Identification of HAX-1 as a Protein That Binds Bile Salt Export Protein and Regulates Its Abundance in the Apical Membrane of Madin-Darby Canine Kidney Cells*

Daniel F. Ortiz‡, James Moseley, German Calderon, Amy L. Swift, Shaohua Li, and Irwin M. Arias

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

ATP-binding cassette (ABC)-type proteins are essential for bile formation in vertebrate liver. BSEP, MDR1, MDR2, and MRP2 ABC transporters are targeted to the apical (canalicular) membrane of hepatocytes where they execute ATP-dependent transport of bile acids, drugs, amphipathic cations, phospholipids, and conjugated organic anions, respectively. Changes in activity and abundance of transporters in the canalicul membrane regulate bile flow; however, little is known regarding cellular proteins that bind ABC transporters and regulate their trafficking. A yeast two-hybrid screen identified HAX-1 as a binding partner for BSEP, MDR1, and MDR2. The interactions were validated biochemically by glutathione S-transferase pull-down and co-immunoprecipitation assays. BSEP and HAX-1 were over-represented in rat liver subcellular fractions enriched for canalicul membrane vesicles, microsomes, and clathrin-coated vesicles. HAX-1 was bound to BSEP, MDR1, and MDR2 in canalicul membrane vesicles and co-localized with BSEP and MDR1 in the apical membrane of Madin-Darby canine kidney (MDCK) cells. RNA interference of HAX-1 increased BSEP levels in the apical membrane of MDCK cells by 71%. Pulse-chase studies indicated that HAX-1 depletion did not affect BSEP translation, post-translational modification, delivery to the plasma membrane, or half-life. HAX-1 depletion resulted in an increased peak of metabolically labeled apical membrane BSEP at 4 h and enhanced retention at 6 and 9 h. HAX-1 also interacts with cortactin. Expression of dominant negative cortactin increased steady state levels of BSEP 2-fold in the apical membrane of MDCK cells, as did expression of dominant negative EPS15. These findings suggest that HAX-1 and cortactin participate in BSEP internalization from the apical membrane.

ABC proteins in the canalicul membrane of hepatocytes generate the driving force that establishes bile flow. The ABC

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† To whom correspondence should be addressed. Tel.: 617-636-3828; Fax: 617-636-6045; E-mail: daniel.ortiz@tufts.edu.

‡ The abbreviations used are: ABC, ATP-binding cassette; BSEP, bile salt export protein; CMV, canalicul membrane vesicles; EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein; ER, endoplasmic reticulum; GST, glutathione S-transferase; HAX-1, HS1-associated protein X-1; MDR, Madin-Darby canine kidney cells; MDR, multidrug resistance protein; RNAi, RNA interference; transporters MDR1 (ABCB1), MDR2 (ABCB4) (MDR3 in humans), and BSEP/SPGP (ABCB11) (bile salt export protein, also called sister of P-glycoprotein), which are closely related in structure and sequence, mediate ATP-dependent transport of biliary constituents. BSEP transports conjugated bile acids (1), and MDR2 is essential for phospholipid transfer into bile (2, 3). MDR1 mediates excretion of hydrophobic, cationic drugs to bile (4), and MDR1 overexpression is an important cause of multidrug resistance in tumor cells (5). MRP2 (ABCC3), which has a different structure and belongs to a different ABC transporter subfamily, mediates secretion of organic anions (6, 7). Mutations that affect expression or activity of these proteins are associated with liver disease. Defects in BSEP cause progressive familial intrahepatic cholestasis type II (8); mutations in MDR3 are manifested by progressive familial intrahepatic cholestasis type III (9), and MRP2 defects give rise to Dubin-Johnson syndrome (6). Disease can also result from defective trafficking of transporters to the canalicular membrane. Mutations that impair MDR3 exit from the endoplasmic reticulum are associated with cholestasis of pregnancy (10); mutations in BSEP that impair expression of the mutant proteins in the apical membrane of MDCK cells are linked to progressive familial intrahepatic cholestasis type II (11); and deletion of two amino acids in MRP2 of a Dubin-Johnson patient resulted in intracellular retention and accelerated degradation of MRP2 (12). Thus, elucidating pathways that govern trafficking of ABC transporters to and from the canalicular membrane can provide critical insight into mechanisms underlying normal biliary secretion and cholestasis (bile secretory failure).

Targeting and trafficking of integral membrane proteins depend on sequence motifs in the proteins, interactions with signaling and trafficking networks, and polypeptides that bind the membrane proteins. Few proteins have been identified that associate with ABC transporters and participate in their mobilization and targeting. Interactions with PDZ domain proteins NHERF/EBP50 and E3Karp (13, 14) are essential for apical expression of cystic fibrosis transmembrane regulator (15). MRP2 retention in the apical membrane requires interaction with radixin (16) and a carboxyl-terminal moiety that binds PDZK1 (17, 18). MDR1, MDR2, and BSEP do not contain obvious PDZ-interacting motifs, and apical expression of MDR proteins is not affected in the liver of radixin knockout mice (16). Thus, trafficking of MDR transporters is controlled by a different subset of proteins. By using a yeast two-hybrid screen, we identified HAX-1 as a binding partner for MDR1, MDR2, and BSEP. HAX-1 is a 34-kDa polypeptide that interacts with a heterogeneous group of proteins that include cortactin (19–
Homogenate 2000 pSL27, respectively (see Table I). 5 mg of protein from a rat liver 3 h with 10 fixed for 10 min in ice-cold methanol. Transwell filters were blocked for ecatAMINE 2000 (Invitrogen). Transwells were washed with PBS and

**EXPERIMENTAL PROCEDURES**

**Materials and Antibodies**—The Cytotrap yeast two-hybrid system and vectors were purchased from Stratagene (La Jolla, CA), and pEGFP-N1 and pEYFP-N2 vectors were from Clontech (Palo Alto, CA). The Silencer Express RNAi kit was from Ambion (Austin, TX), and Easytag NEG-772 [35S]methionine and -cysteine protein labeling mix was from PerkinElmer Life Sciences. Anti-cAM105 Ab anti-body was a gift from S. H. Lin (24). Anti-HAX-1 and anti-calnexin antibodies were purchased from BD Biosciences; C219 antibody was from Signet (Dedham, MA); anti-mannosidase II antibody was from Covance Research (Richmond, CA); anti-clathrin heavy chain TD1 monoclonal antibody was from Sigma; anti-Na/K-ATPase IgG was from United States Biochemical Corp.; anti-cortactin anti-body was from Upstate ImmunoResearch (West Grove, PA); and isotopic-specific goat anti-mouse IgG1 and -mouse IgG2a were from Bethyl Laboratories (Montgomery, TX). LVT90-Tu41 anti-rat IgG (fused with enhanced green fluorescent protein) prepared as described (25). Sulfo-NHS-LC-LC-amidotri-biotin and streptavidin-agarose beads were purchased from Pierce. All other reagents were from Sigma.

Anti-rat HAX-1 (rHAX-1) Tu91 antibodies were raised in rabbits against a full-length rHAX-1 peptide fused to a six-histidine carboxy-terminal tag. Tu91 stained human and rat HAX-1 as 34 and 35-kDa bands, respectively, in extracts derived from transfected HEK293 cells expressing rHAX-1 (see Fig. 4A). Staining was abolished by Tu91 preincubation with purified 6H-HAX-1 protein. Immunofluorescence staining revealed that Tu41 and anti-HAX-1 monoclonal antibodies co-localized with HEK293 cells expressing rHAX-1.

**Yeast Two-hybrid Screen**—A PCR DNA fragment coding for amino acids 620-1278 of rat MDR2 was cloned into the pSOS vector (Stratagene) to generate bait plasmid pSL49. Other pSOS bait plasmids prepared in a similar manner are described below. A liver cDNA library was generated in the pMyr vector from rat liver poly(A) RNA using the Superscript system (Invitrogen). Library plasmid DNA was co-transformed into temperature-sensitivecdc25h yeast with p508 bait plasmids. Yeasts grown for 24–48 h at 25 °C were replica-plated on galactose plates and incubated at the nonpermissive temperature of 37 °C for 3–5 days. Individual colonies that grew at 37 °C were picked and expanded in liquid media at 25 °C. Washed cells were spotted on galactose or glucose solid media and incubated at 37 °C.

**Plasmid Constructs**—The plasmids used in these studies are listed in Table I. The Silencer Express RNAi kit was from Ambion (Austin, TX), and pEGFP-N1 and pEYFP-N2 vectors were from Clontech (Palo Alto, CA). The plasmids used in these studies are listed in Table I. The Cytotrap yeast two-hybrid system (Stratagene) constructs utilized for yeast two-hybrid analyses, protein expression of prey cDNAs in S. cerevisiae, pPK-CMV (Stratagene) for expression of cDNAs in mammalian cells; pEYFP-N1 and pEGFP-C1 (Clontech) for expression of proteins as EYFP or EGFP, respectively, in mammalian cells; pQE60 for expression of His-tagged proteins in E. coli; and pSEC for expression of RNAi duplexes driven by the human U6 promoter. AA refers to the amino acid residues of the designated gene product that are encoded by the DNA fragment in each plasmid; NA indicates not applicable.

| Plasmid | Vector | Gene | Amino acids |
|---------|--------|------|-------------|
| pSL27   | pGEX   | Mdr2 | 620–898     |
| pSL27   | pGEX   | Bsep | 651–741     |
| pSL47   | pSOS   | Bsep | 653–741     |
| pSL49   | pSOS   | Mdr2 | 620–695     |
| pSL55   | pSOS   | Mdr2 | 1–40        |
| pSL56   | pSOS   | Mdr2 | 1248–1278   |
| pSL67   | pSOS   | Mdr1a | 611–704    |
| pJM25   | pSOS   | Mdr2 | 622–680     |
| pJM26   | pSOS   | Mdr2 | 622–656     |
| pJM27   | pSOS   | Mdr2 | 644–700     |
| pJM28   | pSOS   | Mdr2 | 670–728     |
| pJM31   | pSOS   | Mdr2 | 622–638     |
| pJM42   | pSOS   | Mdr2 | 639–656     |
| pJM43   | pSOS   | Mdr2 | 645–656     |
| pJM311  | pSOS   | Mdr2 | 651–7132   |
| pJM33   | pSOS   | Mdr2 | 651–682     |
| pJM34   | pSOS   | Bsep | 680–742     |
| pJM35   | pSOS   | Bsep | 710–742     |
| pJM511  | pSOS   | Bsep | 686–676     |
| pMP94–8 | pMYR   | Rhox–1 | 1–277   |
| pCD04   | PBK-CMV| Rhox–1 | 1–277   |
| pBSEY-EYFP| PEYFP-N1| Bsep | 1–1321     |
| pMDR1-GFP| GFPG-N2| Mdr1a | 1–1277     |
| pQQ-Hax | pQEG60| Rhox–1 | 1–277    |
| p365    | pSec   | Chox–1 | NA        |
| p427    | pSec   | Chox–1 | NA        |
| p623    | pSec   | Chox–1 | NA        |
| p746    | pSec   | Chox–1 | NA        |
| pGFP-acort| GFPG-C1| Cortactin | 492 |

Dubelco’s PBS and then incubated for 1–2 h with anti-HAX-1 antibody diluted 1/25 in IF buffer (3% bovine serum albumin in Dubelco’s PBS). Membranes were washed 10 times in PBS, blocked for 30 min with IF buffer, and incubated with Texas Red-labeled donkey anti-rabbit antibody for 45 min. Filters were washed 10 times with PBS and mounted on glass slides. BSEP-EYFP, MDR1-EYFP, and Texas Red-stained HAX-1 were visualized using a Leica TRS2 laser confocal microscope.

**Preparation of Membrane Fractions, Immunoblot Analysis, and Immunoprecipitation**—Subcellular fractions were prepared from rat liver as indicated. Enrichment of marker proteins was determined by immunoblot analyses, except for lysosomes and mitochondria, which were measured by biochemical enzyme assays. Briefly, rat livers were perfused with SH buffer (250 mM sucrose, 50 mM HEPES-Tris, pH 7.4, 0.1 M NaCl, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 0.5 mM phenylmethyl-sulfonyl fluoride, 5 μg/ml benzamide, 0.1 mM aprotinin) and homogenized in 50 ml of ice-cold SH buffer, and 2000 × g supernatants and pellets were obtained by centrifugation. Canicular membrane vesicles (CMV) were prepared from the 2000 × g pellet as described (29). Immunoblot analyses indicated CMV were enriched 40–50-fold in MDRI and MDR2 (C219 antibody), BSEP (LV790), and CAM-105 (Ab689). The 2600 × g supernatant was used to prepare sinusoidal membrane vesicles (30) that were enriched 20–25-fold in Na/K-ATPase, endoplasmic reticulum microsomes (31) that were enriched 15–20-fold in calnexin, and Golgi membrane vesicles (32) that were enriched 20–25-fold in mannose II. Clathrin-coated vesicles prepared from whole liver homogenates by density centrifugation (33) were enriched 40–50-fold in clathrin heavy chain. Rat liver lysosomes (34) and mitochondria (35) were enriched 40–50-fold in β-hexosaminidase (36) and 30–40-fold in succinate dehydrogenase, respectively.

Immunoprecipitations were performed as described (25). C219 immunoprecipitates prepared in this manner predominantly contained

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MDR1 and MDR2 P-glycoproteins. A small amount of BSEP was also present but was less than 10% that obtained in equivalent immunoprecipitations with LVT90 (our observations and see Ref. 25).

**Biотinylating of Cell Surface Proteins**—Biотinylating was performed essentially as described (37). MDCK cells grown in 12-mm transwell inserts (polyester 0.4-μm pore) were transfected using LipofectAMINE 2000, and media were changed every 1–2 days. 2–3 days after confluency, transwells were washed once with ice-cold DMEM without serum and twice with cold Dulbecco’s PBS containing calcium and magnesium. Cells were incubated twice for 25 min with 0.3 ml of freshly made biotinylation solution (1.5 mg/ml sulfo-NHS-LC-LC-biotin, 10 mM ethanamine, 2 mM CaCl2, 250 mM NaCl, pH 7.5) in either the upper or lower chamber. Transwells were washed twice with Dulbecco’s PBS containing glycine, incubated with Biotinylated BSEP-EYFP, washed twice with Dulbecco’s PBS. Transwell filters were incubated for 1 h in lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5, 1/1000 (v/v) aprotinin, 2 μM leupeptin, 4 μM pepstatin A, 1 mM phenylmethylsulfonyl fluoride), and cells were scraped off. Lysates were centrifuged at 13,000 g for 10 min, and 50 μl of packed streptavidin-agarose beads were added to the supernatants, which were incubated overnight with rotation. The beads were washed three times with lysis buffer, twice with high salt buffer (lysis buffer with 500 mM NaCl), and once with 10 mM Tris, pH 7.5. Proteins were eluted from the beads with 40 μl of SDS-urea loading buffer (4% SDS, 250 mM Tris-HCl, pH 6.8, 10 mM dithiothreitol, 0.5 mM EDTA, 0.5% Triton X-100). Immunoprecipitated BSEP-EYFP was released from anti-rabbit antibody–streptavidin-agarose beads by incubation with streptavidin-agarose beads, which were harvested by centrifugation at 1000 g, and washed three times with lysis buffer. Proteins were eluted with 40 μl of SDS-urea loading buffer and separated by SDS-PAGE. Gels were fixed in 40% methanol, 10% acetic acid and exposed to phosphor screens for 2–7 days.

**Real Time RT-PCR**—Replica 12-mm transwells containing MDCK cells were transfected on the same day and in the same manner as those used for biotinylation. Cells were washed twice with ice-cold PBS, and RNA was isolated once with 250 μl of TRIzol reagent according the manufacturer’s instructions. RNA from each sample was treated with RNase-free DNase (Invitrogen) for 20 min at 37 °C. DNase was inactivated by a 20-min incubation with EDTA at 65 °C. First strand cDNA was generated in a 20-μl reaction containing 0.5 μg of DNA-free RNA and using oligo(dT) primers, SuperScript II reverse transcriptase, and RNAseH. Real time PCR products were monitored in real time for 40 cycles by using Stratagene MX4000 multiplex quantitative PCR instrument. Bsep primers were sense CGATGACCTCAAGAATAATCTCG and antisense GCCGCAATGATCTTAGTGAAGG. Canine β-actin was used as a control with sense primer CGATGACCTCAAGAATAATCTCGG and antisense primer ATCCACGAGGACCTTCAACTCC.

**RESULTS**

**HAX-1 Binds the Linker Domains of MDR2, MDR1, and BSEP in Yeast**—MDR-type proteins are composed of homologous halves that are connected by a cytoplasmic linker domain (Fig. 1A) (reviewed in Ref. 38). A yeast two-hybrid screen, which used the rat MDR2 linker domain as bait, resulted in isolation of full-length HAX-1 cDNAs on three separate occasions. The rat HAX-1 nucleotide sequence exhibited 93 and 85% identity with mouse (GenBank™ accession number NM_011826) and human (GenBank™ accession number NM_006118) HAX-1 genes, respectively. The deduced rat HAX-1 protein (rHAX-1) sequence was 94% and 79% identical to mouse and human HAX-1 amino acid sequences, respectively.

Plasmid pM49-9, which expresses rHAX-1, permitted growth at 37 °C of yeast that harbored plasmids expressing MDR2 (pSL49), BSEP (pSL47), and MDR1 linkers (pSL 67) (Fig. 1B). It did not enable growth of yeast carrying the empty pSOS vector, pSOS-Cell (a negative control that expresses amino acids 148–357 of murine type IV collagenase), or pSOS fusions containing the amino-terminal 40 amino acids of MDR2 (pSL55) or the carboxyl-terminal 35 residues of MDR2 (pSL56). Thus, rHAX-1 specifically interacts in yeast with the linker domains of three MDR subfamily members that reside in the canalicul membrane of hepatocytes.

**HAX-1 Binds Amino Acid Motifs Proximal to the Nucleotide Binding Domains**—Two-hybrid analyses of pSL47 and pSL49 deletion derivatives revealed that rHAX-1 interacted in yeast with portions of the MDR2 and BSEP linkers that are proximal to the amino-terminal nucleotide binding domains (Fig. 1, C and D). The HAX-1 binding region was circumscribed to 20 amino acids in BSEP and to 16 residues in MDR2. These are the only regions of the BSEP and MDR2 linker domains that share significant amino acid sequence identity (see Fig. 1E). Moreover, these linker sequence fragments, particularly the MDR2 linker, exhibit significant similarity with the HAX-1 binding region of the herpesvirus membrane protein K15P (23) (Fig. 1E).

**GST-BSEP and GST-MDR2 Fusion Proteins Bind HAX-1 in Rat Liver Homogenates**—Rat liver homogenates were incubated with GSH-agarose beads adsorbed with chimeric GST proteins containing 91 amino acids of the BSEP linker domain or 79 amino acids of the MDR2 linker. GST-BSEP and GST-MDR2 beads effectively extracted HAX-1 from liver homogenates, whereas GST alone did not (Fig. 2). This experiment indicates that BSEP and MDR2 linker domains efficiently bind HAX-1 in the presence of the myriad proteins of a rat liver extract.
Distribution of HAX-1 in Rat Liver Subcellular Fractions—HAX-1 was primarily associated with 100,000 × g precipitable material (named 100 P) from rat liver homogenates. HAX-1 association with the 100 P fraction was unaffected by high pH or high salt but was dissociated by 1 M urea or 1% Triton (Fig. 3A), indicating strong affinity of HAX-1 for cellular membranes. rHAX-1 was enriched in CMV, clathrin-coated vesicles, and endoplasmic reticulum-enriched microsomal fractions (Fig. 3B). Only trace amounts of rHAX-1 were present in Golgi, lysosomes, mitochondria, or basolateral membrane vesicles. BSEP, MDR1, and MDR2 were also enriched in CMV and clathrin-coated vesicle fractions.

HAX-1 Is Associated with BSEP and MDR1/2 in Cultured Mammalian Cells and Rat Liver—Extracts were prepared from HEK293 cells transfected with plasmids that express BSEP and rHAX-1. BSEP was immunoprecipitated by using the LVT90 antibody (25). Immunoblots revealed that the immunoprecipitates contained HAX-1 in addition to BSEP (Fig. 4A). HAX-1 was not immunoprecipitated from extracts that lacked BSEP, indicating that LVT90 antisera does not cross-react with rHAX-1. Likewise, antibody C219, which recognizes MDR1 and MDR2, co-immunoprecipitated HAX-1 and MDR1a-EGFP (Fig. 4B). These results indicate that HAX-1 interacts with BSEP and MDR1a in mammalian cells.

To determine whether HAX-1 is associated with the transporters in the canalicular membrane, BSEP was immunoprecipitated from CMV by using antibody LV790. Immunoblot analysis indicated that, in addition to BSEP, the immunoprecipitates contained HAX-1 (Fig. 4C). LV790 did not immunoprecipitate HAX-1 from liver fractions that contained HAX-1 but were devoid of BSEP. Likewise, HAX-1 accompanied MDR1 and MDR2 immunoprecipitated from CMV by antibody C219 (Fig. 4D). As
with LVT90, HAX-1 was not detected in C219 immunoprecipitates of liver fractions that did not contain MDRI or MDR2. Thus, HAX-1 is present in protein complexes that contain BSEP, MDRI, or MDR2 in the canalicular membrane of hepatocytes.

**HAX-1 Co-localizes with BSEP-EYFP in the Apical Domain of Polarized MDCK II Cells**—MDCK II cultures were co-transfected with constructs expressing rHAX-1 and BSEP-EYFP. The cells were fixed, immunostained, and visualized by laser confocal fluorescence microscopy. Subcellular co-localization of BSEP-EYFP and HAX-1 varied with cell polarization. In non-polarized MDCK II cells, HAX-1 and BSEP-EYFP were predominantly intracellular, and co-localization was sparse (data not shown). One to 2 days after confluence, cells showed differentiation of apical and lateral membranes. HAX-1 partially co-localized with BSEP-EYFP in the apical membrane; however, most HAX-1 was located immediately below the apical domain, where it partially localized with BSEP-EYFP (Fig. 5, upper panel). In polarized columnar cells, 4–5 days after confluence, BSEP-EYFP was mostly restricted to the apical membrane where it co-localized with HAX-1 (Fig. 5, lower panel).

**Reduction of HAX-1 Expression by RNA Interference Increases Apical Membrane BSEP in MDCK Cells**—RNAi plasmids were generated that express short stem-loop RNAs homologous to 19-bp sequences in the canine HAX-1 mRNA. A matched control that displayed no homology with HAX-1 was also made. MDCK cells co-transfected with pEGFP and RNAi plasmids were purified by fluorescence-activated cell sorting. Transfection efficiency of MDCK cultures varied from 30 to 60%. However, cells transfected with two plasmids displayed greater than 85% co-transfection efficiency (as determined by fluorescence microscopy of cells co-transfected with pEGFP and pECFP plasmids, not shown). Therefore, 85% of cells sorted by EGFP expression were presumed to contain RNAi plasmids. Immunoblots revealed that HAX-1 levels were reduced by 7–75% in EGFP-positive cells expressing different RNAi transcripts (Fig. 6A). Nonfluorescent cells, or cells co-transfected with the control plasmid, displayed HAX-1 levels indistinguishable from nontransfected controls. Transfection with plasmid p365 consistently reduced HAX-1 expression by greater than 70% relative to the pCon control plasmid.

Apical or basolateral surface proteins of MDCK cells transfected with Bsep-EYFP were labeled with a membrane-impermeable biotinylation reagent and purified by binding to streptavidin-agarose. Immunoblots of biotinylated proteins indicated that BSEP-EYFP was predominantly localized to the

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**Fig. 3.** HAX-1 distribution in rat liver fractions. A, HAX-1 is associated with membrane fractions. A rat liver 2000 × g pellet enriched in HAX-1 was homogenized in 2% Triton X-100 (2% Trit), 1% Triton X-100 (1% Trit), 1 mM urea, Na₂CO₃ buffer, pH 11, or 0.5 mM NaCl. Con, control. Immunoblots of 100,000 × g supernatant (S) or pellets (P) of these homogenates indicated that HAX-1 was removed from precipitable material by detergent or chaotropic agents. B, immunoblot analysis of purified liver subcellular fractions. Equal amounts of protein (15 μg) of these homogenates indicated that HAX-1 was removed from precipitated material (Fig. 6–). Immunoblots revealed that HAX-1 levels were reduced by 85% of cells sorted by fluorescence microscopy of cells co-transfected with pEGFP and no insert, Bsep and rHAX-1, no insert and rHAX-1, and Bsep and rHAX-1. Proteins were immunoprecipitated with anti-BSEP antibody (LVT90) or an equivalent amount of nonimmune rabbit serum (N.I.). Immunoblots of LVT90 immunoprecipitates indicated that HAX-1 co-immunoprecipitated with BSEP. HEK-293T cells express human HAX-1, which has a slightly smaller Mr, than the rat ortholog. Thus, two bands were seen in immunoprecipitates from cells expressing human and rat HAX-1. Nonimmune serum does not immunoprecipitate BSEP or HAX-1, and HAX-1 is not immunoprecipitated by LVT90 in the absence of BSEP. B, MDRI and MDR2 are co-immunoprecipitated by the anti-MDR1 C219 antibody. Extracts were prepared from HEK293 cells transfected with the pMDRIa-EGFP plasmid (+) or the pEGFP-N1 empty vector (−). Immunoblots of proteins immunoprecipitated (I.P.) by the C219 monoclonal antibody (C219) or normal mouse IgG2a (N.I.) revealed that HAX-1 specifically co-immunoprecipitates with MDRIa-EGFP. C, immunoblot of proteins immunoprecipitated (Immunopcp.) from CMV by the anti-BSEP LVT90 antibody. LVT90 (LVT) or normal rabbit (n) IgG were incubated with 0.4 μg of rat liver CMV (cmv) or 1 μg of a 2000 × g supernatant (2 k S), which contains HAX-1 but does not contain detectable BSEP or MDR2. Immunoblot analysis revealed that LVT90 immunoprecipitated BSEP from CMV but not from the 2000 × g supernatant fraction (top panel). HAX-1 was present in LVT90 immunoprecipitates derived from CMV, but not from 2000 × g supernatant (bottom panel), indicating that LVT90 precipitates HAX-1 only when BSEP is present. 20 μg of CMV and 50 μg of 2000 × g supernatant protein were loaded in the gel to show relative levels of BSEP and HAX-1. LVT90 was visualized using protein A-horseradish peroxidase conjugates, and the HAX-1 IgG1 monoclonal antibody was visualized with goat anti-mouse IgG-D, IgG1, and IgG2a. Immunoblots of proteins immunoprecipitated with anti-BSEP antibody (LVT90) or an equivalent amount of nonimmune rabbit serum (N.I.) revealed that HAX-1 specifically co-immunoprecipitates with MDRIa-EGFP. C, immunoblot of proteins immunoprecipitated (Immunopcp.) from CMV by the anti-BSEP LVT90 antibody. LVT90 (LVT) or normal rabbit (n) IgG were incubated with 0.4 μg of rat liver CMV (cmv) or 1 μg of a 2000 × g supernatant (2 k S), which contains HAX-1 but does not contain detectable BSEP or MDR2. Immunoblot analysis revealed that LVT90 immunoprecipitated BSEP from CMV but not from the 2000 × g supernatant fraction (top panel). HAX-1 was present in LVT90 immunoprecipitates derived from CMV, but not from 2000 × g supernatant (bottom panel), indicating that LVT90 precipitates HAX-1 only when BSEP is present. 20 μg of CMV and 50 μg of 2000 × g supernatant protein were loaded in the gel to show relative levels of BSEP and HAX-1. LVT90 was visualized using protein A-horseradish peroxidase conjugates, and the HAX-1 IgG1 monoclonal antibody was visualized with goat anti-mouse IgG-D, IgG1, and IgG2a.
channel.

The bleed through of BSEP-EYFP fluorescense into the Texas Red detection channel was avoided with the monochromatic antibody and goat anti-mouse IgG-Texas Red. Localization of HAX-1 at the apical membrane and co-localization with BSEP-EYFP increased with time in culture and polarization of MDCK II cells. The upper panels show cells 2 days after transfection, whereas cells in the lower panels were fixed 5 days after transfection. XZ sections of stained cells were obtained with a Leica TCS2 confocal microscope. Sequential scanning with argon (514 nm) and krypton (568 nm) lasers was used to avoid bleed through of BSEP-EYFP fluorescence into the Texas Red detection channel.

Pulse-chase metabolic labeling was performed in MDCK cells co-transfected with Hax-1 RNAi, or control, plasmids, and BSEP-EYFP. Metabolically labeled Bsep-EYFP was initially detected in whole cell extracts as a 160-kDa isoform that was not observed in biotinylated apical membrane fractions and was chased into higher molecular mass forms of 180–200 kDa (Fig. 8, Total). Metabolically labeled BSEP-EYFP appeared in the apical membrane in 1 h, and the levels peaked in 3–4 h (Fig. 8, Apical). HAX-1 depletion did not affect BSEP-EYFP translation, post-translational modification, or arrival to the apical membrane. However, apical membrane levels of metabolically labeled BSEP-EYFP were significantly higher in HAX-1-depleted cells at 4 (45 ± 14%), 6 (72 ± 15%), and 9 h (103 ± 18%) (Fig. 8, Apical). Total cellular levels of metabolically labeled BSEP did not differ between control and HAX-1-depleted cells (Fig. 8, Total). By 12 h, pulse-labeled apical and total cellular BSEP levels declined similarly in HAX-1-depleted cells and controls. Measurements made at 18, 24, and 30 h revealed no significant difference in BSEP half-life in HAX-1-depleted and control cells (data not shown).

**HAX-1 Associates with Cortactin and Expression of Dominant Negative Cortactin Increases BSEP Levels in the Apical Membrane—**HAX-1 and cortactin co-localize in fibroblast lamellipodia and interact in vitro (19). Analysis of subcellular fractions from rat liver revealed that cortactin is highly enriched in CMV (Fig. 9A). Immunoprecipitation of HAX-1 from CMV also precipitated cortactin (Fig. 9B) indicating that HAX-1 interacts with cortactin in the canalicular membrane.

To determine whether cortactin participates in transporter trafficking, MDCK cells were transfected with plasmids that express BSEP and a dominant negative form of cortactin, which lacks the carboxyl-terminal SH3 domain (39). Cell surface biotinylation indicated that co-expression of dominant negative cortactin was associated with a greater than 2-fold increase in apical membrane BSEP levels (Fig. 10, A and B). Real time RT-PCR of RNA derived from replica transwells revealed no significant differences in Bsep transcript levels in cells expressing GFP or GFP fused to dominant negative cortactin (Fig. 10C).

**Expression of Dominant Negative EPS15 Increases Apical Membrane BSEP—**Cortactin has been implicated in clathrin-
Interaction of HAX-1 with BSEP, MDR1, and MDR2

Fig. 7. HAX-1 depletion increases BSEP abundance in the apical membrane of MDCK cells. A, immunoblot of surface-biotinylated proteins showing that BSEP resides primarily in the apical membrane of MDCK cells. MDCK cells grown in transwells were transfected with a plasmid expressing BSEP-EYFP. Upper or lower transwell chambers were treated with biotinylating reagent. Apical or basolateral (basolat.) membrane proteins were isolated with streptavidin-agarose and immunoblotted with the anti-BSEP LVT90 antibody. B, immunoblots of apically biotinylated proteins (apical) and total extracts (total) derived from cells co-transfected with Bsep-EYFP and with the p365 Hax-1 RNAi plasmid, or the pCon control plasmid. HAX-1 abundance was reduced by 50% in p365 total extracts, which contain proteins from transfected and nontransfected cells, suggesting that HAX-1 expression in transfected cells may be reduced by greater than 70% (see Fig. 6). HAX-1 depletion was accompanied by a 26 ± 5% (*, p < 0.05) increase in total BSEP levels and by a 71 ± 12% (**, p < 0.01) increase in apical membrane levels of BSEP. C, (mean of three experiments in triplicate, and one experiment in quadruplicate, ± S.E., n = 13). D, real time reverse transcription PCR analysis of Bsep RNA in transfected cells. Total RNA was isolated from MDCK cells transfected with plasmids expressing BSEP-EYFP, p365, and pCon. Residual genomic and plasmid DNA was removed by treatment with RNase-free DNase. Real time RT-PCR analysis using two pairs of Bsep-specific primers revealed that Bsep RNA levels were not significantly different in cells containing p365 or pCon (two experiments in triplicate, n = 6). Negative controls included RNA isolated from cells that did not express Bsep and RNA which had been treated with DNase but had not undergone reverse transcription (R.T.). In all cases, real time PCR results were normalized to expression of endogenous canine β-actin RNA.

mediated receptor endocytosis (40, 41). To determine whether clathrin participates in BSEP internalization from the apical membrane, MDCK cells were co-transfected with a plasmid expressing dominant negative EPS15. EPS15 specifically interacts with epsin (42) and the AP-2 adaptor (43). The dominant negative EPS15-EH21 mutant, which lacks the amino-terminal EH (EPS15 homology) domains, inhibits clathrin-coated pit

Fig. 8. HAX-1 depletion extends BSEP residence in the apical membrane. Time course of metabolically labeled apical membrane BSEP in MDCK cells depleted of HAX-1. Cultures were transfected with plasmids that express BSEP-EYFP and Hax-1 RNAi (p365) (I) or control (pCon) (C). Transcripts. Cells were pulse-labeled and chased for the times indicated, and apical surface proteins were biotinylated. BSEP-EYFP was immunoprecipitated from cellular extracts, and aliquots separated by SDS-PAGE were visualized with a PhosphorImager (Total). Numbers on the left indicate the molecular mass in kDa for three BSEP-EYFP bands. Biotinylated BSEP-EYFP was extracted from immunoprecipitates by adsorption to avidin beads and was visualized and quantified by PhosphorImager (Apical). The data are representative of results obtained in three experiments. Apical BSEP-EYFP values from HAX-1-depleted cells (open boxes), which were significantly different from controls (black boxes), are marked with an asterisk (*, p < 0.05; **, p < 0.01, n = 3, except at 2- and 12-h time points in which n = 2).

Fig. 9. HAX-1 associates with cortactin in the apical membrane of hepatocytes. A, immunoblot of rat liver subcellular fractions stained for cortactin. tot, total liver extract; smv, sinusoidal membrane vesicles; cmv, canalicular membrane vesicles; gol, Golgi; mic, endoplasmic reticulum-enriched microsomes; mit, mitochondria; lys, lysosomes; ccv, clathrin-coated vesicles. B, cortactin co-immunoprecipitates with HAX-1 from CMV. Immunoblot of proteins immunoprecipitated from 0.5 mg of CMV protein with anti-HAX-1 Tu91 (Tu91) antiserum or normal rabbit serum (N.I.). The blot was cut and stained with anti-HAX-1 or anti-cortactin monoclonal antibodies. 15 μg of CMV protein were loaded in the cmv lane.
formation at the plasma membrane and receptor endocytosis (27). Expression of EH-21 EPS15 in MDCK cells resulted in a 94 ± 17% increase in apical membrane BSEP (Fig. 11), which indicates that clathrin participates in BSEP internalization from the apical membrane.

DISCUSSION

Trafficking of transporters in hepatocytes is rigorously controlled. Newly synthesized MDR1 and MDR2 traffic directly from the trans-Golgi network to the canalicular membrane (25, 26). BSEP travels from Golgi to intracellular compartment(s) from which it cycles to and from the apical domain (25). Recruitment and removal of canalicular membrane MDR trans-

Fig. 10. BSEP abundance in the apical membrane of MDCK cells is increased by expression of dominant negative cortactin. A, MDCK II cells were co-transfected with plasmids expressing BSEP-EYFP or a fusion protein (GFP-Δcort) consisting of GFP and dominant negative cortactin. Immunoblot of apically biotinylated proteins indicated apical membrane BSEP (apical) was 210 ± 13% (***, p < 0.01) higher in cells expressing GFP-Δcort compared with controls (B) (three experiments in triplicate n = 9). Expression of BSEP in the whole cell extracts (total) was increased by 28 ± 14% (total). C, real time RT-PCR analysis indicated that Bsep RNA levels were not significantly different in cells expressing GFP or GFP-Δcort (two experiments in triplicate, n = 6). Negative controls included RNA isolated from cells that did not express Bsep and RNA that had been treated with DNase but not had not undergone reverse transcription (R.T.). As before, real time PCR results were normalized to expression of the endogenous canine β-actin RNA.

Fig. 11. Dominant negative EPS15 increases BSEP abundance in the apical membrane of MDCK cells. A, MDCK cells were co-transfected with plasmids expressing BSEP-EYFP and fusion proteins containing GFP and dominant negative EPS15 (dnEPS15), GFP, and a noninhibitory EPS15 derivative (EPS15), or GFP alone (GFP). Immunoblots of biotinylated proteins revealed that cells expressing dnEPS15 contained 94 ± 17% (**, p < 0.01) more BSEP in the apical membrane relative to controls expressing GFP alone (n = 9, three experiments in triplicate) (B). Cells expressing EPS15 exhibited 21 ± 12% less apical membrane BSEP, which was not significantly different to controls. C, real time RT-PCR analysis of Bsep RNA. Total RNA isolated from MDCK cells expressing BSEP-EYFP and GFP, GFP-dnEPS15, or GFP-EPS15 was analyzed as described in Fig. 7. No significant differences in Bsep RNA levels were detected between cells expressing GFP, GFP-dnEPS15, or GFP-EPS15 (two experiments in triplicate, n = 6). Negative controls included RNA isolated from cells that did not express Bsep and RNA that had been treated with DNase but not had not undergone reverse transcription (R.T.). Real time PCR results were normalized to expression of the endogenous canine β-actin RNA.
lular fractions, which confirms that HAX-1 binds MDR proteins in mammalian cells.

HAX-1 specifically interacted with the linker domains of MDR1, MDR2, and BSEP and did not bind other intracellular loops. The linker domains were previously thought to represent unstructured connecting loops that span the homologous halves of MDR proteins; however, these regions also have regulatory functions. Linker domain ubiquination of halves of MDR proteins; however, these regions also have unstructured connecting loops that span the homologous loops. The linker domains were previously thought to represent MDR1, MDR2, and BSEP and did not bind other intracellular fractions, which confirms that HAX-1 binds MDR proteins.

The HAX-1 gene codes for a 34-kDa protein that is ubiquitously expressed in mammalian tissues but appears to have no orthologs or paralogs in Drosophila, Caenorhabditis elegans, or yeast. HAX-1 was first identified in a yeast two-hybrid screen as a binding partner for HS-1 (20), a kinase substrate, and a regulator of actin polymerization. HAX-1 also binds the HS-1 paralog cortactin/EMS1 (19) as well as other proteins (21–23, 51, 52).

HAX-1 is closely associated with cellular membranes. Immunofluorescence microscopy of cultured cells revealed that HAX-1 resides predominantly in endoplasmic reticulum, mitochondria, or plasma membrane (19, 20, 23). Analyses of liver subcellular fractions indicated that HAX-1 is predominantly in mitochondria, or plasma membrane (19, 20, 23). Analysis of liver subcellular fractions revealed that HAX-1 resides predominantly in endoplasmic reticulum, mitochondria, or plasma membrane (19, 20, 23).

The subcellular localization of HAX-1 is variable and depends on cell type. HAX-1 is associated with the endoplasmic reticulum of fibroblasts (19) and with mitochondria of B lymphoma cells (20) but not mitochondria purified from rat liver. In nonpolarized MDCK II cells, HAX-1 resides primarily in intracellular membranes and did not co-localize with BSEP or MDR1. With increasing cell polarity, HAX-1 shifted upward to the apical membrane, and co-localization with BSEP increased. Immunoblots of rat liver subcellular fractions revealed that HAX-1 was most highly enriched in CMV, ER microsomes, and clathrin-coated vesicles. HAX-1 co-immunoprecipitated with BSEP, MDR1, and MDR2 from CMV. Thus, HAX-1 associates with MDR transporters in the apical membrane of hepatocytes and polarized cells in culture.

To explore its role in BSEP trafficking, HAX-1 expression was ablated by RNAi. HAX-1 depletion was accompanied by a 71% increase in steady state apical membrane BSEP. Pulse-chase studies revealed no effect of HAX-1 RNAi on BSEP half-life or rates of translation, post-translational modification, or delivery to the apical membrane. However, apical membrane levels of metabolically labeled BSEP continued to rise in HAX-1-depleted cells after they declined in control cells. Apical membrane-labeled BSEP abundance remained higher than controls 4–5 h thereafter, suggesting that HAX-1 depletion retarded BSEP internalization (Fig. 8). Further support that the likely mechanism involves clathrin-mediated endocytosis is as follows: 1) BSEP and HAX-1 are present in clathrin-coated vesicles; 2) expression of dominant negative EPS15, which selectively blocks clathrin mediated endocytosis (27), doubled the apical membrane concentration of BSEP; and 3) expression of dominant negative cortactin also doubled the amount of BSEP in the apical membrane. Cortactin, an actin and HAX-1-binding protein, participates in clathrin endocytosis (41, 53).

Our studies suggest that the HAX-1 participates in previously unrecognized clathrin-mediated endocytosis of BSEP, and possibly other ABC transporters, from the apical plasma membrane. We are currently exploring HAX-1 interaction with specific components of the endocytic machinery.
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