Canalicular Export Pumps Traffic with Polymeric Immunoglobulin A Receptor on the Same Microtubule-associated Vesicle in Rat Liver*

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Basolateral to apical vesicular transcytosis in the hepatocyte is an essential pathway for the delivery of compounds from the sinusoidal blood to the bile and to traffic newly synthesized resident apical membrane proteins to their site of function at the canalicular membrane front. To characterize this pathway better, microtubules in a hepatocyte homogenate were polymerized by addition of taxol, and associated membrane-bound vesicles were isolated. This fraction was enriched in polymeric immunoglobulin A receptor and contained apical membrane proteins. Immunoelectron microscopy demonstrated that polymeric immunoglobulin A receptor was localized predominantly on vesicles ranging from 100 to 160 nm and that the multidrug resistance protein 2 and the bile salt export pump co-localized on these vesicles. The minus-end microtubule motor, dynein, was highly enriched in the fraction, and its intermediate chain could be released effectively by incubation with 1 mM ATP or GTP. However, the association of the transcytotic vesicles with the microtubules was not sensitive to hydrolyzable or non-hydrolyzable nucleotides. This study characterizes a fraction of microtubule-associated vesicles from rat hepatocytes and demonstrates that several resident apical membrane transport proteins and the polymeric immunoglobulin A receptor traffic on the same vesicle.

Hepatocytes are highly polarized epithelial cells in the liver that are specialized for the transport of nutrients, bile salts, chemotherapeutic drugs, and toxins from the blood into the bile. The polarized nature of the hepatocyte is defined by numerous primary, secondary, and tertiary active transport proteins that localize to either the sinusoidal or the canalicular membrane (4). This is in contrast to other cell systems that utilize a direct pathway from the Golgi network to the apical membrane (5–7). Therefore, do not usually involve an alteration in biosynthesis or degradation of the transporter. The process of regulated exocytosis/endocytosis has become increasingly recognized as a method of rapid regulation of membrane transport systems (8–12). This mechanism of regulation requires the existence of a submembranous pool of vesicle-bound transport proteins that can be recruited to and fuse with the secretary membrane, thereby bringing additional transporters to the site of function. Upon removal of the stimulus, the transport proteins are usually endocytosed back into intracellular vesicles to wait for the next round of stimulation.

Bile secretion, which is dependent on membrane transport systems, is also a regulated process (13–16), and recent data suggest that secretion at the hepatocyte canalicular domain can be regulated by exocytic insertion of transporters from an intracellular pool of vesicles (13, 15, 17). This occurs rapidly, is accompanied by an increase in the bile canalicular lumenal size, and is significantly inhibited by pretreatment with nucodazole. Immunocytochemistry has demonstrated that canalicular membrane proteins are seen in a pericanalicular pool of vesicles that largely disappear upon treatment with dibutyryl cAMP (Bt2cAMP) (13, 18). In addition, Kubitz et al. (19, 20) have demonstrated a reversible redistribution of multidrug resistance-associated protein 2 (Mrp2) in intact, perfused livers subjected to osmoregulation and lipopolysaccharide. Furthermore, the activity of ATP-dependent canalicular transport proteins has been shown to be increased in membrane vesicles isolated from livers treated with Bt2cAMP (21). The recent cloning of canalicular membrane transport proteins has led to a submembranous pool of vesicle-bound transport proteins that can be recruited to and fuse with the secretary membrane, thereby bringing additional transporters to the site of function. Upon removal of the stimulus, the transport proteins are usually endocytosed back into intracellular vesicles to wait for the next round of stimulation.

Bile secretion, which is dependent on membrane transport systems, is also a regulated process (13–16), and recent data suggest that secretion at the hepatocyte canalicular domain can be regulated by exocytic insertion of transporters from an intracellular pool of vesicles (13, 15, 17). This occurs rapidly, is accompanied by an increase in the bile canalicular lumenal size, and is significantly inhibited by pretreatment with nucodazole. Immunocytochemistry has demonstrated that canalicular membrane proteins are seen in a pericanalicular pool of vesicles that largely disappear upon treatment with dibutyryl cAMP (Bt2cAMP) (13, 18). In addition, Kubitz et al. (19, 20) have demonstrated a reversible redistribution of multidrug resistance-associated protein 2 (Mrp2) in intact, perfused livers subjected to osmoregulation and lipopolysaccharide. Furthermore, the activity of ATP-dependent canalicular transport proteins has been shown to be increased in membrane vesicles isolated from livers treated with Bt2cAMP (21). The recent cloning of canalicular membrane transport proteins has led to...
a better understanding of the physiology of hepatic apical secretion and has provided the tools for studying the regulation of this process (22–25).

In this paper we have sought to characterize further these putative transport vesicles. We have chosen the isolated rat hepatocyte couplet (IRHC) model system because in these cells the canicular lumen is a closed vacuole, thus mimicking a cholestatic liver in which there is an increase in the number of intracellular vesicles moving to, but not yet fusing with, the canicular membrane (26, 27). We have taken advantage of the association of transcytotic vesicles with microtubules in order to obtain an enriched fraction of vesicles. Western blot analysis of this fraction has demonstrated the enrichment of the transcytotic marker, polymeric immunoglobulin A receptor (pIgARec), as well as the presence of a number of apical membrane transport proteins. Immunoelectron microscopy was used to identify specifically vesicles on which Mrp2 and the bile salt export pump (Bsep) co-localize with pIgARec.

**EXPERIMENTAL PROCEDURES**

### Materials

Type B collagenase was purchased from Roche Molecular Biochemicals. Leibovitz’s (L-15) medium, penicillin, and streptomycin were from Life Technologies, Inc., and fetal bovine serum was from Gemini Bioproducts, Inc. (Calabasas, CA). Faclastaxel (taxol equivalent) was purchased from Molecular Probes (Eugene, OR). ATP, ATP-γS, GTP, GTP-γS, and AMP-PNP were from Calbiochem. Secondary, fluorescent antibodies were from Jackson Laboratories (West Grove, PA) and protein A gold was prepared by Jan Slot (Utrecht, The Netherlands). All other chemicals were the highest purity available commercially.

### Antibodies

Rabbit polyclonal antiserum to pIgARec was kindly provided by Janet Larkin, Barnard College. Antibody to Mrp2 (EAG15) was provided by Dietrich Keppler, Heidelberg, Germany, and was raised to the carboxyl terminus of rat Mrp2. Polyclonal antisera to the bile salt export pump (Bsep) was raised to an oligopeptide containing the carboxyl-terminal 13 amino acids and was provided by Bruno Stieger, Zurich, Switzerland. Monoclonal antibody C219 to P-glycoprotein was from Sigmet Laboratories, and monoclonal antibody to dipeptidyl peptidase IV was from BIOSOURCE, Inc (Camarillo, CA). Monoclonal antibody to β-tubulin (TUB2.1), dynein intermediate chain (70.1), p58 (58K-9), control asctes (NS-1), and normal rabbit serum were purchased from Molecular Probes (Eugene, OR). Antibodies to EGFRec and other proteins detected with the 5 nm PAG include the Golgi p58 protein and EGFRec. In the case of the monoclonal antibody to p58 and other proteins detected with their specific polyclonal antiserum and 5 nm PAG. Other proteins detected with the 5 nm PAG include the Golgi p58 protein and EGFRec. In the case of the monoclonal antibody to p58 and other proteins detected with their specific polyclonal antiserum and 5 nm PAG.

### SDS-PAGE

Polyacrylamide gel electrophoresis was carried out according to Laemmli (31). Protein concentration was determined using Bio-Rad Bradford protein assay. Samples from the PNS, 40K P, 40K SN, 16K P, and 16K SN were resuspended in MEPS buffer to achieve the same final concentration of protein as found in the 16K P. After the addition of Laemmli sample buffer, equal amounts of protein were loaded into each lane (generally 10–20 μg/lane). Proteins were transferred to nitrocellulose for 1.2 h at 250 mA and subsequently blocked with 5% milk and 0.15% glycine in Tris-buffered saline. Primary antibody was added in the blocking buffer overnight at 4 °C and subsequently was detected with a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence reagents from Amersham Pharmacia Biotech.

### Electron Microscopy

#### Negative Staining—

In order to view the polymerized microtubules with attached vesicles, the 16K P was gently resuspended in 0.25 μm sucrose in 0.1 M Tris and attached to carbon, formvar-coated EM grids. Contrast was provided by 0.4% uranyl acetate.

#### Transmission Electron Microscopy—

The 16K P was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate, pH 7.4, post-fixed with 1% OsmO4, dehydrated, and embedded in Epon resin. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed on a Zeiss 910 transmission electron microscope.

### Immunoelectron Microscopy—

Monoclonal antibodies and associated vesicles were resuspended and attached to grids as described above. They were fixed in 1.6% paraformaldehyde or PLP fixative (32) for 3 min, quenched with 0.15% glycine, and nonspecific sites blocked with 1% fish skin gelatin, 1% bovine serum albumin in phosphate-buffered saline. Double antibody labeling using protein A gold (PAG) was conducted according to Slot et al. (10). Canalicul transporters, Mrp2 and Bsep, were detected with their specific monoclonal antiserum and 5 nm PAG. Other proteins detected with the 5 nm PAG include the Golgi p58 protein and EGFRec. In the case of the monoclonal antibody to p58 and the ascites negative control of rabbit IgG was used prior to the identification of transcytotic vesicles with an antibody to the cytoplasmic domain of the pIgARec and 10 nm PAG. Nonspecific background labeling was determined by an equal dilution of non-immune rabbit serum (NRS) or control ascites. After incubation with PAG the grids were washed with phosphate-buffered saline, followed by distilled water, and embedded and dried with 2% molybdenum and 0.4% uranyl acetate. Grids were coded by a computer and were analyzed in a blinded fashion on a Zeiss 910 transmission electron microscope. Quantitative double labeling immunoelectron microscopy was conducted by photographing at ×50,000 magnification any vesicle labeled for pIgARec (10 nm PAG) and then counting the number of 5 nm PAG on the vesicle prior to breaking the code.

**Nucleotide Sensitivity Assay**

In order to ascertain the effect of nucleotides upon vesicle binding to microtubules, 1 μM of various nucleotides or nucleotide analogues was added to the 40K SN/taiux mixture (made to 5 mg protein/ml) either immediately or after 15 min of microtubule polymerization, and polymerization was continued for an additional 30 min. Creatine phosphate (10 mM) and creatine kinase (50 μg/ml) were added to regenerate ATP and acetate phosphate (10 mM), and acetate kinase (0.05 unit/ml) were added to regenerate GTP. The microtubule-associated vesicle pellet was recovered at 16,000 g and was subjected to SDS-PAGE analysis for recovery of various proteins. Densitometry was performed using the software GelPro from Media Cybernetics (Silver Spring, MD), and all lanes were normalized to their content of β-tubulin.

**Isolation and Culture of IRHC**

Hepatocyte couplets were isolated from rat liver as described previously from this laboratory (28). Prior to culture, centrifugal elutriation with a Beckman JE-6B rotor was performed in order to enrich further for couplets. Briefly, isolated hepatocytes were pelleted and resuspended in 30 ml of Liebovitz’s (L-15) medium containing 10% calf serum. Cells were loaded into the rotor at a flow rate of 10 ml/min at 4 °C, with a rotor speed of 1000 rpm. Seven fractions of 100 ml each were collected at flow rates of 10, 16, 24, 30, 35, 46, and 60 ml/min. Couplets were enriched to approximately 50% in fractions collected at 38, 46, and 60 ml/min. After pelleting at 50 × g, the couplets were washed in serum-free L-15 and resuspended in L-15 containing 10% fetal bovine serum at an approximate concentration of 0.2–0.4 × 106 cells/ml. Cells were plated in 100-mm Petri dishes (10–100 cells/cm2) and incubated at 37 °C for 4 h in an air atmosphere.

**Isolation of Microtubule-associated Vesicles**

Microtubule-associated vesicles were isolated according to a previously reported protocol based upon polymerization of endogenous tubulin (29, 30). After incubation at 37 °C for 4 h, the couplets were washed 3 times with serum-free L-15 at 4 °C. The cells were then scraped into a microtubule stabilizing buffer (MEPS, 5 mM MgSO4, 5 mM EGTA, 35 mM K PIPEES, 142 mM sucrose) containing 1 mM dithiothreitol and a mixture of protease inhibitors (phenylmethylsulfonyl fluoride, benzamide, aprotenin, leupeptin, and antipain) and homogenized at 4 °C by shearing through two 27-gauge needles attached to each other via PE20 tubing. A post-nuclear supernatant (PNS) was collected after centrifugation at 1000 × g to remove unbroken cells and nuclei, and this was subsequently subjected to a 40,000 × g (40K) centrifugation to pellet plasma membrane sheets and large organelles. The 40K SN was recovered and taxol (1 mM stock in Me2SO) was added to a final concentration of 20 μM. Microtubule polymerization was carried out at 37 °C for 15–60 min. Microtubules and associated vesicles were pelleted at 16,000 × g (16K) for 30 min, resuspended, and subjected to analysis by SDS-PAGE or electron microscopy.
Immunofluorescent demonstration of the localization of canalicular membrane transport proteins in isolated rat hepatocyte couplets. Indirect immunofluorescence was performed as described under “Experimental Procedures.” A, the canalicular organic anion transporter, Mrp2, is localized to the canalicular membrane and to a pericanalicular pool of vesicles under basal conditions. B, after 30 min treatment with Bt2cAMP (100 μM) and isobutylmethylxanthine (500 μM) the transporter is restricted to the expanded apical membrane and to apical remnants at the peripheral membrane. C, pretreatment of the hepatocytes with 20 μM nocodazole for 2 h prior to fixation causes much of the Mrp2 protein to be retained at the peripheral, basolateral membrane. D, specificity of the labeling is demonstrated by the absence of staining in hepatocytes from the TR2 rat that genetically lacks the canalicular transporter. A pericanalicular punctate staining is seen also when untreated hepatocytes couplets were labeled for Bsep (E) and P-glycoprotein (F) (Mdr gene product).

RESULTS

Immunofluorescence

Isolated hepatocyte couplets were cultured for 4 h on glass coverslips as described above. Following fixation/permeabilization with cold methanol, cells were subjected to indirect immunofluorescence as described previously (13). Fluorescence was viewed on a Bio-Rad MRC600 confocal scanning microscope and digital images recorded on an Iomega Zip disc. Images were processed using Adobe Photoshop.

Immunolocalization of Apical Membrane Proteins in IRHC—

Under basal conditions, resting IRHC demonstrate the Mrp2 protein both on the canalicular membrane and in an intracellular vesicular compartment (Fig. 1A). After treatment with Bt2cAMP there is a loss of the intracellular vesicle compartment labeled for Mrp2 coincident with an enlargement of the bile canaliculus (Fig. 1B). Nocodazole pretreatment of the IRHC results in retention of Mrp2 on the basolateral plasma membrane (Fig. 1C), demonstrating the microtubule dependence of vesicle movement to the apical membrane. The labeling of the canalicular membrane under these conditions reflects the transporter that existed at the site of joining of the two cells following isolation. These data confirm that the apical transporter, Mrp2, resides in an intracellular vesicular compartment under basal conditions and that movement to the apical membrane can be regulated. The kinetics of this protein kinase A-stimulated redistribution has been described in detail recently (18). Under basal conditions a similar pericanalicular, vesicular distribution is seen also when untreated hepatocytes couplets were labeled for Bsep (E) and P-glycoprotein (F) (Mdr gene product).

Morphologic Characterization of the Microtubule-associated Vesicle Pellet—

The protocol utilized to isolate these transcytotic vesicles takes advantage of their association with microtubules. After 4 h in culture the IRHC have fully established an
apical membrane domain which contains the transport proteins Mrp2, P-glycoprotein, Bsep, and Ca\textsuperscript{2+}Mg\textsuperscript{2+}-ecto-ATPase (Fig. 1 (13)). Centrifugation of the PNS at 40,000 × g pelleted intact plasma membrane sheets and large organelles (40K P), while allowing for the retention of all cytosolic factors as well as tubulin monomers in the 40K SN. Subsequent addition of 20 μM taxol to the 40K SN resulted in efficient polymerization of tubulin monomers; 90–100% of the tubulin was recovered in the 16K P after 15 min of polymerization as determined by SDS-PAGE (data not shown). Negative staining of the recovered material revealed long microtubules with many vesicles attached along their lengths (Fig. 2A). Transmission electron microscopy of the 16K P revealed a heterogeneous population of membrane-bound vesicles (Fig. 2B). Round vesicles with double membranes were prominent, and multivesicular elements and tubular structures were also seen. Vesicle sizes ranged from 30 to 300 nm. The compacted, pelleted microtubules appear as amorphous background material in the micrographs.

Biochemical Characterization of the Microtubule-associated Vesicle Pellet—The PNS contained 6–14 mg of protein at a concentration of 7–10 mg/ml. Centrifugation at 40,000 × g removed 1–2 mg of protein in the plasma membrane-enriched pellet. The final 16K P was a small, pure white pellet that contained 100–200 μg of protein that was resuspended in 50–100 μl of MEPS. Due to differences in protein concentration, the various fractions were normalized to the same protein concentration (mg/ml) as the 16K P, and equal amounts of protein (10–20 μg) were loaded into the PAGE lanes. When densitometry was performed, only the 40K P and 16K P were compared with the PNS since the concentration of specific protein in some subfractions was too small for accurate quantitation.

Many vesicle and organelle compartments within the cell are associated with microtubules and, therefore, would be expected to be found in the 16K P. To determine if the microtubule-associated vesicle fraction contained members of the transcytotic pathway, we analyzed the fractions by Western blot for the presence of pIgARec detected all three forms of the receptor, 105, 116, and 120 kDa, in the PNS and 40K P. However, only the 116- and 120-kDa forms were found in the 16K P containing the microtubule-associated vesicles. B, the canalicular membrane proteins Mrp2, Bsep, P-glycoprotein, dipeptidyl peptidase IV, and Ca\textsuperscript{2+}Mg\textsuperscript{2+}-ecto-ATPase were also detected in varying amounts in the 16K P. As expected, all these proteins were found in highest concentration in the 40K P-containing plasma membrane.

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FIG. 2. Transmission electron microscopy of the microtubule-associated vesicle fraction. A, after gentle resuspension, the 16K P was attached to coated EM grids, and negative staining was performed with 0.4% uranyl acetate. Vesicles are seen along the length of the microtubule. B, heterogeneity in vesicle size and morphology is appreciated when thin sections of Epon-embedded material are examined in the electron microscope. The material seen in the background represents the pelleted, compacted microtubules. Bars = 200 nm.

FIG. 3. Western blot analysis of centrifugal fractions from rat hepatocytes examining the distribution of canalicular membrane proteins. A, equal amount of total protein (10–20 μg) from each of the fractions was loaded into lanes of a 7.5% gel, and PAGE and Western blotting were conducted as described under “Experimental Procedures.” A polyclonal antibody to the cytoplasmic domain of pIgARec detected all three forms of the receptor, 105, 116, and 120 kDa, in the PNS and 40K P. However, only the 116- and 120-kDa forms were found in the 16K P containing the microtubule-associated vesicles. B, the canalicular membrane proteins Mrp2, Bsep, P-glycoprotein, dipeptidyl peptidase IV, and Ca\textsuperscript{2+}Mg\textsuperscript{2+}-ecto-ATPase were also detected in varying amounts in the 16K P. As expected, all these proteins were found in highest concentration in the 40K P-containing plasma membrane.
The 16K P was also examined for the presence of other proteins known to reside on intracellular vesicular compartments. Western blotting using an antiserum to EGFRec revealed a significant amount of the protein in the 16K P (Fig. 5). Based upon previous studies, the presence of this non-recycling receptor is believed to represent early and late endocytic compartments (39, 40). Microtubules also have an intimate association with the Golgi apparatus and may tether this organelle within the cytosol. Bloom and Brashear (41) have described a 58-kDa peripheral membrane protein that binds to the cytoplasmic face of the Golgi and that may act to anchor the Golgi to microtubules. When a monoclonal antibody to this 58-kDa protein was used in Western blotting of the liver fractions, equal concentration of the protein was found in the 16K P and 16K SN (Fig. 5).

The minus-ended microtubule motor, dynein, is also abundant in the hepatocyte (42) and is the primary motor involved in the microtubule-dependent endocytic pathway (30). The intermediate chain of dynein was efficiently recovered in the 16K P after 30 min of tubulin polymerization (Fig. 5). The plus-ended microtubule motor, kinesin, was also found in the 16K P; however, the abundance of this protein was very low (data not shown).

**Nucleotide Sensitivity**—To determine the nucleotide sensitivity of these microtubule-associated proteins, nucleotides and their analogues (1 mM) were added to the 40K SN either at the time of addition of taxol or after 15 min of polymerization. Microtubule polymerization was then carried out for an additional 30 min in their presence. No significant difference in recovery of β-tubulin was noted in any of the conditions, as determined by SDS-PAGE and Western blotting. Densitometric analysis of Western blots for plgARec, dynein, and Mrp2 was performed by normalizing for the amount of β-tubulin loaded per lane. Recovery of each protein is reported as the percentage of the control lane that received no treatment. A representative blot and densitometric analysis are shown in Fig. 6. There was significant loss of the intermediate chain of dynein from the 16K P after treatment with ATP and GTP (13.75 ± 7.9 and 4.81 ± 3.25% recovery, respectively). Both ADP and ATP/S treatment resulted in a loss of approximately 50%, whereas there was full recovery of dynein after GTP/S treatment. In contrast, there was no significant difference in recovery of the transcytotic protein, plgARec, or Mrp2 after any treatment. These data suggest the following: 1) attachment of transcytotic vesicles containing plgARec and Mrp2 is not sensitive to nucleotides; 2) the association of the intermediate chain of dynein with the vesicle membrane is not required for the continual binding of transcytotic vesicles to microtubules; and 3) ATP is not required for the attachment of transcytotic vesicles to microtubules.

**Immunoelectron Microscopic Characterization of Microtubule-associated Vesicles**—Immunoelectron microscopy was performed on the 16K P in order to examine individual vesicles for their membrane proteins. Because Western blotting data confirmed that several vesicle populations were present in the 16K P, transcytotic vesicles were first identified by the presence of plgARec. In initial experiments, any vesicle with 3 or more 10 nm PAG (representing plgARec) was photographed and PAG quantified and the vesicle diameter determined. Single labeling experiments established that an antibody to the cytoplasmic domain of the plgARec efficiently labeled vesicles ranging in size from 80 to 260 nm. Double labeling with the EAG15 antibody to the carboxyl terminus of Mrp2 along with the antibody to the plgARec demonstrated that Mrp2 could be found on approximately 74% (63/85) of the vesicles labeled for the plgARec. The mean number of 5 nm gold particles per vesicle was significantly higher for Mrp2 than the NRS control (2.08 ± 0.93 versus 0.16 ± 0.13, p < 0.0001) (Table I). These vesicles had a mean diameter of 138 ± 38 nm, with 76% of them found between 100 and 160 nm (see Fig. 7 for representative vesicles). It was noted that there was consistently less labeling for the plgARec when Mrp2 was used for double labeling, regardless of which antibody was applied first. Although this was not quite statistically significant (p = 0.066), its consistency suggests that some steric hindrance may be occurring due to the proximity of the two canalicular proteins. This observation further supports the finding that the organic anion transporter, Mrp2, can travel to the canalicular membrane on the same vesicle as the plgARec.

Immunofluorescence experiments using formaldehyde fixation revealed that preservation of Bsep immunogenicity required the presence of periodate and lysine, as utilized in PLP fixative. Results from double labeling experiments for Bsep, Mrp2, NRS (5 nm PAG), and plgARec (10 nm PAG) using PLP fixative can be seen in Table I. Significantly greater labeling is seen for Bsep as compared with NRS (1.26 ± 1.40 versus 0.23 ± 0.57, p < 0.0001), and the mean diameter of the vesicles was 130 ± 40 nm (see Fig. 7 for representative vesicles). Thus, another apical transport protein, Bsep, co-localizes with plgARec on these putative transcytotic vesicles. When double labeling of microtubule-associated vesicles was carried out for Bsep (5 nm PAG) and Mrp2 (10 nm PAG), co-localization occurred at similar rates of labeling (Table I).

In contrast, when the isolated microtubule-associated vesicles were labeled for p58, the Golgi peripheral protein, the labeling was found on a different population of vesicles with a mean diameter of 51 ± 10 nm (Fig. 7). The smaller size of these vesicles is consistent with the size of vesicles seen budding off the Golgi in transmission electron micrographs of IRHC and with those previously reported to be intra-Golgi transport vesicles (43, 44). Intact Golgi complexes were not labeled with the p58 antibody and were not seen by transmission electron mi-
croscopy in the Epon-embedded material. Double labeling for pIgARec and p58 revealed that 81% of these vesicles were 100 nm and that these small vesicles had only an average of 0.60 ± 1.83 of the 10 nm PAG representing pIgARec. In contrast, the remaining 18% of the vesicles were >100 nm (mean diameter 146 ± 35 nm) and had an average of 5.3 ± 3.25 of the 10 nm PAG representing pIgARec (Table II). Only 5/14 of these larger vesicles demonstrated any labeling for p58. Thus, the larger vesicles that were predominantly labeled for Mrp2 and pIgARec were most likely transcytotic vesicles and/or vesicles in the endocytic pathway. To distinguish vesicles further from these pathways, double labeling was also performed with the antibody to EGFRec. In this case, 65.6% of the total vesicles labeled had diameters of 100 nm (mean diameter 74 ± 11 nm). These vesicles had an average of 0.88 ± 1.3 large gold particles representing pIgARec. The remaining 34.4% had a mean diameter of 132 ± 25 nm and had an average of 4.19 ± 2.46 of the 10 nm PAG representing pIgARec (Table II). These vesicles also demonstrated significant labeling for EGFRec. Large (>250 nm) vesicles representing multivesicular bodies or lysosomes were not seen, as would be expected from reports that the EGFRec is completely internalized within these vesicles and not accessible to labeling antibodies (40). Thus, these data suggest that we can distinguish between vesicle populations in the 16K P by employing specific antibodies as follows: Golgi-associated transport vesicles have a mean diameter of 51 nm and contain the p58 protein; endocytic vesicles in the lysosomal pathway contain EGFRec and have an intermediate diameter of 74 nm; vesicles in the transcytotic pathway can be labeled with antibodies to pIgARec, Mrp2, and Bsep and have an average diameter of 134 nm.

**TABLE I**
Quantitation of immunogold labeling of microtubule-associated vesicles

Double labeling immunogold was performed on the 16K pellet fraction as described under "Experimental Procedures." The first antibody was detected with 5 nm protein A gold (PAG), and the second antibody was detected with 10 nm PAG. Vesicles were fixed with paraformaldehyde (top section) or PLP fixative (bottom section). The number of vesicles counted is indicated in parentheses, and all analysis was done in a blinded fashion. Data represents the mean ± S.D. of six experiments in top section and two experiments in bottom section.

| Antibodies (n) | Mean number gold/vesicle, 1st antibody | Mean number gold/vesicle, 2nd antibody | Mean diameter |
|---------------|--------------------------------------|--------------------------------------|---------------|
| NRS/pIgARec (80) | 0.16 ± 0.13                          | 5.92 ± 1.42                         | 132 ± 31      |
| Mrp2/pIgARec (85) | 2.08 ± 0.93*                         | 3.73 ± 2.23                        | 138 ± 38      |

| Antibodies (n) | Mean number gold/vesicle, 1st antibody | Mean number gold/vesicle, 2nd antibody | Mean diameter |
|---------------|--------------------------------------|--------------------------------------|---------------|
| NRS/pIgARec (44) | 0.23 ± 0.57                          | 7.02 ± 5.13                         | 134 ± 46      |
| Bsep/pIgARec (42) | 1.26 ± 1.40*                        | 5.74 ± 4.59                        | 130 ± 40      |
| Mrp2/pIgARec (22) | 2.23 ± 1.48*                        | 5.09 ± 4.24                        | 130 ± 35      |
| Bsep/Mrp2 (10) | 1.40 ± 1.17                          | 2.70 ± 1.06                        | 143 ± 34      |

* p < 0.0001, NRS compared with Mrp2 or Bsep, Student’s paired t test.

**FIG. 6.** Nucleotide sensitivity of vesicles and dynein binding to polymerized microtubules. A, prior to the addition of 20 μM taxol, the 40K SN was adjusted to a protein concentration of 5 mg/ml. Endogenous tubulin was allowed to polymerize for 15 min before addition of 1 mM nucleotide or nucleotide analogue. Creatine phosphate (10 mM) and creatine kinase (50 μg/ml) or acetate phosphate (10 mM) and acetate kinase (0.05 unit/ml) were added to regenerate ATP or GTP, respectively. Polymerization proceeded for 30 min, and the microtubule-associated vesicles were then pelleted at 16,000 × g, and PAGE and Western blotting were carried out as described previously. B, densitometric analysis was conducted using the software GelPro, and values were normalized for the amount of β-tubulin loaded per lane. Despite the significant loss of dynein from the 16K P after treatment with ATP and GTP (13.76 ± 7.9 and 4.81 ± 3.25% recovery, respectively), vesicles containing pIgARec and Mrp2 were not dissociated from the microtubules. Data represents the mean ± S.D. of eight experiments for pIgARec and dynein and three experiments for Mrp2.
FIG. 7. Immuno-electron microscopic visualization of different vesicle populations. The 16K P was gently resuspended and allowed to attach to coated EM grids. Double labeling immuno-electron microscopy was performed as described under "Experimental Procedures" utilizing 5 and 10 nm PAG. A and B, Mrp2 (5 nm PAG) and pIgARec (10 nm PAG) co-localize on putative transcytotic vesicles with a mean diameter of 137 nm. C and D, similar co-localization was seen for Bsep (5 nm PAG) and pIgARec (10 nm PAG). E, double labeling for EGFRec (5 nm PAG) and pIgARec (10 nm PAG) revealed that 66% of the labeled vesicles had a mean diameter of 74 nm and demonstrated only a low level (<1 PAG/vesicle) of labeling for pIgARec. The remaining 34% of the vesicles had diameters comparable with the transcytotic vesicles and demonstrated increased labeling for pIgARec (4 PAG/vesicle). F, a third population of vesicles was detected by double labeling for p58 (5 nm PAG) and pIgARec (10 nm PAG). The arrowhead indicates a putative transcytotic vesicle that labels only for pIgARec, whereas the small arrow shows the smaller vesicles labeling only for p58, demonstrating the discrimination of the two populations. Bar, 100 nm.

TABLE II
Quantitation of immunogold labeling of microtubule-associated vesicles

| Antibodies         | Mean number gold/vesicle | Mean number gold/vesicle | Mean diameter |
|--------------------|--------------------------|--------------------------|---------------|
|                    | (n)                      | 1st antibody             | 2nd antibody  |
| nm                 |                          |                          |               |
| p58/pIgARec        | <100 (86)                | 5.49 ± 1.83              | 0.60 ± 1.83   | 51 ± 10 |
|                    | >100 (20)                | 1.10 ± 3.58              | 5.30 ± 3.25   | 146 ± 35 |
| EGFRec/pIgARec     | <100 (40)                | 7.23 ± 3.35              | 0.88 ± 1.30   | 74 ± 11 |
|                    | >100 (21)                | 5.86 ± 7.6               | 4.19 ± 2.46   | 132 ± 25 |

DISCUSSION

The isolated rat hepatocyte couplet system provides a non-transformed cell model in which liver-specific functions and proteins are maintained in short term (4–6 h) culture (28, 45, 46). Over a 2–4 h period, two adjoining cells will reorganize their plasma membranes such that a bile canalicular (apical) domain will be separated from the basolateral domain by tight junctions, thus establishing a polarized bile secretory unit. For proper biliary secretion to occur, the hepatocyte must be able to transcytose efficiently the major canalicular membrane ABC transport proteins, Mrp2, Bsep, and Mdr1, from the basolateral membrane to their site of function at the apical membrane.

The importance of microtubules in apically directed vesicle movement and secretion in the hepatocyte is well established (13, 17, 18). Therefore, we utilized a method of taxol-induced polymerization of endogenous tubulin to obtain a microtubule-associated vesicle fraction (16K P) from cultured hepatocytes enriched in functional couplets. Recovery of the transcytotic pathway was confirmed by the presence of the mature, 120-kDa form of the pIgARec precursor may represent the exocytic pathway that delivers pIgARec to the basolateral membrane or an early endocytotic or sorting compartment. Both of these vesicular compartments are known to be associated with microtubules (47, 48) and would, therefore, be expected to be present in this fraction. Although the 116-kDa form is also largely found on the basolateral membrane, Western blotting for Na$^{+}$K$^{-}$-ATPase and CE9 revealed significant depletion of these basolateral membrane markers from the 16K P. Thus, the centrifugation at 40,000 × g was capable of removing most of the plasma membrane fraction prior to polymerization of the microtubules. The small amount of the basolateral proteins in the 16K P may represent components of the exocytic pathway, as the proteins travel from the Golgi/trans-Golgi network to the basolateral membrane. Alternatively, Casciola-Rosen and Hubbard (49) have reported finding Na$^{+}$K$^{-}$-ATPase on early endosomes in rat hepatocytes which could also account for the small amount of this protein in the 16K P fraction.

Functional integrity of the hepatocyte is dependent upon the numerous membrane transport systems localized specifically to either the apical or the basolateral plasma membrane. In vivo, numerous membrane transport systems localized specifically to either the apical or the basolateral plasma membrane. In vivo, the plasma membrane fraction prior to polymerization of the microtubules. The small amount of the basolateral proteins in the 16K P may represent components of the exocytic pathway, as the proteins travel from the Golgi/trans-Golgi network to the basolateral membrane. Alternatively, Casciola-Rosen and Hubbard (49) have reported finding Na$^{+}$K$^{-}$-ATPase on early endosomes in rat hepatocytes which could also account for the small amount of this protein in the 16K P fraction.

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This paper confirms that these transport proteins are also found in the microtubule-associated vesicle fraction. More importantly, immunoelectron microscopy has showed that putative transcytotic vesicles that are identified by labeling for pIgARec demonstrate co-localization for Mrp2 and Bsep. This is the first demonstration at the ultrastructural level that apical membrane transport proteins travel in the same vesicle as pIgARec. Previously Barr et al. (50) have demonstrated that a fraction of vesicles immunosorbed for pIgARec also contained dipeptidyl peptidase IV, an apical membrane enzyme; however, they did not show co-localization of the two proteins to the same vesicle. Although the labeling for Mrp2 and Bsep was significantly higher than the normal serum control, it was always lower than the labeling for the pIgARec. Scott and Hubbard (51) have shown that the rate of synthesis of pIgARec is much higher than that for other apical membrane proteins and that the receptor is released into bile at a rate of 30% per h. If the rate of traffic of Mrp2 and Bsep are similar to the other apical markers, it is not surprising that each vesicle contains less of the transport proteins than the pIgARec.

The sinusoidal membrane of the hepatocyte contains many receptors responsible for binding various physiologic ligands for the purpose of internalization by receptor-mediated endocytosis. The ultimate fate of these receptors vary; both the transferrin receptor and the asialoglycoprotein receptor largely recycle back to the basal membrane after releasing their ligands in the acidic environment of the early endosome (52–54), whereas the EGFRec is trafficked predominantly to the lysosome (39, 40). This is in contrast to the plgARec that separates from these other receptors, probably in the sorting compartment known as compartment of uncoupling of receptors and ligands (CURL) (55), and is trafficked efficiently to the apical membrane. It is not surprising, therefore, that vesicles from other intracellular compartments would also be recovered in the microtubule-associated vesicle fraction. The finding of EGFRec finding the presence of endosomes in the fraction and immunoelectron microscopy for EGFRec identified predominantly vesicles with smaller mean diameters (74 ± 11 nm) than the majority of vesicles with plgARec. However, the larger vesicles that labeled for plgARec (mean diameter of 132 ± 25 nm) also showed significant co-labeling for EGFRec. These vesicles may represent a fraction of recycling endosomes, which recently have been shown to also contain dIgA and which contain dipeptidyl peptidase IV, an apical membrane enzyme; however, they did not show co-localization of the two proteins to the same vesicle. Although the labeling for Mrp2 and Bsep was significantly higher than the normal serum control, it was always lower than the labeling for the pIgARec. Scott and Hubbard (51) have shown that the rate of synthesis of pIgARec is much higher than that for other apical membrane proteins and that the receptor is released into bile at a rate of 30% per h. If the rate of traffic of Mrp2 and Bsep are similar to the other apical markers, it is not surprising that each vesicle contains less of the transport proteins than the pIgARec.

The Golgi peripheral membrane protein p58 was also found in significant quantity in the 16K P by Western blotting. However, it is found in the soluble, non-bound fraction in approximately equal concentration, and this may be because of a tenuous, peripheral association with the Golgi membrane. Double labeling immunoelectron microscopy for plgARec and p58 demonstrated labeled vesicles that had a mean diameter of 51 ± 10 nm, with 81% of the vesicles labeled for p58 <100 nm. This size is more comparable with that reported for intra-Golgi trafficking (43, 44), although our value is slightly smaller than previously reported. One explanation that we cannot discount is that these "vesicles" represent vesiculations of the Golgi membrane. These vesicles had a low level of labeling for plgARec; however, this may again reflect the limitations imposed by the small size of the vesicle as opposed to the size of the gold particle. Only 5/14 of the double-labeled vesicles >100 nm had labeling for p58. Therefore, in the 16K P fraction of microtubule-associated vesicles we can distinguish vesicles of different sizes that are involved in intra-Golgi trafficking, endocytic trafficking, and transcytotic trafficking.

It is well established that vesicle movement in cells can occur along tracks of microtubules and that force and directionality are determined by specific minus- and plus-ended motors (57–60). It has been previously reported that the liver contains a large amount of dynein, with the ratio of dynein to microtubules being 15-fold higher than in brain (42). Furthermore, in the hepatocyte, like other polarized epithelial cells, it is believed that the minus end of the microtubule is located toward the Golgi-rich pericanalicular region (61). Accordingly we found this minus-ended motor significantly enriched in the 16K P. The specificity of the association of this microtubule motor was demonstrated by a significant loss (85–95%) from the pellet upon incubation with 1 mM ATP or GTP. Dynnein binding in our system was more sensitive to the trinucleotides than reported by Oda et al. (29), where approximately 50% was lost from the 16K P. This difference may reflect our use of an ATP- and GTP-regenerating system with the addition of the trinucleotides. Kinesin, the plus-ended microtubule motor, was found in very low amounts in the 16K P, confirming previous reports (29). Marks et al. (62) have demonstrated that kinesin represents only 0.3% of the total protein in rat liver homogenate.

Oda et al. (29) showed that following a round of receptor-mediated endocytosis, ligand-containing vesicles bound for lysosomes, but not receptor-containing recycling vesicles, are released along with dynein intermediate chain following treatment with trinucleotides. Such trinucleotide sensitivity has also been demonstrated for lysosomes (63) and endocytic transport carrier vesicles labeled with horseradish peroxidase (64). In contrast, we found that plgARec and Mrp2 were not lost from the 16K P upon incubation with 1 mM ATP or GTP, despite the significant loss of the dynein intermediate chain. Exocytic vesicles traveling from the trans-Golgi to the plasma membrane have also been reported to be insensitive to ATP treatment (47). Although microtubule binding of endocytic transport carrier vesicles has been shown to be sensitive to trinucleotides, the binding of the vesicles to microtubules is not dependent upon the presence of dynein but requires epithelial derived cytosolic factors, vesicle membrane proteins, and microtubule-binding proteins other than the classic motor proteins (47, 60, 64). Proteins that have been shown to be important in vesicle binding and dynein-dependent vesicle transport include CLIP 170 (65, 66) and dynactin p150glued (67, 68). Perhaps in our study the transcytotic vesicles associated with the microtubule rapidly, and subsequent ATP hydrolysis released the intermediate chain of dynein, while leaving the vesicle bound to the microtubule through other proteins yet to be defined.

In summary, endogenous microtubule polymerization with taxol allows recovery of vesicles associated with this cytoskeletal system. A heterogeneous population of membrane-bound vesicles is seen by transmission electron microscopy. Immunoelectron microscopy has identified putative transcytotic vesicles that are generally >100 nm in size, as well as putative intra-Golgi transport vesicles and endocytic elements that have smaller dimensions. Furthermore, double labeling immunoelectron microscopy has demonstrated that the canalicular membrane transport proteins, Mrp2 and Bsep, co-localize on the same vesicle with plgARec and thus must traffic together. ATP and GTP displace dynnein but not the putative transcytotic vesicles from the microtubules. Future work will be conducted to clarify the linkage of these vesicles with the microtubules and to define potential regulatory proteins that control this secretory process that is fundamental to bile formation.

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