Rab11a and myosin Vb are required for bile canalicular formation in WIF-B9 cells

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Hepatocytes polarize by forming functionally distinct sinusoidal (basolateral) and canalicular (apical) plasma membrane domains. Two distinct routes are used for delivery of membrane proteins to the canaliculus. Proteins having glycosylphosphatidylinositol anchors or single transmembrane domains are targeted to the sinusoidal plasma membrane from where they transcytose to the canalicular domain. In contrast, apical ATP-binding-cassette (ABC) transporters, which are required for energy-dependent biliary secretion of bile acids (ABCB11), phospholipids (ABCB4), and nonbile acid organic anions (ABCC2), lack initial residence in the basolateral plasma membrane and traffic directly from Golgi membranes to the canalicular membrane. While investigating mechanisms of apical targeting in WIF-B9 cells, a polarized hepatic epithelial cell line, we observed that rab11a is required for canalicular formation. Knockdown of rab11a or overexpression of the rab11a-GDP locked form prevented canalicular formation as did overexpression of the myosin Vb motorless tail domain. In WIF-B9 cells, which lack bile canaliculi, apical ABC transporters colocalized with transcytotic membrane proteins in rab11a-containing endosomes and, unlike the transcytotic markers, did not distribute to the plasma membrane. We propose that polarization of hepatocytes (i.e., canalicular biogenesis) requires recruitment of rab11a and myosin Vb to intracellular membranes that contain apical ABC transporters and transcytotic markers, permitting their targeting to the plasma membrane. In this model, polarization is initiated upon delivery of rab11a-myosin Vb-containing membranes to the surface, which causes plasma membrane at the site of delivery to differentiate into apical domain (bile canaliculus).

hepatocyte polarization | plasma membrane segregation

Polarization of hepatocytes involves formation of functionally distinct sinusoidal (basolateral) and canalicular (apical) plasma membrane domains that are separated by tight junctions. Bile is secreted across the canalicular membrane. Apical ATP binding cassette (ABC) transporters are required for energy-dependent biliary secretion of bile acids [bile salt export pump (BSEP) and ABCB11] (1), phospholipids (MDR3 and ABCB4) (2), sterols (ABCG5 and ABCG8) (3), and nonbile acid organic anions (MRP2 and ABCC2) (4, 5). Insufficient amounts of ABC transporters in the canalicular membrane impair biliary secretion, resulting in cholestasis (i.e., bile secretory failure) (6).

Canalicular membrane proteins use two distinct delivery routes from Golgi membranes. Proteins having glycosylphosphatidylinositol anchors or single transmembrane domains are targeted to the sinusoidal plasma membrane and subsequently undergo transcytosis to the canalicular domain (7, 8). In contrast, newly synthesized apical ABC transporter traffic from the Golgi membranes to the canalicular membrane without initial residence in the sinusoidal plasma membrane (9–11). Studies in WIF-B9 cells, a polarized hepatic epithelial cell line (12, 13), revealed that apical ABC transporters colocalize with rab11a and constitutively cycle between rab11a-positive endosomes and the canalicular membrane (14).

Because rab11a has been implicated in apical targeting in polarized epithelial cells (15–17), we used WIF-B9 cells to determine its role in constitutive apical cycling and canalicular biogenesis.

Materials and Methods

Construction and Generation of Recombinant Adenovirus. Rab11aS25N-cyan fluorescent protein (CFP) was provided by Juan Bonifacino (National Institutes of Health). KIAA1119, which encodes human myosin Vb, was obtained from the Kazusa DNA Institute, Chiba, Japan. The myosin Vb tail domain (18) was constructed by PCR and subcloned into CFP-C1 (Clontech). Recombinant adenovirus was generated (19) and purified (20). RNA interference was performed by cloning the annealed sequences 5′-caagaagcatccaggttga-3′ and 5′-tcaacctggatgct-tcttg-3′ into RNAi-Ready pSIREN-RetroQ Vector (Clontech).

Cell Culture, Transfection, and Adenoviral Infection. WIF-B9 cells were cultured in a humidified 7% CO_2 incubator at 37°C (14). Unless otherwise specified, cells were plated on a 35-mm glass bottom dish (MatTek) at 1.4×10^5 cells per dish in complete medium. Infections with recombinant adenoviruses encoding rab11aS25N-CFP, myosin Vb tail CFP, or BSEP-yellow fluorescent protein (YFP) were performed as described in ref. 14. From 30–50% of cells were infected. To generate rab11a knockdown WIF-B9 stable cell lines, cells were plated on a 10-cm dish and transfected by using Lipofectamin 2000 (Invitrogen) according to the manufacturer's instructions. After isolation of stable colonies, rab11a expression was determined by immunoblot analysis.

Antibodies and Immunofluorescence. Mouse monoclonal antibodies against HA321 and 5′ nucleotidase (5′NT) were provided by Ann Hubbard (The Johns Hopkins University School of Medicine, Baltimore), Rabbit antiserum against cell adhesion molecule 105 (cCAM105) was provided by Sue-Hwa Lin (University of Texas, M. D. Anderson Cancer Center, Houston). Rabbit antiserum against myosin Vb was provided by John Hammer (National Institutes of Health). Other primary antibodies were mouse monoclonal antibodies to MRP2 (M2III-6, Kamiya Biomedical), ZO-1 and E-cadherin (BD Biosciences), and rabbit polyclonal antibody to rab11a (Zymed Laboratories). Affinity-purified secondary antibodies were from Jackson ImmunoResearch or Molecular Probes. Other chemicals were commercial products. Immunofluorescence was performed as described in ref. 14.

Confocal Fluorescence Microscopy. Live and fixed cell image analysis was performed by using a Zeiss 510 inverted confocal microscope with a ×63 oil immersion objective NA1.4. Live cells

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Abbreviations: ABC transporter, ATP binding cassette transporters; BSEP, bile salt export pump; cCAM105, cell adhesion molecule 105; CFP, cyan fluorescent protein; 5′NT, 5′nucleotidase; YFP, yellow fluorescent protein.

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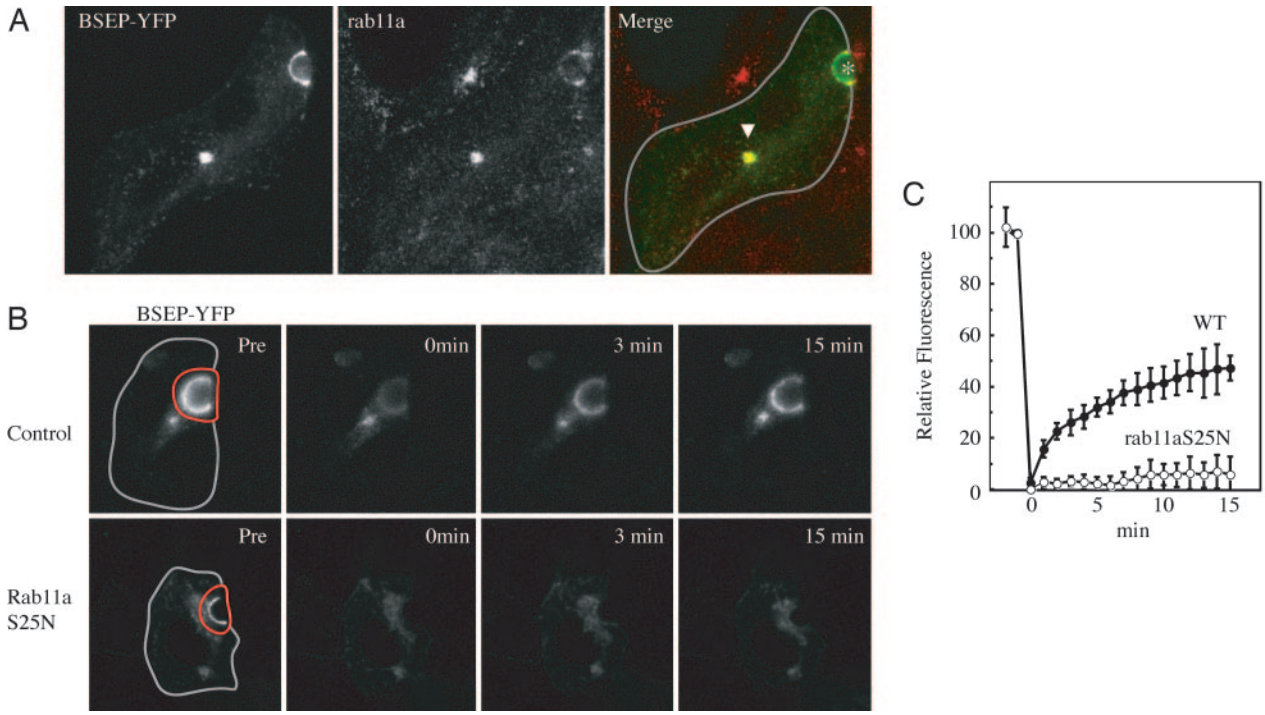

Fig. 1. Rab11a requires constitutive apical cycling in polarized WIF-B9 cells. (*A*) BSEP-YFP localized canalicular membrane and rab11a-positive endosomes. WIF-B9 cells infected with BSEP-YFP were immunostained with rab11a antibody. BSEP localized in canalicular membrane (asterisk) and rab11a-positive endosomes (arrowhead). Gray line shows the basolateral membrane. (*B*) Rab11aS25N inhibit constitutive BSEP apical cycling. On the third day in culture, WIF-B9 cells were infected with adenovirus encoding BSEP-YFP, which was abundantly expressed in the bile canaliculus within 3 days, at which time the cells were infected with or without adenovirus that encoded rab11aS25N-CFP. WIF-B9 cells infected with adenovirus, which encoding BSEP-YFP and/or rab11aS25N-CFP, were used for live cell imaging by confocal fluorescent microscopy at 37°C. The area enclosed by the red line was photobleached. Time in *Center Left*, *Center Right*, and *Right* denotes minutes after bleach. Gray line identifies the basolateral membrane. (*C*) Quantification of BSEP-YFP recovery in the canalicular membrane region after photobleaching. Mean fluorescence intensity was determined at the times indicated. $n = 10 \pm$ SD. Fluorescence recovery after photobleaching was abolished by rab11aS25N-CFP expression in polarized WIF-B9 cells.

were maintained at 37°C by using an ASI 400 Air Stream Incubator (Nevtek). For photobleaching experiments, the confocal pinhole was fully open. BSEP-YFP molecules were excited at 514 nm and imaged at 520–600 nm. Selective photobleaching was performed with 488 and 514 nm laser line at full power. Images were captured at 60–120 sec intervals. For imaging of vesicle movement, the confocal pinhole was set 2 Airy. Images were captured at 1-sec intervals. For imaging of fixed cells, the confocal pinhole was set 1 Airy.

Image Analysis. Sequence images were exported as single TIFF files. Quantification of mean fluorescence intensity in selected regions of the bile canaliculus was performed by using NIH IMAGE 1.62. A Zeiss LSM image examiner was used to analyze endosome area (pixel number) in a selected area of the perinuclear region. All measurements of fluorescence intensity were corrected for background. Movies were produced by using NIH IMAGE 1.62 or QUICKTIME 5.0 (Apple Computer). Contrast of original images was increased in some experiments. Images were processed with PHOTOSHOP 6.0 (Adobe Systems).

Analysis of Canalicular Formation. As a measure of polarization, canalicular formation was assessed by counting the number of canaliculi as visualized by bright field microscopy and belt-like tight junctions identified by ZO-1 staining (12). Adherens junctions identified by E-cadherin staining were present throughout the plasma membrane in polarized and nonpolarized cells (Fig. 5, which is published as supporting information on the PNAS web site), as was observed in ref. 13. In WIF-B9 cells, canalicular formation is progressive (see Fig. 2*E*) (13). To examine the role of rab11a and myosin Vb in canalicular biogenesis, WIF-B9 cells were cultured for 3 days, at which time ≈30% of cells formed bile canaliculi. Cells were then infected with adenovirus encoding BSEP-YFP, rab11aS25N-CFP, or myosin Vb tail-CFP. At day 7, at which time ≈70% of wild-type WIF-B9 cells formed bile canaliculi, cells were fixed and stained for ZO-1. Adenovirus-infected cells were examined by using bright field imaging and fluorescence imaging of belt-like tight junctions based on ZO-1 staining, including Z sectioning, which permitted examination of the entire cell. Results with both methods agreed within 5%. Data from at least six independent experiments were averaged, and standard deviation was calculated.

Results

Rab11a Is Required for Constitutive Apical Cycling in WIF-B9 Cells. Because WIF-B9 cells are resistant to transient transfection, we used adenovirus encoding BSEP-YFP and rab11aS25N-CFP GDP-locked form, which acts as a dominant negative (17, 21), to determine the role of rab11a in constitutive apical cycling. Polarized WIF-B9 cells showed steady-state colocalization of BSEP-YFP and rab11a adjacent to the canalicular membrane and in intracellular sites, including a juxtanuclear structure adjacent to the microtubular organizing center (Fig. 1*A*) (14). On the third day in culture, WIF-B9 cells were infected with adenovirus-encoding BSEP-YFP, which was abundantly expressed in the bile canaliculus within 3 days, at which time the cells were infected with adenovirus, which encoded rab11aS25N-CFP. Photobleaching experiments were performed 24 h later, at which time canalicular BSEP-YFP fluorescence had not substantially declined. Photobleaching of BSEP-YFP in the cana-

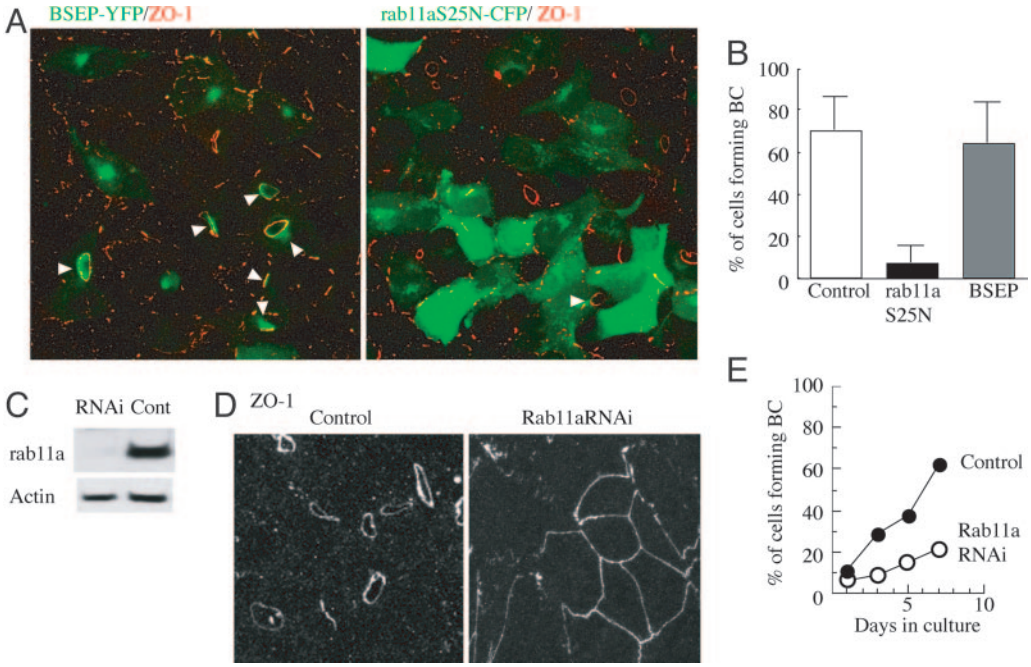

Fig. 2. Rab11a is required for canalicular formation. (*A*) Effect of rab11aS25N expression on canalicular formation. At day 3 in culture, WIF-B9 cells were infected with adenovirus encoding rab11aS25N-CFP or BSEP-YFP. At day 7 in culture, the cells were immunostained with ZO-1, a tight junction marker. White arrowheads denote bile canaliculi in cells infected with adenovirus encoding rab11aS25N-CFP or BSEP-YFP. BSEP-YFP expression was a control for adenoviral infection. Percentage of WIF-B9 cells expressing rab11aS25N or BSEP-YFP ranged from ≈30% to ≈50% and averaged ≈40%. (*B*) Rab11aS25N expression reduced canalicular formation. The percentage of WIF-B9 cells manifesting bile canaliculi on day 7 after infection with adenovirus-encoding rab11aS25N-CFP or BSEP-YFP was determined. BSEP-YFP expression was a control for adenoviral infection. Percentage of cells forming BC refers to all cells in the control and only infected cells expressing rab11aS25N or BSEP. $n = 6 \pm$ SD. (*C*) WIF-B9 cells transfected with rab11a RNA interference (RNAi) vector were cloned. Equal protein loadings of homogenates from control cells or cells expressing short hairpin RNAs directed against rab11a were analyzed by SDS/PAGE before immunoblotting with the indicate antibody. β-actin served as the internal control. (*D*) The effect of rab11a knockdown on canalicular formation. WIF-B9 control and rab11a knockdown cells were cultured for 7 days and immunostained for ZO-1. Canalicular formation was evident in control cells but was rarely detected in rab11a knockdown cells. (*E*) Rab11a knockdown cells have decreased canalicular formation. WIF-B9 control and rab11a knockdown cells were immunostained for ZO-1 at day 1, 3, 5, and 7. Percentage of cells forming BC refers to all cells.

licular area was followed by delayed fluorescence recovery in rab11aS25N-CFP expressing cells compared with control cells (Fig. 1 *B* and *C*) (14). The steady-state distribution of BSEP-YFP was not qualitatively changed.

Live-cell imaging revealed that BSEP-positive endosomes trafficked throughout the cytoplasm. Endosome movement was abolished by nocodazole but not cytochalasin treatment (data not shown), as previously observed in control cells (14). These results demonstrate that rab11a is required for targeting of BSEP to the canalicular membrane and suggest its role in constitutive apical cycling.

The Role of rab11a in Canalicular Biogenesis. To examine the consequences of impaired apical targeting for canalicular biogenesis, WIF-B9 cells in culture for 3 days were infected with adenovirus-encoding rab11aS25N-CFP or BSEP-YFP. At day 7, no significant differences in growth characteristics were observed among these cells. Quantification of the number of polarized cells based on the presence of bile canaliculi by bright field examination and ZO-1 staining revealed that ≈70% of control or BSEP-YFP expressing cells were polarized. By contrast, only 10% of rab11aS25N-CFP-expressing cells were polarized (Fig. 2 *A* and *B*). Expression of rab11aS25N decreased the percentage of cells forming canaliculi to levels below that of day 3 cultured WIF-B9 cells, 30% of which form canaliculi. These results suggest that rab11a is required for canalicular biogenesis and, perhaps, maintenance.

To examine further the role of rab11a in canalicular biogenesis, we developed stable rab11a knockdown WIF-B9 cells by using short hairpin RNA expression constructs. Rab11a knockdown cells continued to grow at the same rate as did wild-type cells (data not shown), even when rab11a protein expression was severely reduced (Fig. 2*C*). Using bright field examination and ZO-1 staining (Fig. 2 *D* and *E*), we quantified canalicular formation in rab11a knockdown cells. In control cells, ZO-1 staining revealed belt-like tight junctions that delineated the canaliculus (Fig. 2*D*). In rab11a knockdown cells, belt-like tight junctions were absent. ZO-1 staining appeared as a complete or partial "chicken-wire" pattern (Fig. 2*D*), which suggests a change in tight junction organization and appearance. In control WIF-B9 cells, the percentage of cells having canaliculi increased with time in culture (Fig. 2*E*) (13), whereas in rab11a knockdown cells, canalicular formation was greatly reduced (Fig. 2*E*). These results confirm that rab11a is required for canalicular biogenesis.

To investigate the role of rab11a in canalicular biogenesis, we examined the distribution of apical, basolateral, and transcytotic membrane proteins in polarized and nonpolarized WIF-B9 cells. In polarized WIF-B9 cells, the plasma membrane pools of apical ABC transporters, BSEP and MRP2, were localized exclusively to the canalicular membrane (Figs. 1*A* and 3*A*), (14, 22). Transcytotic membrane proteins, such as 5′NT, which is a glycosylphosphatidylinositol anchor membrane protein, and cCAM105, which is a single transmembrane protein, localized in both canalicular and basolateral domains, consistent with their indirect trafficking to the canalicular membrane (Fig. 3*A*, see also Fig. 6, which is published as supporting information on the PNAS web site) (23). HA321 was restricted to the basolateral membrane (Fig. 3*A*) (23).

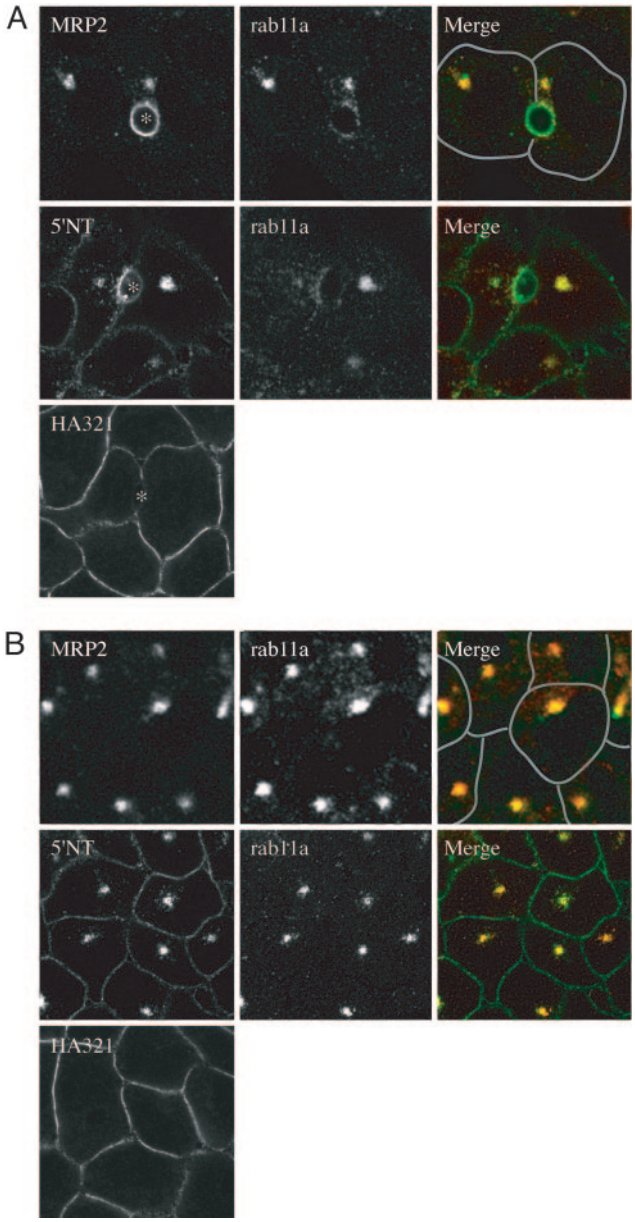

Fig. 3. Before polarization, apical ABC transporters reside in rab11a-positive endosomes. (*A*) Localization of MRP2, 5′NT, and HA321 in polarized WIF-B9 cells. WIF-B9 cells were immunostained for endogenous MRP2, HA321, and 5′NT. MRP2 localized to the canalicular membrane and rab11a-positive endosomes, whereas HA321 localized in the basolateral membrane. 5′NT localized in both canalicular and basolateral membrane and rab11a-positive endosomes. Asterisks indicate bile canaliculus. Gray lines show basolateral membrane. (*B*) Localization of MRP2, 5′NT, and HA321 in nonpolarized WIF-B9 cells. MRP2 was localized in rab11a-positive endosomes. HA321 was distributed in the plasma membrane. 5′NT was distributed in plasma membrane and rab11a-positive endosomes. Gray line shows basolateral membrane.

MRP2, 5′NT, and BSEP colocalized with rab11a-positive endosomes (Figs. 1*A* and 3*A*). In nonpolarized WIF-B9 cells, HA321, 5′NT, and cCAM105 distributed throughout the plasma membrane (Figs. 3B and 6). In contrast, BSEP-YFP and MRP2 were restricted to intracellular sites that colocalized with rab11a and the transcytotic markers, 5′NT and cCAM105 (Fig. 3*B*; see also Fig. 7, which is published as supporting information on the PNAS web site). These observations indicate that, before canalicular formation, apical ABC transporters which, in polarized cells, traffic by the direct route from Golgi membranes, are segregated from basolateral proteins and restricted to rab11a-positive endosomes.

Myosin Vb Is Required for Canalicular Formation. Rab11a binds to several proteins, including myosin Vb (24), a motor protein, which promotes transit along the actin cytoskeleton, binds rab11a and rab11-FIP2 (18, 25), and is implicated in actin-dependent vesicle trafficking. Because the canalicular domains of hepatocytes and WIF-B9 cells are rich in actin (12, 26), actin-dependent cargo traffic may required before membrane targeting. Therefore, we studied the effect of myosin Vb motorless tail domain, which acts as a dominant negative (18, 27), on canalicular formation. Polarized and nonpolarized WIF-B9 cells endogenously express myosin Vb, which colocalized with BSEP-YFP (Fig. 4 *A* and *B*). Overexpression of myosin Vb motorless tail domain did not affect cell growth but prevented canalicular formation similar to that observed in rab11a knockdown cells or after expression of rab11aS25N (Fig. 4*C*). In addition, the area (pixel number) of perinuclear rab11a-positive endosomes containing MRP2 and BSEP-YFP was five times larger than in control cells (9.4 μm^2 vs. 2.0 μm^2) which suggests an accumulation of endosome, some of which appears aggregated (Fig. 4*E*; see also Fig. 8, which is published as supporting information on the PNAS web site). These endosomes also contained 5′NT and cCAM105 (Fig. 8) but not HA321 (Fig. 4*E*). These results demonstrate that, in addition to rab11a, myosin Vb is required for canalicular biogenesis.

Discussion

In response to cues provided by cell–cell and cell–substrate contacts, polarization is thought to require regulated coordination between sorting of plasma membrane proteins into post-Golgi vesicles and endosomal transport vesicles, delivery to specific plasma membrane sites, and redistribution into respective polarized domains. Largely based on studies of MDCK cells, the current polarization paradigm is that, before polarization, basolateral and apical direct pathway proteins, which are present in different intracellular compartments (28, 29), are targeted to the plasma membrane, where they are homogeneously distributed, and after cell–cell and cell–substrate contacts, redistribute to their appropriate domains (30, 31). Our studies suggest a different mechanism in WIF-B9 cells. Before tight junction formation, basolateral and apical direct pathway proteins were in different locations. The former were in the plasma membrane, whereas the latter were in rab11a-positive endosomes that, upon canalicular formation, trafficked to the canalicular membrane.

In the present study, WIF-B9 cells manifesting bile canaliculi are designated as polarized. Cells lacking bile canaliculi are said to be nonpolarized; however, they may represent a different form of polarization. Previous studies of WIF-B9 cells in culture indicate that before development of the type of polarity present in hepatocytes, WIF-B9 cells may undergo transitional formation of apical-basal polarity as observed in columnar cells (13, 32). The distribution of apical and basal-lateral plasma membrane markers in WIF-B9 cells that have lost hepatocyte polarity after rab11a knockdown or expression of rab11a or myosin Vb dominant negative constructs resembles that described in apical-basal polarity (Figs. 3 and 4). Whether the observed loss of "hepatocyte" polarity in the present studies represents reversion to columnar-type apical-basal polarity could not be determined. No intermediate cytoplasmic structures of the type described in other cell lines (28, 29) were observed in the putative apical-basal polarity stage of WIF-B9 cell polarization.

In WIF-B9 cell and other polarized mammalian epithelial cells, delivery of canalicular membrane proteins requires an intact microtubule structure (14) that is affected by PAR-1, a

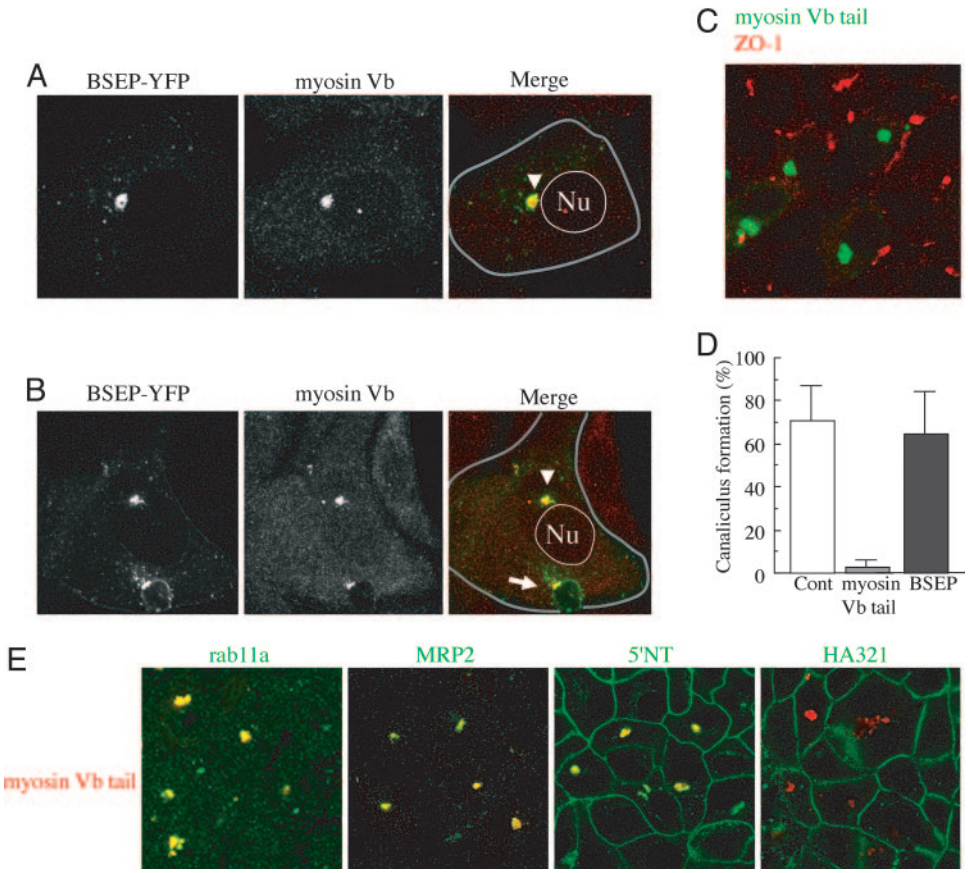

Fig. 4. Expression of myosin Vb tail domain inhibited canalicular formation. Distribution of endogenous myosin Vb in nonpolarized (*A*) and polarized (*B*) WIF-B9 cells. WIF-B9 cells infected with adenovirus encoding BSEP-YFP were immunostained with myosin Vb antibody. (*A*) In nonpolarized cells, endogenous myosin Vb localized with BSEP-YFP in perinuclear endosomes (arrowhead). Gray line shows plasma membrane. Nu, nuclei. (*B*) In polarized cells, endogenous myosin Vb colocalized with BSEP-YFP in perinuclear (arrowhead) and pericanalicular endosomes (arrow). Gray line shows basolateral membrane. Nu, nuclei. (*C*) The effect of myosin Vb tail expression on canalicular formation. At day 3 in culture, WIF-B9 cells were infected with adenovirus encoding myosin Vb tail-CFP. At day 7 in culture, the cells were immunostained with ZO-1. Percentage of WIF-B9 cells expressing myosin Vb tail or BSEP-YFP ranged from ≈30% to ≈50% and averaged ≈40%. Myosin Vb tail expressing cells did not form canalicular domains. (*D*) Myosin Vb tail expression reduced canalicular formation. The percentage of WIF-B9 cells manifesting bile canaliculi on day 7 after infection with adenovirus encoding myosin Vb-CFP or BSEP-YFP was determined. BSEP-YFP expression was a control for adenoviral infection. Myosin Vb tail expression decreased canalicular formation. Percentage of cells forming BC refers to all cells in the control and only infected cells expressing myosin Vb tail or BSEP. $n = 6 \pm$ SD. (*E*) Distribution of rab11a, MRP2, 5′NT, and HA321 in myosin Vb tail expressing WIF-B9 cells. Cells were immunostained for endogenous rab11a, MRP2, 5′NT, and HA321. Myosin Vb tail expression resulted in accumulation of perinuclear rab11a-positive endosomes. MRP2 and 5′NT colocalized with myosin Vb tail in perinuclear endosomes. 5′NT was also present in the plasma membrane. HA321 was restricted to the plasma membrane.

kinase for microtubule-associated proteins. Inhibition of PAR-1 prevented canalicular formation in WIF-B9 cells in association with altered microtubular orientation (32). Specific vesicle trafficking machinery is also required for establishment of membrane polarity. Here we demonstrate that rab11a and myosin Vb are required for canalicular formation. We propose that rab11a and myosin Vb positive endosomes constitute a reservoir for proteins that, in concert with unidentified polarization cues, are required for canalicular formation and trafficking of apical ABC transporters and transcyotic membrane proteins to the canalicular membrane.

Absence of polarity was associated with disrupted trafficking of proteins that use the direct trafficking pathway from Golgi membranes to the canalicular membrane and cycle through rab11a-positive endosomes. In nonpolarized and rab11a- or myosin Vb-inhibited WIF-B9 cells, proteins that, in polarized cells, traffic to the basalateral membrane (HA 321) or undergo transcytosis (5′NT and cCAM105) were present in the plasma membrane, whereas apical ABC transporters were restricted to intracellular sites. Membrane proteins undergoing transcytosis were also associated with intracellular rab11a-positive endosomes in nonpolarized and polarized WIF-B9 cells.

Expression of the GDP locked form of rab11a increasingly reduced canalicular formation. Confocal fluorescence image analysis performed in cells that still retained a canaliculus revealed abundant microtubule-dependent movement of BSEP-YFP-containing endosomes throughout the cell, as previously observed in control cells (14). However, recovery of BSEP-YFP after photobleaching the canalicular domain was markedly reduced (Fig. 1). These observations suggest that rab11a identifies an endosomal complex that traffics on microtubules but cannot traverse the pericanalicular actin and fuse with the canalicular membrane. Membrane proteins undergoing transcytosis were also associated with intracellular rab11a-enriched structures in nonpolarized WIF-B9 cells. It is not known whether rab11a and myosin Vb are required for trafficking of these membrane proteins to the canalicular membrane.

Expression of the motorless tail of myosin Vb also prevented polarization. Myosin Vb, an actin motor protein, colocalizes with rab11a-positive endosomes that are enriched in apical ABC

transporters and transcytotic membrane proteins (Fig. 4). In polarized MDCK cells, myosin Vb is required for targeting rab11a-positive endosomes to the apical plasma membrane (18). Our results suggest that myosin Vb, which binds rab11a (18), is required for trafficking endosomes that contain apical ABC transporters and transcytotic membrane proteins to sites that initiate polarization. Myosin Vb may serve as a motor for delivery of endosomal cargo through the actin network in WIF-B9 cells.

Several possible mechanisms may account for the role of rab11a and myosin Vb in canalicular biogenesis. In polarized mammalian cells, rab11a has been implicated in targeting of apical proteins from endosomes to the apical surface (15–17). In *Drosophila* and *Caenorhabditis elegans*, rab11a participates in morphogenesis (33–35). Both rab11a and myosin Vb may be required for directing critical junctional, cytoskeletal, targeting, or other proteins or lipid components to the plasma membrane. In the absence of rab11a or myosin Vb, proteins that normally traffic to the canalicular membrane remain intracellular, and normal turnover of canalicular membrane proteins and lipids may result in loss of polarity. These endosomes may contain or be linked to specific targeting molecules, such as SNAREs, which facilitate membrane targeting and fusion. BSEP and MRP2 are not determinants of polarization because hepatocytes remain polarized in mammals that lack either of these apical ABC transporters (5, 6). Alternatively, restoration of the direct pathway, whereby canalicular proteins traffic from intracellular rab11a-positive sites to sites that initiate polarize cues on the plasma membrane, may be the major determinant in establishing bile canalicular polarity.

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