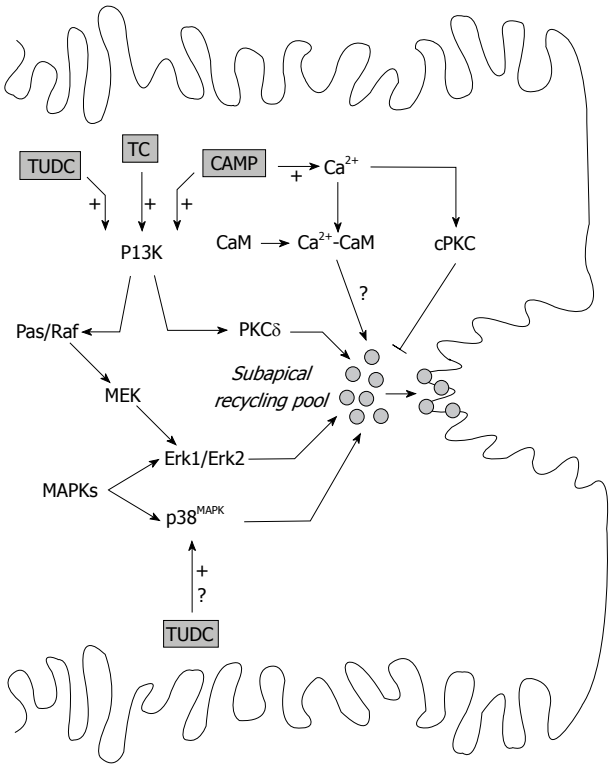

Figure 4 Signaling pathways involved in the exocytic insertion of canalicular transporters promoted by cAMP and by TC and TUDC. cAMP effect involves elevation in cytosolic Ca^{2+} and activation of the PI3K-dependent pathway. Formation of the CaM complex promotes apical insertion of transporters *via* unidentified mediators, and is counter-regulated by activation of cPKC. PI3K promotes exocytic insertion of canalicular transporters by activation of PKCδ and Erk-1 and Erk-2 of MAPK, *via* the Ras/Raf- MAPK kinase (MEK)-Erk-1/2 pathway. TC and TUDC also evoke the PI3K-dependent signaling pathway and promote insertion of canalicular transporters *via* the Ras/Raf-MEK-Erk-1/2 pathway. TUDC also stimulates canalicular carrier insertion by activation of MAPKs of the p38MAPK type, by an unknown mechanism.

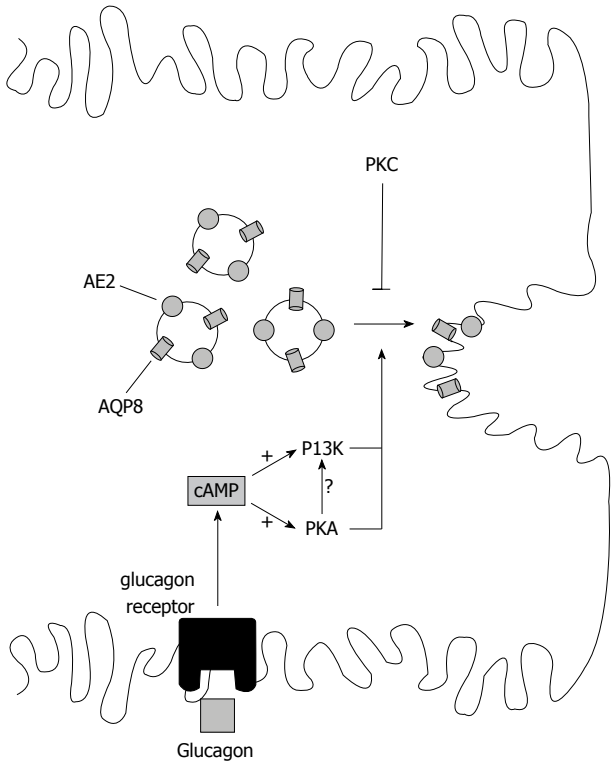

Figure 5 Signaling pathways involved in the co-stimulation of the canalicular targeting of AE2 and AQP8 by cAMP. AE2 and AQP8 are co-localized in the same population of pericanalicular vesicles, thus explaining common signaling modulation. cAMP stimulates AE2 and AQP8 targeting *via* activation of PKA. The PI3K pathway mediates the cAMP-stimulated, PKA-dependent targeting of AQP8, and probably that of AE2. cAMP effect on both transporters is counteracted by activation of PKC.

as a possible effector of the cAMP-dependent, PI3K-mediated pathways that leads to Mrp2 insertion[38]. The endogenous bile salt taurocholate (TC), which, as does cAMP, evokes the PI3K-dependent signaling pathway[61] and activates PKCδ[62], also promotes insertion of ABC transporters into the canalicular membrane in a PI3K-sensitive manner[61].

Another bile salt that stimulates exocytic insertion of canalicular transporters is tauroursodeoxycholate (TUDC)[63], but its action mechanism seems to involve another set of signaling molecules (Figure 4). TUDC activates within minutes mitogen-activated protein kinases (MAPKs) of both the $p38^{MAPK}$ type[63] and of the extracellular signal-regulated kinase (Erk) type (Erk-1 and Erk-2)[64]. These effects are causally linked to increased biliary excretion of bile salts and canalicular insertion of Bsep; the latter event having been demonstrated only for $p38^{MAPK}$[63]. The stimulus induced by TUDC on Erk-1/2, but not on $p38^{MAPK}$, is dependent on the sequential activation of PI3K and Ras/Raf[65]. The two MAPK-dependent pathways seem to act in parallel, and dual activation is required[63]. Studies in human hepatoblastoma HepG2 cells and in rat hepatocytes have shown that TUDC-stimulated insertion of BSEP involves not only increased targeting from the subapical compartment to the canalicular membrane, but also enhanced trafficking from the Golgi complex to the subapical compartment, and that $p38^{MAPK}$ may be a key signaling molecule in mediating this latter effect[66]. Coincidently, hypo-osmotic cell swelling, which shares with TUDC several downstream signaling effectors, also stimulates bile salt excretion by activation of Erk-1/2 and $p38^{MAPK}$[67], and both types of MAPKs are involved in hypotonicity-stimulated, microtubule-sensitive bile salt excretion[68,69].

AE2/Ae2: Apart from its functional localization in the canalicular membrane, the canalicular Cl^-/HCO_3^- exchanger AE2/Ae2 is present in pericanalicular vesicles[21,70], which migrate to the canalicular membrane on demand (Figure 5). The sorting in polarized liver cells of the three Ae2 variants, Ae2a, Ae2b1 and Ae2b2, has been studied using collagen-sandwiched primary rat hepatocytes[21]. After 72-96 h, GFP constructs from each recombinant Ae2 isoform co-localize in the canalicular membrane and in subapical, vesicular structures, and no signal is detected at the basolateral pole. This shared sorting of Ae2 isoforms is sensitive to the microtubule-disrupting agent colchicine, which suggests microtubule-dependent vesicular transport and exocytotic insertion of these transporter isoforms in the canalicular membrane.

Microtubule-dependence of Ae2 trafficking has been confirmed by functional studies. Ae2-mediated Cl^-/HCO_3^- exchange is increased in rat hepatocytes exposed to a bicarbonate-containing medium or in response to cAMP, and this increased activity is blocked with colchicine[59]. The cAMP-elevating hormone glucagon also stimulates this activity through a microtubule- and a cAMP-dependent, PKA-mediated mechanism[71]. The stimulation of Cl^-/HCO_3^- exchange activity by cAMP or glucagon is inhibited by PKC agonists[59,71], which suggests the existence of a counter-regulatory mechanism similar to that occurring for the targeting of Ntcp and ABC canalicular transporters (see above).

AQP8: This water canalicular channel is largely localized in intracellular vesicles in hepatocytes, as demonstrated by both subcellular fractionation[23], confocal immunofluorescence[23] and immunoelectron microscopy studies[72]. As a result of this property, it can be quickly inserted in the canalicular membrane on demand[24,73]. The cell-permeable cAMP analog dibutyryl cAMP induces redistribution of AQP8 to the canalicular membrane, and increases hepatocyte membrane water permeability in a microtubule-dependent manner[22,23]. Further studies in isolated rat hepatocytes[74] have shown that, as with AE2, AQP8 is inserted in the canalicular membrane by the cAMP-elevating hormone glucagon, by a process that involves both PKA and PI3K activation[75]., Immunofluorescent co-staining studies in WIF-B cells have shown intracellular co-localization of AQP8 and AE2, which suggests that these transporters are expressed in the same population of pericanalicular vesicles[76] (Figure 5). This explains the similar behavior of both transporters in response to a similar regulatory stimulus. Thus, apart from modulating the biliary secretion of osmotically-active solutes to the bile canaliculus *via* exocytic insertion of relevant carriers (e.g., BSEP, MRP2, AE2), hepatocytes can also modulate their canalicular membrane water permeability by inserting AQP8, thus facilitating the osmotic movement of water under choleretic stimulus.

ALTERATIONS OF THE DYNAMIC LOCALIZATION OF TRANSPORTERS IN LIVER DISEASE

Endocytic internalization of hepatocellular transporters is a common feature in liver disease. This applies mainly to those liver diseases that involve primary impairment in the capability of hepatocytes to produce bile (hepatocellular cholestasis). In these cases, changes in transporter localization may become a major pathomechanism that explains the secretory failure. Alternatively, changes in carrier localization can occur as a secondary consequence of a cholestatic manifestation caused by mechanical impediments to deliver bile to the duodenum (obstructive cholestasis). In this case, transporter mis-localization may aggravate/perpetuate the primary secretory halt. We summarize here the current evidence in the literature that alterations in the dynamic localization of transporters occur in experimental and human cholestatic liver disease.

Endocytic internalization of transporters in animal models of cholestasis

Endocytic internalization of the main canalicular transporters was first described in experimental models of cholestasis in rodents. Internalization of Mrp2 and Bsep into intracellular vesicles, mainly at the pericanalicular domain, has been shown to occur in experimental models of both obstructive and hepatocellular cholestasis.

Bile duct ligation (BDL): Experimental ligation of the common bile duct in the rat is an accepted model of obstructive cholestasis. BDL leads to a marked alteration in the pattern of staining of both Mrp2 and Bsep, as detected by indirect immunofluorescence microscopy. Paulusma *et al*[77] have found that, 48 h after BDL in rats, immunostaining of these transporters at the canalicular level becomes fuzzy, contrasting with the well-delimited detection in sham-operated controls. The authors have assumed that this represents mis-localization of the transporters to intracellular vesicles at a subapical compartment, next to the canaliculus. These alterations are accompanied by a severe impairment of the biliary excretion of model solutes. For example, Mrp2-mediated transport of the model substrate dinitrophenyl glutathione is substantially impaired in isolated hepatocytes from rats with BDL[77]. Endocytic internalization seems not to be circumscribed to Mrp2 or Bsep, as a similar phenomenon was observed for the canalicular enzymes dipeptidyl peptidase IV[78] and Ca^{2+}/Mg^{2+}-ATPase[79]. Altered localization of Mrp2 and Bsep may represent aggravation of the secretory dysfunction caused by the parallel decrease in the hepatocellular content of the carriers that also occurs in this disease[80,81], or even to be a causal factor of this reduction[77,82-85]. Indeed, Paulusma *et al*[77] have also found that, in contrast to that which is observed for Mrp2 protein content, mRNA levels are preserved after BDL, which suggests post-transcriptional downregulation of Mrp2 expression. They have postulated that endocytic internalization may represent the primary step toward enhanced breakdown of the endocytosed carriers. If maintained with time in chronic cholestatic conditions, this may cause redirection of the protein to the lysosomal compartment, followed by degradation.

The events leading to endocytic internalization of Mrp2 and Bsep in BDL rats remain uncertain. It is likely that accumulation of bile salts or other endogenous, potentially toxic compounds in the liver represents a causal factor. Bile salts are able to trigger oxidative stress[86,87], which in turn may explain the release of pro-inflammatory cytokines in BDL rats[88]. Both events have been involved in canalicular transporter internalization, as described below. We have found that the alteration in the normal pattern of localization of Mrp2, and that of the tight-junctional protein occludin, does not occur until 4 h after BDL in rats[78], in contrast

to the immediate response observed in drug-induced cholestasis (see next section). This suggests that BDL alterations are secondary to intracellular accumulation of deleterious endogenous compounds.

Drug-induced cholestasis: Administration to laboratory animals of drugs known to induce functional, hepatocellular cholestasis, or administration of endogenous compounds thought to be the etiological factors of human cholestatic liver diseases, has been used as an experimental tool to study the mechanisms of the disease. Administration of the cholestatic, naturally-occurring estrogen estradiol-17β-d-glucuronide ($E_2$17G)[89,90], the cholestatic monohydroxylated bile salt taurolithocholate (TLC)[91,92] and the cholestatic immunosuppressor drug cyclosporine A[93] all induce cholestasis in a short-term fashion, accompanied by endocytic internalization of Mrp2 and Bsep.

We have characterized in detail the mechanisms of transporter internalization in E_2-17G-induced cholestasis, an experimental model that reproduces in part pregnancy-induced cholestasis. After a single, i.v. administration of this compound, bile flow decreases in a dose-dependent fashion with a nadir at 20 min, and spontaneously recovers to normality by 2 h post-injection[94]. The cholestatic phase is associated with endocytic internalization of Mrp2 and Bsep, whereas the recovery phase occurs in parallel with the spontaneous re-insertion of subapical vesicles into the canalicular membrane[89,90]. While the internalization process is microtubule-independent, re-insertion is microtubule-dependent, and stimulated by cAMP[95]. We also found that repeated administration of E_2-17G to rats leads to both a deeper internalization of Mrp2 and an abnormal localization of a small fraction to the lateral membrane[78]. The latter phenomenon likely reflects loss of the fence between apical and basolateral domains caused by the simultaneous alteration of the tight-junctional structures[95,96]. Unlike Mrp2 and Bsep, AQP8 has a preserved localization in E_2-17G-induced cholestasis, and, like Mrp2 and Bsep, this water channel has a dual (intracellular plus plasma membrane) localization[97].

Lipopolysaccharide (LPS)-induced cholestasis: LPS is an endotoxin localized in the outer membrane of Gram-negative bacteria. The toxin induces cholestasis mainly by the release of pro-inflammatory cytokines, such as tumor necrosis factor-α and interleukin-1 by monocytes/macrophages and, in the liver, Kupffer cells[98]. Administration of LPS to laboratory animals represents, therefore, a good experimental model of inflammatory cholestatic diseases, not only of those caused by endotoxemia, but also those related to hepatitis caused by alcohol, autoimmune disease or drug intake.

LPS administration leads to endocytic internalization of Mrp2 and Bsep, which relocalizes in intracellular vesicular structures[99-101]. The time-dependency of the effect of LPS on Mrp2 internalization has been characterized by Kubitz *et al*[101]. These authors have found that, 3 h after LPS treatment, Mrp2 is found in intracellular vesicles in the vicinity of the canalicular membrane, and that these vesicles are deeply internalized after 6-12 h treatment. Endocytic internalization of ABC canalicular transporters seems to be specific, as localization of the canalicular enzyme dipeptidyl peptidase IV is not affected by the treatment. Mrp2 internalization is reversed by perfusing the liver with a hypo-osmotic buffer, a maneuver known to stimulate exocytic insertion of canalicular transporters under normal conditions[99,102]. However, this rescue of transporters occurs within 3 h of LPS administration, but not later on. It is possible that reversibility of the endocytic process depends on the degree of internalization of Mrp2, and that sustained internalization leads to delivery of the protein to the lysosomal compartment, followed by degradation. LPS effects can be prevented by administration of glucocorticoids[101] or by heat stress[103,104], two maneuvers that cause a decrease in synthesis and/or release of pro-inflammatory cytokines.

Oxidative-stress-induced cholestasis: Oxidative stress is a common feature in most liver diseases[105]. Radical oxygen species induce biliary secretory failure and cholestasis, even at low, pre-necrotic levels[106], and endocytic internalization of canalicular transporters may play a key role. We have shown that Bsep undergoes endocytic internalization into intracellular vesicles in isolated rat hepatocyte couplets when exposed to low levels of the pro-oxidizing compound tert-buthylhydroperoxyde (*t*BOOH)[107]. This is accompanied by a reduced capability to accumulate the fluorescent bile salt analogue CLF in their canalicular vacuoles. A similar phenomenon has been described for Mrp2 after exposure of isolated perfused rat livers to the pro-oxidant agents *t*BOOH[108], chloro-dinitrobenzene[108] and ethacrynic acid[109,110], or after hepatic ischemia-reperfusion[111].

Endocytic internalization of transporters in human cholestatic liver disease

Changes in canalicular export pumps have been shown to occur in many human cholestatic liver diseases. Unlike the situation in rodents, downregulation of the expression of these transporters in human cholestatic disease is mostly post-transcriptional in nature, therefore, internalization of these transporters followed by degradation may represent a crucial mechanism to explain the disease in humans.

Internalization of canalicular export pumps has been observed in virtually all kinds of human cholestasis, including: (1) obstructive extrahepatic cholestasis[112,113]; (2) inflammatory cholestasis associated with autoimmune hepatitis[113]; (3) mixed (obstructive plus inflammatory) cholestatic disease, such as primary biliary cirrhosis[114] and primary sclerosing cholangitis[113]; and (4) acute cholestasis induced by drugs, such as that triggered by

antibiotics, tiopronin, chlorpromazine and non-steroidal anti-inflammatory drugs[113,115]. Patients with obstructive cholestasis that are subjected to percutaneous transhepatic biliary drainage show different degrees of transporter dyslocalization, depending on the efficacy of the biliary drainage[112,113], which points to a central role for retained endogenous compounds in this pathomechanism.

Mechanisms of endocytic internalization in cholestasis: role of signaling pathways

The mechanisms by which endocytosis of canalicular transporters occurs in cholestasis remains poorly understood. At least in part, this may be because they are multifactorial.

Alterations of actin-cytoskeletal integrity by administration of the F-actin poison phalloidin[116], or secondary to the administration of pro-oxidant compounds, such as *t*BOOH[107] or the hydrophobic bile salts taurochenodeoxycholate[117], triggers canalicular transporter endocytosis. This may be related to the fact that actin cytoskeleton is involved in transcytosis processes by operating as a bridge between microtubules and the apical membrane itself, in a coordinated action of the microtubule- and the F-actin-based motor proteins, kinesin and myosin, respectively[118]. However, internalization of canalicular transporters also occurs with preserved actin organization, e.g. in E_2-17G-[89,90] or TLC-[92]induced cholestasis. In these cases, components of the microfilament network other than actin, but associated with it, may be independently affected. Actin can interact with, and possibly regulate, transmembrane proteins *via* binding to plasma membrane actin cross-linking proteins, such as the ezrin-radixin-moesin (ERM) family of proteins, or by binding to interacting-partner proteins, such as PDZK1 and HAX-1. These cytoskeleton-associated proteins are required for the biosynthetic targeting of transmembrane proteins from the *trans*-Golgi network to the proper membrane domain, and for their further cell-surface retention[119-121]. Mice that lack radixin, the main ERM protein in liver, develop conjugated hyperbilirubinemia associated with retrieval of Mrp2[122]. Furthermore, downregulation of radixin using interfering RNA technology in collagen-sandwich-cultured rat hepatocytes disturbed the normal development of canalicular structures, and dissociated canalicular export pumps from their normal location at the apical membrane. Inside the cell, the transporters are found to be largely associated with Rab11-containing endosomes[123]. Furthermore, a disturbed co-localization of MRP2/Mrp2 and radixin associated with endocytic internalization of the carrier is apparent in obstructive and estrogen-induced cholestasis in rats[124], and in several cholestatic liver diseases in humans, including primary biliary cirrhosis stage Ⅲ, drug-induced liver injury, obstructive jaundice, primary sclerosing cholangitis and autoimmune hepatitis[113,114]. On the contrary, alteration in cholestasis of the localization/function of interacting-partner proteins, such as PDZK1 (for Mrp2) and HAX-1 (for Bsep, Mdr2 Mrp1) remains

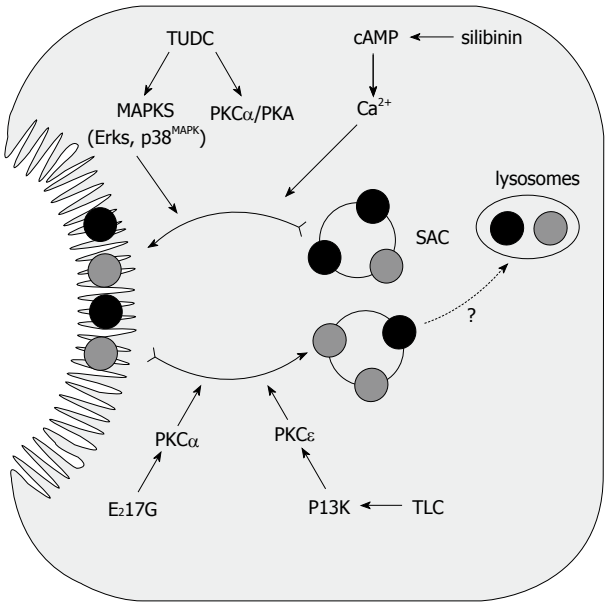

Figure 6 Endocytic internalization of canalicular transporters in $E_2$17G and in TLC-induced cholestasis. Protection from these cholestatic agents by the anticholestatic agents cAMP and TUDC is also shown. $E_2$17G and TLC induce endocytic internalization of canalicular transporters into the subapical compartment (SAC); this may lead to delivery to the lysosomal compartment, followed by degradation. $E_2$17G-induced activation of PKCα and TLC-induced, phosphatidylinositol 3-kinase (PI3K)-dependent activation of PKCε have been proposed to mediate this retrieval. Elevation of intracellular cAMP levels induced by administration of the permeant cAMP analogue DBcAMP, or by the phosphodiesterase inhibitor silibinin, prevents internalization, and accelerates re-insertion, *via* cytosolic Ca^{2+} elevations. On the other hand, TUDC prevents transporter endocytosis probably *via* co-stimulation of PKCα- and PKA-dependent pathways.

to be confirmed. This possibility however exists, since retention of Mrp2[122] and Oatp1a1[125] in the apical and the basolateral membranes, respectively, requires interaction with the PDZ-domain protein, PDZ1. In addition, there is evidence that HAX-1 participates in clathrin-mediated Bsep endocytosis from the canalicular plasma membrane[126].

Accumulating evidence indicates that changes in canalicular transporter localization that occur in cholestasis also depend on activation of critical intracellular signaling pathways (Figure 6). Representative examples are cPKC (mainly, PKCα in hepatocytes). Selective activation of cPKC induces endocytic internalization of Bsep from the canalicular membrane and cholestasis in the isolated perfused rat liver[58]. Coincidently, pan-specific activation of PKC also induces redistribution of MRP2 from the canalicular to the basolateral membrane in HepG2 cells[127]. A critical participation of cPKC in the endocytic internalization of Bsep and the associated bile-salt secretory failure has recently been demonstrated by our group in E_2-17G-induced cholestasis in rats[128]. A similar role for cPKC has also been reported in cholestasis associated with *t*BOOH-induced oxidative stress[107] (Figure 7). However, under oxidative stress, the type of canalicular protein that is internalized and the signaling molecule involved seem to depend on the magnitude of the oxidative challenge. Low concentrations of the oxidizing

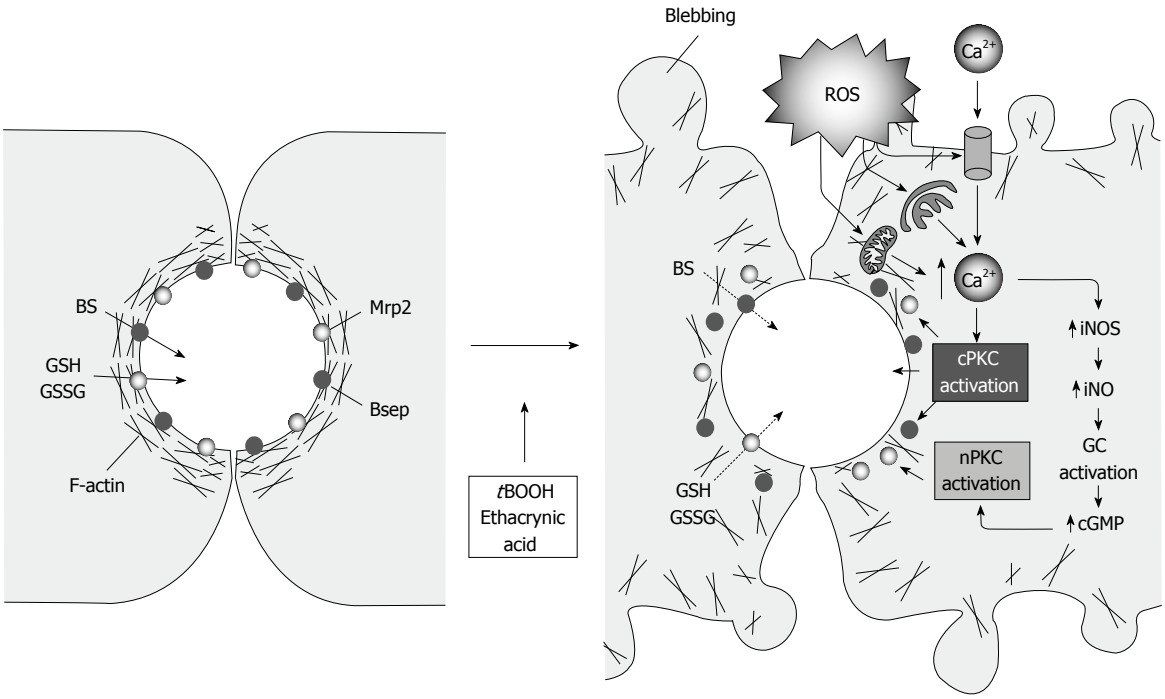

Figure 7 Endocytic internalization of canalicular transporters under oxidative stress. In normal cells, the pericanalicular arrangement of F-actin allows for the appropriate insertion of the canalicular transporters in their membrane domain. Reactive oxygen species produced by the administration of oxidizing compounds, such as tBOOH or ethacrynic acid, induces mobilization of Ca^{2+} across the plasma membrane and membranes of the calciosome (smooth ER and mitochondria), and the subsequent activation of cPKC. cPKC activation induces blebbing and redistribution of F-actin from the pericanalicular region to the cell body. This rearrangement, in turn, leads to canalicular transporter internalization. Moderate Ca^{2+} elevations may also activate iNOS, which induces NO-mediated guanylate cyclase activation and further cGMP-mediated activation of nPKC, which may internalize selectively Mrp2.

compound, ethacrynic acid, does not translocate cPKC, but novel PKC isoforms (nPKC). Under these conditions, the compound internalizes selectively Mrp2 without affecting Bsep, by a mechanism that probably involves Ca^{2+}-dependent activation of inducible nitric oxide (NO) synthase (iNOS), followed by NO-mediated cGMP increase, and further cGMP-activation of nPKC[110]. However, higher doses of ethacrynic acid, sufficient to activate cPKC isoforms, induce internalization of Bsep and Mrp2[110].

The nPKC isoform PKCε is also activated in TLC-induced cholestasis, and has been suggested to be involved in the TLC cholestatic effect[129]. This phenomenon occurs in a PI3K-dependent manner, which is consistent with the finding that PI3K products are potent activators of PKCε[130] (Figure 6). Since PI3K has been also shown to have pro-insertion properties (see above), this may be regarded as paradoxical. However, pro-exocytic and pro-endocytic effects of PI3K have been inferred by using pan-specific inhibitors of PI3K, and different isoforms of this kinase may have accounted for by these different effects.

Anticholestatic therapeutic approaches based upon modulation of dynamic carrier localization

As illustrated above for E2 17G-induced cholestasis, internalization of hepatocellular transporters in cholestasis is spontaneously reversed if the cholestatic insult is transient. This spontaneous recovery occurs by a microtubule-dependent re-targeting of the endocytosed transporters to the canalicular membrane[95]. Some experimental therapeutic approaches have been designed to prevent transporter internalization and/or to accelerate this re-insertion, so as to avoid irreversible consequences of sustained internalization (Figure 6). The therapeutic agents studied include the following.

cAMP: This second messenger partially prevents the impairment of bile flow and internalization of ABC transporters in experimental cholestasis, consistent with its capability to stimulate vesicle-mediated targeting of canalicular transporters[54,55,60]. The drop in bile flow and transport activity of Bsep[90] and Mrp2[89] in the acute phase of E2 17G-induced cholestasis can be partially prevented by cAMP. More significantly, cAMP shortens spontaneous recovery to normality of bile flow, Mrp2 function and Mrp2 localization[89]. A similar acceleration of the re-insertion of endocytosed transporters has been described by our group for Bsep in TLC-induced cholestasis[92]. In isolated rat hepatocyte couplets, a preventive effect of cAMP has been observed in E2 17G-[90,131] and TLC[92,131]-induced Bsep mis-localization. In this case, however, prevention by cAMP is complete. This protective effect is significantly blocked by the Ca^{2+} chelator, BAPTA/AM, but not by the PKA inhibitor, KT5720, which suggests involvement of Ca^{2+}-dependent signaling pathways. A similar anticholestatic mechanism in terms of the signaling modulators involved is afforded by silibinin, the active component of the hepatoprotector silymarin[131]. This most likely

results from the capability of silibinin to inhibit cAMP phosphodiesterase, thus increasing endogenous cAMP intracellular levels[131].

TUDC: This taurine-conjugate bile salt stimulates exocytic insertion of canalicular export pumps as part of its choleretic effect[63], and counteracts endocytic internalization of Bsep[134] and Mrp2[91] in TLC-induced cholestasis (Figure 6). The Ca^{2+}-sensitive, PKC isoform, PKCα, has been proposed to mediate its anticholestatic effect[91], *via* a cooperative PKCα/PKA-dependent mechanism[133]. This is in apparent contradiction with more recent findings that PKCα is cholestatic rather than hepatoprotective[58]. However, the biological response evoked by the interplay between different protein kinases (PKCα/PKA) may be different from that evoked by just one of them (PKCα). Furthermore, TUDC activates Erk[64] and $p38^{MAPK}$[63], and the cholestatic effect of PKCα may be overridden by the choleretic effects of these signal transduction pathways.

4-Phenylbutyrate (4-PBA): This compound has been shown to restore the reduced cell surface expression of cystic fibrosis transmembrane conductance regulator in cystic fibrosis patients, who have mutated forms of the protein, which suggests improved targeting of the transporter to its membrane domain. When the 4PBA-proinserting property was tested for Bsep in normal rats, it was observed that canalicular expression and bile-salt transport function were improved by this compound[134]. A possible mechanism that 4PBA treatment increases the cell-surface-resident Bsep is the interruption of the internalization process from the cell surface to the intracellular compartment, or promotion of recycling from the intracellular compartment back to the cell surface[134]. Stabilization of Bsep in the membrane by 4PBA has also been confirmed in MDCK cells for wild-type Bsep and Bsep with E297G and D482G mutations, which occurs in progressive familiar intrahepatic cholestasis type 2 (PFIC2). Since trafficking of these Bsep-mutated proteins is impaired in PFIC2[135], this agent may be a potential candidate to halt the progression of this genetic disease. Its efficacy in acquired cholestatic diseases remains to be ascertained.

FUTURE DIRECTIONS

The overwhelming progress in molecular biology techniques and the availability of *in vitro*, polarized cell models for the study of hepatobiliary function has greatly facilitated the characterization at a molecular level of the mechanism involved in the sorting of hepatobiliary transport systems from their sites of synthesis, and their recycling from/to endosomal compartments available on demand. However, the increasing number of new cytoskeletal, motor and signaling proteins that are being discovered as a result of these technological developments makes the characterization of their role in transporter trafficking an endless challenge.

Advances in the molecular field have promoted a parallel progress in the understanding of the consequences that the alterations in the mechanisms of trafficking have in liver disease. It is becoming increasingly evident that impairment in the dynamic localization of hepatocellular transporters is a common feature in hepatocellular cholestasis. However, the characterization of the molecular mechanisms that underlie this alteration is in its infancy. Many crucial questions remain to be answered, for example: (1) which are the signaling mediators that trigger endocytosis of canalicular transporters in each kind of cholestasis; (2) which are the molecular targets of these cholestatic mediators that ultimately govern carrier internalization?; and (3) can changes in localization of these transporters be not only prevented but, what is more important from the therapeutic point of view, reversed by factors that counteract these dysfunctions? Satisfactory answers to these questions would allow the design of new therapeutic strategies in cholestatic liver diseases to assure proper localization of transporters in an attempt to prevent their accelerated degradation. We hope that progress in experimental therapeutics based on this current information encourages clinical researchers to apply this knowledge to envisage better, innovative therapeutic alternatives for the treatment of human cholestatic liver disease.

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