Myosin II Regulatory Light Chain Is Required for Trafficking of Bile Salt Export Protein to the Apical Membrane in Madin-Darby Canine Kidney Cells*

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BSEP, MDR1, and MDR2 ATP binding cassette transporters are targeted to the apical (canalicular) membrane of hepatocytes, where they mediate ATP-dependent secretion of bile acids, drugs, and phospholipids, respectively. Sorting to the apical membrane is essential for transporter function; however, little is known regarding cellular proteins that bind ATP binding cassette proteins and regulate their trafficking. A yeast two-hybrid screen of a rat liver cDNA library identified the myosin II regulatory light chain, MLC2, as a binding partner for BSEP, MDR1, and MDR2. The interactions were confirmed by glutathione-S-transferase pulldown and co-immunoprecipitation assays. BSEP and MLC2 were overrepresented in a rat liver subcellular fraction enriched in canalicular membrane vesicles, and MLC2 colocalized with BSEP in the apical domain of hepatocytes and polarized WifB, HepG2, and Madin-Darby canine kidney cells. Expression of a dominant negative, non-phosphorylatable MLC2 mutant reduced steady state BSEP levels in the apical domain of polarized Madin-Darby canine kidney cells. Pulse-chase studies revealed that Blebbistatin, a specific myosin II inhibitor, severely impaired delivery of newly synthesized BSEP to the apical surface. These findings indicate that myosin II is required for BSEP trafficking to the apical membrane.

Localization of MDR1 (ABCB1), MDR2 (ABCB3 in humans) (ABCB4), and BSEP/SPGP (ABCB11) in the plasma membrane of hepatocytes is restricted to the apical domain, where the transporters mediate ATP-dependent secretion of essential biliary constituents. BSEP transports bile salts (1), MDR1 secretes small cationic hydrophobic drugs (2), and MDR2 mediates phospholipid transfer into bile (3, 4). Genetic defects in BSEP produce progressive familial intrahepatic cholestasis type II (PFIC II) (5), and transfer into bile (3, 4). Genetic defects in BSEP produce progressive familial intrahepatic cholestasis type II (PFIC II) (5), and transfer into bile (3, 4). Genetic defects in BSEP produce progressive familial intrahepatic cholestasis type II (PFIC II) (5), and transfer into bile (3, 4).

Materials and Antibodies—Cytotrap yeast two-hybrid system and vectors were purchased from Stratagene (La Jolla, CA), pEGFP-N1 and pEYFP-N2 vectors from Clontech (Palo Alto, CA), and pMal-p2x from New England Biolabs (Beverly, MA). Monomeric pDsRed-N1 vector was a gift from Dr. Roger Tsien. Easytag NEG-772 [35S] methionine and cysteine protein labeling mix were from PerkinElmer Life Sciences (Boston, MA). c219 monoclonal antibody was obtained from Signet (Dedham, MA); fluorescently labeled secondary antibodies were from Jackson Immunoresearch (West Grove, PA) and Molecular Probes (Eugene, OR). Goat anti-MLC2 antibody (MLCA-20) and rabbit anti-phospho MLC (MLCP) were from Santa Cruz Biotechnology (Santa Cruz, CA). anti-non-muscle myosin II heavy chain BT561 antiserum was from Biomedical Technologies (Stoughton, MA). Anti-FLAG M5 monoclonal antibody was from Sigma, and anti-MBP rabbit antibodies were from New England Biolabs. LVT90/Tu41 anti-BSEP antibodies

1 The abbreviations used are: MDCK, Madin-Darby kidney cells; BSEP, bile salt export protein; ABC, ATP binding cassette; CMV, canalicular membrane vesicles; DsRed, monomeric Discosoma sp. Red fluorescent protein; EYFP, enhanced yellow fluorescent protein; GSH, glutathione; GST, glutathione S-transferase; MBP, maltose-binding protein; MDR, multidrug resistance protein; Mlc2, myosin II regulatory light chain; MHC, myosin II heavy chain; PBS, phosphate-buffered saline; WT, wild-type.
were prepared (12). Streptavidin-agarose beads, sulfo-NHS-LC-LC-biotin, and dithiobis(succinimidylpropionate) were purchased from Pierce. Biotinylated GST was obtained from Calbiochem. All other reagents were from Sigma.

Plasmids—Plasmids used in yeast two-hybrid analyses and GST fusion proteins have been described (15). The pMP47–5 plasmid isolated in the yeast two-hybrid screen contains a full-length rat Mlc2 cDNA (GenBank™ accession X05566). Mlc2 cDNA was excised from pMP47–5 with XbaI and Xhol and transferred to pFLAG-CMV to generate pMP47–5. Mlc2 cDNA excised with EcoR1 and Sall was subcloned into pMAL-p2X to produce pMAL-Mlc2 cDNA inserts from pMal-EYFP, pMal2-WT-GFP, and pMal2-AA-GFP (18) were transferred to pmDSRed-N1, which encodes a monomeric DsRed fluorescent protein (19), to generate pBsep-DeRd, pMlc2-WT-DeRd, and pMlc2-AA-DeRd. Yeast Two-hybrid Screen—A PCR DNA fragment coding for amino acids 653–741 of rat Bsep was cloned into pSOS vector (Stratagene) to generate bait plasmid pSL49. 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-sensitive cdc25h yeast with pSOS bait plasmids. Yeast grown for 24–48 h at 25 °C were replica-plated on galactose plates and incubated at non-permissive temperature of 37 °C for 3–5 days. Individual colonies that grew at 37 °C were picked and expanded in liquid medium at 25 °C. Washed cells were spotted on galactose or glucose solid medium and incubated at 37 °C.

GST Pulpdolvents—GST-agarose beads (Amersham Biosciences) were used to purify GST, GST-MDR2, and GST-BSEP fusion proteins from BL-21 Escherichia coli harboring plasmids pGEX5–3, pS.L27, or pS.L25 (15). For binding to GST-MDR2, 5 mg of protein from a rat liver homogenate 2000 × g pellet were solubilized for 30 min in binding buffer containing 1% Triton X-100, protease inhibitors (10 µg/ml pepstatin, 1 µg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 5 µM benzamidine, 0.1 µM aprotinin), 50 mM KPO4, and either 250, 350, or 450 mM NaCl and centrifuged at 13,000 × g. Supernatants were incubated for 2–3 h with 10 µg of GST or GST-MDR2 proteins bound to GSH-Sepharose beads. The beads were washed four times with the corresponding binding buffers, and bound proteins were eluted with SDS loading buffer and subjected to Western blot analyses (20). Maltose-binding protein (MBP) and MBP-MLC2 were purified from Escherichia coli according to the manufacturer’s instructions. GSH-Sepharose beads coated with 1 µM of GST or GST-BSEP were incubated for 2 h with 100 ng of MBP or MBP-MLC2 in PBS 0.1% Triton X-100. The beads were washed three times with PBS 0.1% Triton X-100. Bound proteins were separated by SDS-PAGE and immunoblotted with anti-MBP antibodies (New England Biolabs).

Immunofluorescence Microscopy and Culture and Transfection of Mammalian Cells—Rat liver cryosections were prepared and immunostained (20). Semi-thin sections were fixed in cold methanol and incubated with MLC2-P and e219 antibodies or normal rabbit serum and normal mouse IgG. Primary antibodies were labeled with Alexa-488 anti-IgG and Alexa-594 anti-mouse secondary antibodies. WI69 and HepG2 cells were cultured on poly-l-lysine-coated glass coverslips, which were washed in PBS, fixed in methanol, and blocked for 1 h in 3% bovine serum albumin and 3% normal donkey serum in Dulbecco’s PBS. Cells were then incubated 1–2 h with goat anti-MLC-A20 and mouse anti-MDR1 (e219) antibodies diluted in IF buffer (3% bovine serum albumin in Dulbecco’s PBS). Coverslips were washed ten times in PBS, blocked for 30 min with IF buffer, and incubated with Texas red-labeled donkey anti-goat and fluorescein-labeled donkey anti-mouse antibodies for 45 min. Coverslips were washed ten times in PBS and mounted using Prolong anti-quench mounting medium. Mounted tissue sections and cells were viewed with a Hamamatsu digital camera on an Axiovert 10 epifluorescence microscope. MDCKII cells (gift from Dr. Enrique Rodriguez-Boulan) were grown in transwells (Corning) and transfected using Lipofectamine 2000 (Invitrogen). Transwells were washed with PBS and fixed for 10 min in ice-cold methanol. Filters were excised, washed ten times with PBS, and mounted on glass slides. Fluorescent proteins were viewed with a Leica TIR2 laser confocal microscope.

Preparation of Membrane Fractions, Immunoblot Analysis, and Immunoprecipitation of Rat Liver Subcellular Fractions—Membrane, immunoblotting, and immunoprecipitations from rat liver fractions were performed as indicated (20). Enrichment of marker proteins was determined by immunoblot analyses, except for lysosomes and mitochondria, which were measured by biochemical enzyme assay. Rat liver lysosomes (21) and mitochondria (22) were a gift from Dr. Ana Maria Cuervo. MDCK cells cultured in six-well plates were transfected with pBsep-DeRd, or pmDeRd, pMal2-FLAG, or pFLAG-CMV. Two days later cells were washed twice with PBS and incubated for 30 min at room temperature with 100 µM dithiobisuccinimidylpropionate. Cells were washed with 50 mM glycine in PBS and lysed for 1 h in 2 × PBS 1% Triton X-100, 10 mM glycine. Homogenates were prepared by douncing 10 strokes in a glass homogenizer and centrifuging for 30 min at 10,000 × g. Supernatants were incubated for 1 h with protein G-Sepharose beads, which were removed by centrifugation. Supernatants were incubated with 10 µl of Tu41 antisera or normal rabbit serum overnight. Antibodies were recovered with 50 µl of protein G-Sepharose beads, which were washed four times with 2 × PBS 0.25% Triton X-100. Immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted.

Biotinylation and Pulse-Chase Metabolic Labeling—Cell surface biotinylation and pulse-chase labeling of proteins in MDCK cells were performed as described (20, 23) for the time periods indicated. Blebbistatin was dissolved in Me2SO to produce a 10–mM stock solution that was stored under nitrogen. For myosin II inhibition, transwells were preincubated for 30 min before the pulse in medium containing 100 µM Blebbistatin or Me2SO. Inhibitor was maintained throughout the pulse and chase periods.

**FIG. 1. Interaction of MLC2 with BSEP, MDR1, and MDR2 in yeast.** A, diagram outlining the structure of MDR-type proteins. Hatched bars represent the twelve transmembrane helices (TM), shaded boxes indicate the two nucleotide binding domains (NBD), and the black box represents the linker region (Link). B, yeast two-hybrid interaction of MLC2 with different bait chimeras. The pM47–5 plasmid, which contains the full-length Mlc2 cDNA in pMYR, was transformed into yeast with pSOS plasmids carrying rat BSEP (pSL47), MDR2 (pSL49), and MDR1A (pSL67) linker domains. Yeast grown in liquid medium at 25 °C were spotted on galactose (gal) or glucose (gld) solid medium and incubated at 37 °C, which is a non-permissive temperature for growth of the cdc25h temperature-sensitive mutant. Pry expresion is under control of the Gad1 promoter, and no growth is expected on glucose medium. Controls included empty pSOS vector and pSOS containing 40 amino acids from the amino terminus (pSL55) or 30 residues of the carboxyl terminus of MDR2 (pSL65). SOS bait plasmids were also transformed into yeast in the company of the empty pMyr vector. C, mapping of the MLC2 binding region in the BSEP linker domain. Two hybrid analyses were done with constructs expressing truncated versions of the BSEP linker opposite the pM47–5 plasmid, which expresses full-length MLC2. The thick boxes represent the 90-amino acid BSEP linker. Plus and minus signs designate the relative growth of yeast at 37 °C. All deletion constructs were co-transformed into yeast with the pMYR empty vector to test for false positive growth. D, mapping of the MLC2 binding region in the MDR2 linker.
RESULTS

The Linker Domains of MDR1, MDR2, and BSEP Interact with MLC2 in Yeast—A yeast two-hybrid screen of a rat liver cDNA library was performed using the BSEP linker domain as bait. The linker resides immediately downstream of the first nucleotide binding domain and connects the homologous halves of BSEP (Fig. 1A) (24). The screen identified a full-length cDNA that encodes the rat non-muscle regulatory myosin II light chain MLC2 as a BSEP interacting protein. Mlc2 cDNAs were isolated on three separate occasions.

Plasmid pM47–5, which contains the full-length Mlc2 cDNA, permitted growth at 37 °C of yeast that harbored plasmids expressing BSEP (pSL47), MDR2 (pSL49), and MDR1a linkers (pSL47) (Fig. 1B). It did not permit growth of yeast carrying the empty pSOS vector, pSOS-Coll (a negative control that expresses amino acids 148–357 of murine type IV collagenase), or pSOS fusions that contain the amino-terminal 40 amino acids of MDR2 (pSL55) or the carboxyl-terminal 35 residues of MDR2 (pSL56). Thus, MLC2 interacts with the linker domains of three MDR subfamily members. Two-hybrid analyses of pSL47 and pSL49 deletion derivatives revealed that MLC2 binds GST-BSEP but not GST alone (lower panel). MLC2 binds directly with BSEP interacting protein GST-BSEP (GST fused to 90 amino acids of the BSEP linker domain). GST-MDR2 and GST-BSEP fusion proteins were used in the assay (of the immunoblot membrane confirmed that equivalent amounts of MLC2 and MBP were loaded in the indicated that MBP-MLC2 binds GST-BSEP but not GST alone (separated by SDS-PAGE). Immunoblotting with anti-MBP antibodies alone (GST-BSEP, or GST alone, were incubated with MBP-MLC2 (chimera (MBP-MLC2) binds GST-BSEP fusion protein (GST fused to 90 amino acids of the MDR2 linker domain) were separated by SDS-PAGE. Immunoblotting with anti-MBP antibodies indicated that MBP-MLC2 binds GST-BSEP but not GST alone (lower panel). MBP alone does not bind GST-BSEP or GST. Purified MBP-MLC2 and MBP were loaded in the first two lanes. Coomassie staining of the immunoblot membrane confirmed that equivalent amounts of GST and GST-BSEP were used in the assay (upper panel).

FIG. 2. MLC2 binds GST-MDR2 and GST-BSEP fusion proteins. A, proteins extracted from rat liver homogenates by agarose beads adsorbed with GST alone (G) or with GST-MDR2 (M) (GST fused to 79 amino acids of the MDR2 linker domain) were separated by SDS-PAGE. Immunoblotting revealed that GST-MDR2 extracted MLC2 from liver homogenates but GST alone did not (lower panel). Because myosin II complexes precipitate out of solution at salt concentrations below 200 mM, GST beads were incubated with liver extracts and washed in the presence of 250, 350, and 450 mM NaCl. Coomassie staining of the nylon membrane (upper panel) indicated that equivalent amounts of GST and GST-MDR2 were used in the pulldown assays. A rat liver homogenate (total lane was included as control. B, a maltose-binding protein-MLC2 chimera (MBP-MLC2) binds GST-BSEP fusion protein (GST fused to 90 amino acids of the BSEP linker domain). Agarose beads adsorbed with GST-BSEP, or GST alone, were incubated with MBP-MLC2 (lc) or MBP alone (mb) purified from E. coli. Proteins bound to washed beads were separated by SDS-PAGE. Immunoblotting with anti-MBP antibodies indicated that MBP-MLC2 binds GST-BSEP but not GST alone (lower panel). MBP alone does not bind GST-BSEP or GST. Purified MBP-MLC2 and MBP were loaded in the first two lanes. Coomassie staining of the immunoblot membrane confirmed that equivalent amounts of GST and GST-BSEP were used in the assay (upper panel).

GST-MDR2 and GST-BSEP Fusion Proteins Bind MLC2—GSH-agarose beads adsorbed with a chimeric GST protein containing 79 amino acids of the MDR2 linker, or with GST alone, were incubated with rat liver homogenates. GST-MDR2 beads effectively extracted MLC2 from liver homogenates, whereas GST alone did not (Fig. 2A). The interaction was robust, and GST-MDR2 was capable of binding MLC2 in the presence of 0.5 M salt. GSH-agarose beads coated with GST-BSEP, which contains 90 amino acids of the BSEP linker, were incubated with purified MBP or an MBP-MLC2 chimera. Immunoblots of bound proteins revealed that MBP-MLC2 interacted with GST-BSEP, but not with GST alone (Fig. 2B). MBP alone did not bind GST-BSEP or GST alone. These experiments, together with the yeast two-hybrid results, indicate that MDR2 and BSEP linker domains interact directly with MLC2.
MDR1 and MDR2 transporters interact with the myosin II, which is a heterohexamer composed of two heavy chains, two essential light chains, and two regulatory light chains, two essential light chains, and two regulatory light chains. Immunoprecipitates of the transporters also precipitated myosin heavy chain (Fig. 5, top panel). MLC2 detection in cell lines, MLC2 colocalized with MDR1 in abundant pericanalicular vesicles. Close proximity of these vesicles to the canalicular membrane prevented resolution as to whether MLC2 and the transporters colocalize in the canalicular membrane.

Myosin II Co-immunoprecipitates with BSEP from Mammalian Cell Extracts—BSEP was immunoprecipitated from MDCK cells co-transfected with plasmids expressing BSEP-DsRed and MLC2 tagged with the FLAG peptide. Immunoblot analysis revealed that immunoprecipitates contained MLC2 in addition to BSEP (Fig. 3). MLC2 was not immunoprecipitated from cells that did not express BSEP, indicating that anti-BSEP Tu41 antibody does not cross-react with MLC2. Co-immunoprecipitation indicates that MLC2 and BSEP interact in mammalian cells.

MLC2 Is Enriched in the Canalicular Domain of Hepatocytes, WifB9, and HepG2 cells—Immunofluorescence microscopy of rat liver sections revealed that MLC2 was enriched in the apical domain of hepatocytes. Colocalization of MLC2 and MDR1/2 fluorescence was most pronounced in pericanalicular vesicles rather than in the canalicular membrane (Fig. 4A). The hepatoma-derived WifB9 (25) and HepG2 (26, 27) cell lines polarize in culture to form canalicular structures between cells that are functionally and structurally analogous to the bile canaliculus of hepatocytes. MLC2 is enriched in the apical domain of WifB9 (Fig. 4B) and HepG2 (Fig. 4C) cells. In both hepatocytes and cell lines, MLC2 colocalized with MDR1 in abundant pericanalicular vesicles. Close proximity of these vesicles to the canalicular membrane prevented resolution as to whether MLC2 and the transporters colocalize in the canalicular membrane.

Myosin II Co-immunoprecipitates with BSEP and MDR1/2 from Rat Liver CMV—Immunoblot analyses of rat liver subcellular fractions indicated that BSEP, MDR1, MDR2, MLC2, and myosin II heavy chain were enriched in canalicular membrane vesicles (CMV) (Fig. 5A). BSEP or MDR1/2 were immunoprecipitated from CMV using Tu41 or c219 antibodies, respectively. Immunoprecipitates were examined for subunits of myosin II, which is a heterohexamer composed of two heavy chains, two essential light chains, and two regulatory light chains. Immunoprecipitation of the transporters also precipitated myosin heavy chain (Fig. 5, B and C). MLC2 detection in immunoprecipitates was obscured by IgG light chains, which co-migrated with MLC2 in SDS-PAGE gels. Tu41 and c219 antibodies did not precipitate myosin heavy chain from liver fractions that contained myosin II but were devoid of BSEP, indicating that anti-BSEP and anti-MDR1 antibodies do not cross-react with myosin II. These data indicate that BSEP, MDR1, and MDR2 transporters interact with the myosin II holoenzyme in hepatocytes.

Expression of a Non-phosphorylatable MLC2 Mutant Decreases Apical Membrane BSEP in MDCK Cells—MLC2 phosphorylation at threonine 18 and serine 19 residues positively regulates myosin II activity (28). Plasmids expressing a non-phosphorylatable MLC2 mutant (MLC-AA-GFP), in which Thr-18 and Ser-19 were replaced by alanine, or wild-type MLC2 (MLC-WT-GFP) fused to GFP, were cotransfected into MDCK cells with constructs expressing BSEP-DsRed. Confocal fluorescence microscopy of polarized MDCK monolayers revealed that more BSEP-DsRed resides within cells expressing MLC-AA-GFP than in controls transfected with MLC-WT-GFP, in which BSEP displays a predominantly apical localization. MLC-WT-GFP exhibits an apical punctate distribution, which is not seen with MLC-AA-GFP, and colocalizes more extensively with BSEP-DsRed than does the non-phosphorylatable mutant.

Biotinylation of surface proteins in transfected MDCK cells revealed that plasma membrane BSEP-EYFP resides exclusively in the apical domain (15). Apical BSEP-EYFP levels in cells co-transfected with MLC-AA-GFP were less than half (45 ± 6%) of those observed in controls expressing MLC-WT-GFP (Fig. 7A and B). Expression of MLC-AA-GFP did not
Figure 7. Non-phosphorylatable MLC-AA-GFP decreases BSEP abundance in the apical membrane of MDCK cells but does not affect transepithelial permeability. A, immunoblot of surface-biotinylated proteins isolated from MDCK cells transfected with plasmids expressing BSEP-EYFP and MLC-WT-GFP or MLC-AA-GFP. Apical surface proteins were labeled by addition of biotinylation reagent to upper transwell chambers. Total extracts (Total) and apical membrane proteins (Apical), which were isolated by adsorption to streptavidin-agarose, were immunoblotted with the anti-BSEP Tu41 antibody. Densitometric analyses of immunoblots indicated that MLC-AA-GFP significantly decreased apical membrane BSEP-EYFP expression levels, and biotinylated apical membrane BSEP in transfected MDCK cells incubated with 100 μM Blebbistatin (Blebsntn +) or with Me2SO (−). Cells were pulse-labeled, chased for the time periods indicated, and apical surface proteins were biotinylated. BSEP-EYFP was immunoprecipitated from cellular extracts, and aliquots separated by SDS-PAGE were visualized by phosphorimaging (Total). Biotinylated BSEP-EYFP was extracted from immunoprecipitates by adsorption to avidin beads and was visualized and quantified by phosphorimaging (Apical). B, relative surface BSEP-EYFP values (values are relative to the mean of MLC-WT-GFP measurements in each of three experiments done in triplicate, n = 9, means ± S.E., p values are from a Student’s t test; **, p < 0.005). C, MLC-AA-GFP expression did not increase transepithelial mannitol permeability of MDCK monolayers. 3H-Mannitol values from cells incubated with Blebbistatin (black boxes) were significantly different from controls (gray boxes) after 2, 3, and 4 h of chase (**, p < 0.005, n = 3). Values are relative to apical levels in control cells after 2 h of chase. Total labeled BSEP-EYFP values were not significantly different in Blebbistatin-treated and control cells.

**DISCUSSION**

The MLC2 gene product was identified as a binding partner for BSEP, MDR1, and MDR2 in a yeast two-hybrid screen. Interactions were confirmed in vitro by GST pulldown assays and in mammalian cells by co-immunoprecipitation. MLC2 encodes a 20-kDa regulatory light chain subunit of the non-muscle myosin II holoenzyme, which consists of two heavy chains, two regulatory light chains, and two essential light chains, two regulatory light chains, and two essential light chains. The MLC2 gene product was identified as a binding partner for BSEP, MDR1, and MDR2 in a yeast two-hybrid screen. Interactions were confirmed in vitro by GST pulldown assays and in mammalian cells by co-immunoprecipitation. MLC2 encodes a 20-kDa regulatory light chain subunit of the non-muscle myosin II holoenzyme, which consists of two heavy chains, two regulatory light chains, and two essential light chains, two regulatory light chains, and two essential light chains.
MLC2 Regulates BSEP Trafficking

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