

# Role of Glycosylation in Trafficking of Mrp2 in Sandwich-Cultured Rat Hepatocytes

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## ABSTRACT

The multidrug resistance-associated protein (MRP) family plays a major role in the hepatic excretion of organic anions. The expression, localization, and function of Mrp2 (Abcc2), a canalicular multispecific organic anion transport protein, were studied in sandwich-cultured rat hepatocytes. The amount of Mrp2 protein remained constant in sandwich-cultured rat hepatocytes over 4 days in culture, but the molecular mass increased ~10 kDa from 190 to 200 kDa. Mrp2 was internalized initially after hepatocyte isolation and was gradually sorted to the canalicular membrane. Disposition of 5-(6)-carboxy-2',7'-dichlorofluorescein (CDF), an Mrp2 substrate, confirmed the changes in Mrp2 localization. CDF was localized predominantly inside hepatocytes at day 0 and gradually localized to the canalicular domain over time in culture. By day 4 in culture,

CDF was localized exclusively in the canalicular networks. Tunicamycin, an inhibitor of glycosylation, decreased the molecular mass and simultaneously impaired the trafficking of Mrp2 to the canalicular membrane. Treatment of lysates from both day 0 (Mrp2, 190 kDa) and day 4 (Mrp2, 200 kDa) sandwich-cultured rat hepatocytes with peptide *N*-glycosidase F, a deglycosylation agent, resulted in a band of 180 kDa, suggesting that Mrp2 from both day 0 and day 4 was glycosylated, but Mrp2 on day 4 was more glycosylated than on day 0. In conclusion, these data support the hypothesis that glycosylation of Mrp2 is responsible for the increase in molecular mass and may be involved in directing the canalicular localization of Mrp2 in sandwich-cultured rat hepatocytes over days in culture.

Mrp2 (Abcc2), previously designated the canalicular multispecific organic anion transporter, has been characterized as an ATP-dependent membrane transport protein responsible for the biliary excretion of organic anions, including both conjugated and unconjugated amphiphilic anions (Konig et al., 1999; Borst et al., 2000; Gerk and Vore, 2002). In the liver, Mrp2 is located exclusively on the canalicular membrane, with a molecular mass of 190 kDa (Ogawa et al., 2000).

Dedifferentiation and loss of many liver-specific properties are well known to occur in primary hepatocytes cultured in a conventional configuration. For example, Na<sup>+</sup>-dependent taurocholate cotransporting polypeptide activity in primary rat hepatocytes cultured on a gelled collagen substratum has been shown to be reduced to 2 to 7% of basal activity after 72 h in culture, whereas Mrp2 activity was maintained at

50% of its original value (Rippin et al., 2001). However, hepatocytes cultured in a sandwich configuration (between two layers of gelled collagen) exhibit a more normal physiological morphology, including extensive bile canalicular networks and maintenance of transport protein expression and function compared with hepatocytes cultured in a conventional configuration (Liu et al., 1998,1999b,c). The sandwich-cultured hepatocyte system has been used as an *in vivo*-like model to study biliary excretion (Liu et al., 1999c).

Nascent proteins contain signals that determine their ultimate destination within the cell, and protein glycosylation is one strategy used by cells to assist in sorting proteins to the proper membrane domain. MRP2/Mrp2 has at least two glycosylation sites, one of which is located in the N terminus and the other in the third extracellular loop of the C terminus (Borst et al., 2000). Nonfunctional MRP2 identified in a patient with Dubin-Johnson syndrome was found to contain two amino acid deletions (Keitel et al., 2000, 2003). This mutant protein was less glycosylated, retained in the endoplasmic reticulum, and was sensitive to endoglycosidase, suggesting impaired stability and trafficking of MRP2 (Keitel et al., 2000). Trafficking experiments with a series of chi-

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**ABBREVIATIONS:** Mrp, multidrug resistance-associated protein; PNGase F, peptide *N*-glycosidase F; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; BSA, bovine serum albumin; CDF, 5-(6)-carboxy-2',7'-dichlorofluorescein; HBSS, Hanks' balanced salt solution; PBS, phosphate-buffered saline; TBS/T, Tris-buffered saline containing 0.05% Tween 20.

meric Mrp1/Mrp2 proteins suggested that the critical domain determining Mrp2 trafficking was in the N-terminal fragment (Konno et al., 2003). Liu et al. (1999b) reported that the molecular mass of Mrp2 in rat hepatocytes cultured for 5 days in a sandwich configuration was increased by ~10 kDa. These data were consistent with the hypothesis that Mrp2 underwent glycosylation in primary sandwich-cultured rat hepatocytes over time in culture.

Tunicamycin, a hydrophobic analog of UDP-*N*-acetylglucosamine, is an antibiotic that inhibits *N*-glycosylation by blocking the addition of *N*-acetylglucosamine to dolichol phosphate, the first step in the formation of the core oligosaccharide (Christensen et al., 2000; Kamitani and Sakata, 2001). In the current study, tunicamycin was used to block Mrp2 glycosylation and to explore the influence of glycosylation on the localization of this transport protein in sandwich-cultured rat hepatocytes.

## Materials and Methods

**Chemicals and Antibodies.** Complete protease inhibitor cocktail tablets were obtained from Roche Diagnostics (Mannheim, Germany). PNGase F was obtained from New England BioLabs (Beverly, MA). Collagenase (type I, class I) was obtained from Worthington Biochemicals (Lakewood, NJ). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and insulin were from Invitrogen (Carlsbad, CA). Rat tail collagen (type I) and insulin/transferrin/selenium culture supplement were obtained from BD Biosciences (Bedford, MA). Bovine serum albumin (BSA), trypsin inhibitor, and tunicamycin were purchased from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal anti-Mrp2 (M<sub>2</sub>III-6) antibody was purchased from Alexis Biochemicals Corporation (Pittsburgh, PA), and mouse anti-actin antibody was purchased from Chemicon International (Temecula, CA). Horseradish peroxidase-conjugated anti-mouse IgG antibody was obtained from Amersham Biosciences Inc. (St. Louis, MO), and Alexa Fluor 488-conjugated anti-mouse and rabbit IgG antibodies, Rhodamine Red-X-conjugated anti-mouse and -rabbit IgG antibodies, and CDF were purchased from Molecular Probes (Eugene, OR). Rabbit polyclonal anti-calreticulin and mouse anti-Golgi antibodies were purchased from AbCam (Cambridge, UK). Rabbit antiserum anti-Mrp2 was a kind gift from Dr. Sugiyama (University of Tokyo, Tokyo, Japan). Electrophoresis reagents were obtained from Invitrogen. LumiGlo chemiluminescence substrate kit was purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

**Animals.** Male Wistar rats from Charles River Laboratories, Inc. (Raleigh, NC) were used as liver donors. Rats were housed in an alternating 12-h light/dark cycle for at least 1 week before the study

was performed and were allowed food and water ad libitum. All animals received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences (National Institutes of Health publication 86-23, revised 1985). The Institutional Animal Care and Use Committee approved all procedures.

**Sandwich-Cultured Hepatocytes.** Hepatocytes were isolated as described previously (Chandra et al., 2001). In brief, rats were anesthetized with ketamine and xylazine (60 and 12 mg/kg i.p., respectively). The liver was perfused in situ with oxygenated Ca<sup>2+</sup>-free Krebs-Henseleit bicarbonate buffer containing 5.5 mM glucose for 10 min at 37°C followed by perfusion with the same buffer containing 5 mM CaCl<sub>2</sub> and 0.5 mg/ml collagenase type I for 10 min. After perfusion, the liver was removed, and hepatocytes were released into 100 ml of DMEM by gently tearing the capsule of the liver. The cells were filtered through a nylon mesh and centrifuged at 50g for 2 min. The pellet was resuspended in 25 ml of DMEM and 25 ml of 90% isotonic Percoll and centrifuged at 70g for 5 min. The cells were washed again with DMEM, and the viability of hepatocytes was determined by trypan blue exclusion. Only those hepatocytes with viability greater than 90% were seeded onto dishes.

Plastic culture dishes (60 mm) from Nalge Nunc International (Rochester, NY) were precoated with neutralized rat tail collagen solution at least 1 day before isolating the hepatocytes. Twenty-four hours later, the gelled collagen was hydrated with 3 ml of DMEM. After isolation, the hepatocytes were resuspended in DMEM containing 5% FBS. The hepatocytes were seeded onto these precoated dishes at a density of  $3 \times 10^6$ /dish; 1 to 2 h later, the medium was replaced with 3 ml of fresh medium supplemented with 5% FBS + 0.1  $\mu$ M dexamethasone (day 0). After 24 h, neutralized collagen (0.2 ml, 1.5 mg/ml, pH 7.4) was overlaid on top of the hepatocytes. Fresh DMEM supplemented with 1% insulin/transferrin/selenium culture supplement (v/v) + 0.1  $\mu$ M dexamethasone was added on top of the gelled collagen. The culture medium was changed daily.

**Tunicamycin Treatment.** Hepatocytes were treated with 0.1  $\mu$ g/ml tunicamycin or vehicle (0.1% methanol) by supplementing the culture medium for designated periods on days 1 to 3 for Western blot and for 48 h for immunohistochemical studies. In preliminary experiments, a tunicamycin concentration of 1  $\mu$ g/ml caused morphological alterations in sandwich-cultured rat hepatocytes after 24 h. Based on preliminary experiments, a 10-fold lower concentration of tunicamycin (0.1  $\mu$ g/ml) was selected for investigation in this study. This concentration is well below that used in other studies with Madin-Darby canine kidney cells (Fernandez et al., 2002).

**Deglycosylation of Mrp2.** Sandwich-cultured rat hepatocytes on day 0 and day 4 were lysed in 1× Complete protease inhibitor cocktail in 0.5% SDS/0.5 mM EDTA. The lysate was incubated with PNGase F according to the manufacturer's instructions with slight modification for either 20 or 40 min at 37°C and then loaded onto a 4 to 12% Bis-Tris gel for Western blot analysis.

**CDF Disposition Assessed by Microscopy.** Hepatocytes were preincubated in Hanks' balanced salt solution (HBSS) at 37°C for 10 min. Thereafter, cells were incubated with 10  $\mu$ M CDF diacetate at 37°C for 10 min and rinsed three times with 3 ml of HBSS to remove extracellular substrate before viewing with an Axiovert 100TV inverted microscope (Carl Zeiss, Thornwood, NY).

**Biliary Excretion Measurements.** Biliary excretion measurements were carried out in sandwich-cultured rat hepatocytes from day 1 to day 4 as described previously (Chandra et al., 2001). In brief, sandwich-cultured rat hepatocytes were rinsed twice with HBSS  $\pm$  Ca<sup>2+</sup> and incubated with the same buffer for 10 min at 37°C. Thereafter, the cells were incubated with 10  $\mu$ M CDF diacetate in HBSS for 10 min and then washed three times with 3 ml of ice-cold HBSS. Hepatocytes were lysed with 2 ml of 1% Triton X-100 in PBS for 20 min at room temperature. CDF concentrations were analyzed by a microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT). The biliary excretion index, calculated using B-CLEAR technology (Qualyst, Inc., Research Triangle Park, NC), was determined by



**Fig. 1.** Representative Western blot of Mrp2 protein in sandwich-cultured rat hepatocytes over time in culture. Actin was used as the internal control. The molecular mass increased from ~190 kDa on day 0 to ~200 kDa on day 4 in culture.

dividing the difference in CDF accumulation in hepatocytes preincubated in HBSS<sub>(cells + bile)</sub> and hepatocytes preincubated in Ca<sup>2+</sup>-free HBSS<sub>(cells)</sub> by CDF accumulation in hepatocytes preincubated in HBSS<sub>(cells + bile)</sub> (Liu et al., 1999a). The CDF biliary clearance (Cl<sub>bile</sub>) was determined based on the following equation:

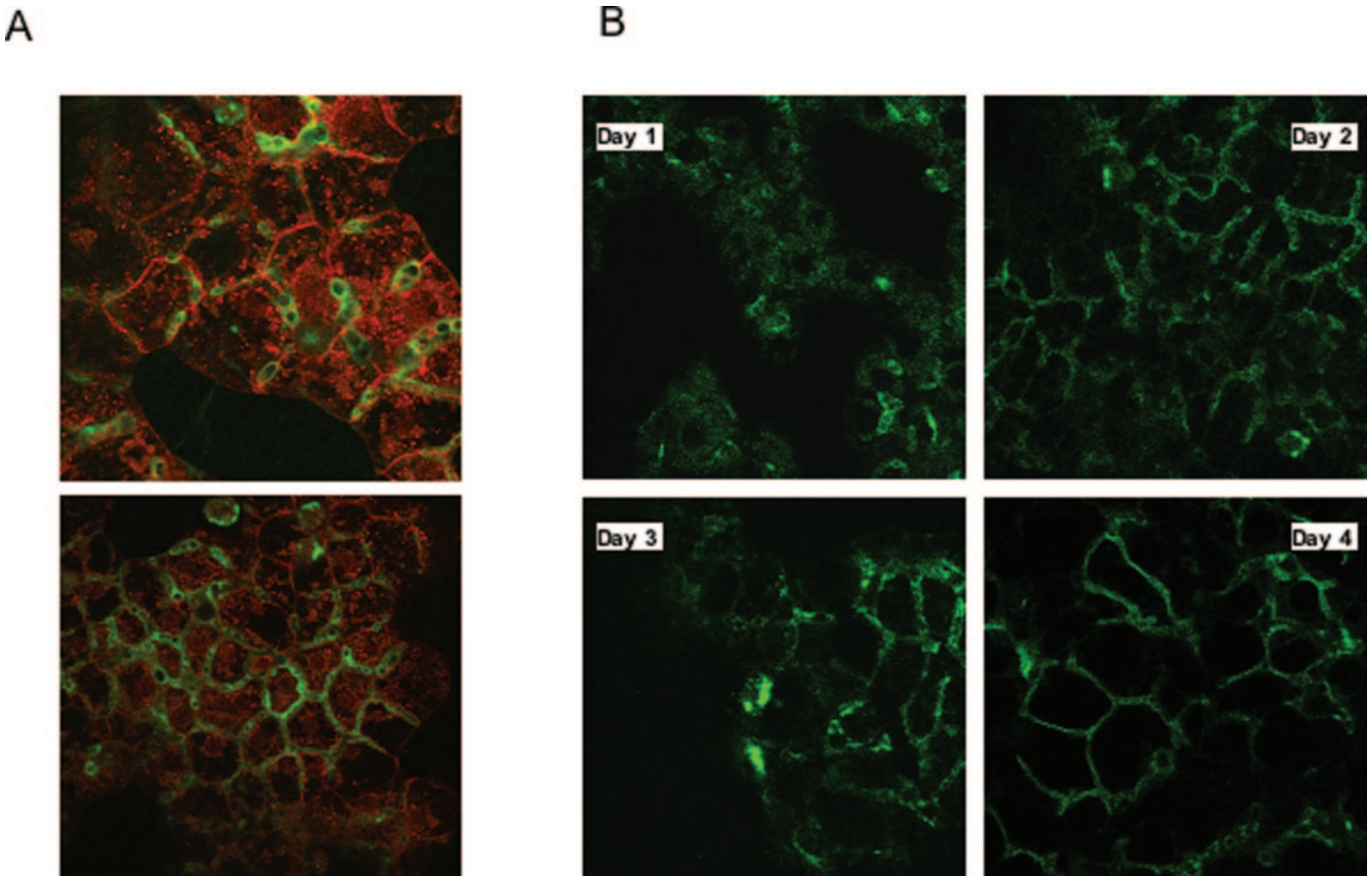
$$Cl_{bile} = \frac{\text{accumulation}_{(cells + bile)} - \text{accumulation}_{(cells)}}{\text{incubation time} \times \text{incubation medium concentration}} \times 100\%$$

**Sample Preparation for Western Blot.** The method for the preparation of total cellular membranes and cytosolic fractions from sandwich-cultured rat hepatocytes was adapted from Almquist et al. (1995). Sandwich-cultured rat hepatocytes in 60-mm dishes on day 0 or day 4 were washed with ice-cold 1× phosphate-buffered saline (PBS) (Sigma-Aldrich), harvested, snap frozen in liquid nitrogen, and stored at −80°C for subsequent use. Frozen cells were thawed and suspended in membrane preparation buffer (10 mM Tris-HCl, pH 7.6, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, and 1 mM EDTA) plus Complete protease inhibitor cocktail. Cells were homogenized (80 vigorous strokes, in ice-cold homogenizer) and disrupted with a sonic dismembrator (model 100; Fisher Scientific Co., Pittsburgh, PA) (4 × 15-s bursts with 15-s intervals on ice). Homogenate was spun at 800g at 4°C for 20 min to remove nuclei and unbroken cells. The supernatant was then spun at 130,000g at 4°C for 60 min. The separated total cellular membrane pellet and supernatant (cytosol) were snap frozen in liquid nitrogen and stored at −80°C for Western blot analysis. Whole cell lysate from sandwich-cultured rat hepatocytes was prepared with 1% SDS/1 mM EDTA.

**Western Blot.** Aliquots containing 50 μg of protein from whole cell lysate, total cellular membranes, or cytosol in sample buffer and 50 mM dithiothreitol were loaded onto a 10-well 4 to 12% Bis-Tris gel

(Invitrogen). Electrophoresis was carried out in the Novex mini-gel system from Invitrogen, at constant voltage (150 V) for 2 h. Proteins were electrophoretically transferred onto polyvinylidene difluoride membranes. The blots were blocked overnight at 4°C in 5% dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBS/T, pH 7.4), and incubated with anti-Mrp2 antibody (1:2000) in TBS/T for 1 h at room temperature. After three washes, secondary antibody anti-mouse IgG (1:1000) in TBS/T was incubated with the blots for 1 h at room temperature. Mrp2 was detected using the LumiGlo chemiluminescence reagent kit. Membranes were stripped and reprobed with mouse anti-actin antibody (1:2000) and secondary antibody anti-mouse IgG (1:10000). Densitometric analysis was performed using Quantity One version 4.40 software (Bio-Rad, Hercules, CA), and all data were normalized for actin.

**Immunohistochemical Assay.** Sandwich-cultured rat hepatocytes were fixed with ice-cold acetone for 10 min at 4°C and air-dried. Small sections were excised and stored at −80°C. Samples were rehydrated with ice-cold blocking buffer (PBS containing 5% goat normal serum and 1% BSA) for 40 min at room temperature. Fixed sandwich-cultured rat hepatocytes were incubated with different primary antibodies, mouse IgG, or rabbit serum in PBS containing 1% BSA at room temperature for 1 h. The primary antibodies were either mouse monoclonal anti-Mrp2 alone (1:400) or mouse monoclonal anti-Mrp2 (1:400) plus rabbit polyclonal anti-calreticulin (1:600) or rabbit anti-Mrp2 serum (1:600) plus mouse monoclonal anti-Golgi (1:600). Samples were washed three times with PBS for 10 min and incubated with fluorescence-conjugated goat anti-mouse IgG (~1:150–200) and/or fluorescence-conjugated goat anti-rabbit IgG (1:200) in PBS containing 1% BSA for 1 h at room temperature. After washing three times with PBS for 10 min, the fluorescent images



**Fig. 2.** Confocal microscopy images revealing immunofluorescent localization of Mrp2 (green) on the canalicular domain and Mrp3 (red) on the basolateral domain in day 4 sandwich-cultured rat hepatocytes (A), and Mrp2 in sandwich-cultured rat hepatocytes over time in culture (B). The duration of culture time before the cells were fixed is indicated.



were obtained with a Leica TCS-NT inverted confocal microscope (Leica, Heidelberg, Germany).

## Results

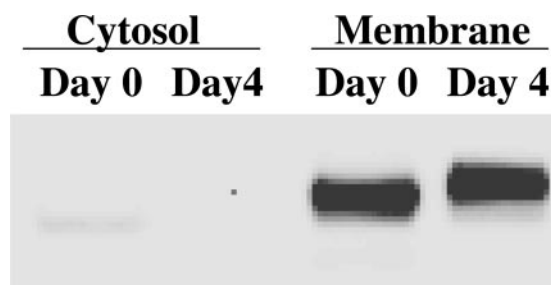
**Mrp2 Expression in Sandwich-Cultured Rat Hepatocytes.** The expression of Mrp2 protein was determined by Western blot analysis of total cell lysates from day 0 through day 4 in sandwich-cultured rat hepatocytes (Fig. 1). Mrp2 protein levels seemed to remain constant during the 4-day culture period, although the molecular mass of Mrp2 increased during culture by  $\sim 10$  kDa from  $\sim 190$  kDa (day 0) to  $\sim 200$  kDa (day 4). The band representing Mrp2 was  $\sim 190$  kDa in day 0 hepatocytes, which was similar to the molecular mass of Mrp2 in freshly isolated rat liver (Fig. 7).

**Localization and Function of Mrp2 in Sandwich-Cultured Rat Hepatocytes.** Day 4 sandwich-cultured rat hepatocytes exhibited distinct canalicular and basolateral domains, as evidenced by the immunofluorescent localization of canalicular Mrp2 and basolateral Mrp3 (Fig. 2A). In Fig. 2A, top, the image from one section of day 4 sandwich-cultured rat hepatocytes clearly showed that Mrp2 localized to the tubular structures of the bile canaliculi (green), whereas the staining pattern of Mrp3 revealed fluorescence in the basolateral membrane (red). In some areas, the red staining (Mrp3) occurred underneath the green staining (Mrp2), but they were not colocalized. Fig. 2A, bottom, presents an image from another section of day 4 sandwich-cultured rat hepatocytes that clearly reveals the intensive canalicular networks. Despite the fact that Mrp2 protein levels were relatively consistent from day 0 through day 4 in culture, Mrp2 was not localized properly to the canalicular domain early in culture. Immunofluorescent localization of Mrp2 detected by confocal microscopy (Fig. 2B) indicated that on day 1, Mrp2 was localized intracellularly. Over time in culture, Mrp2 progressively translocated to the canalicular domain, and by day 4, Mrp2 was localized almost exclusively to this site. To investigate the localization of Mrp2 after hepatocyte isolation, total cellular membranes and cytosolic fractions were prepared by ultracentrifugation and analyzed by Western blot. Most Mrp2 in day 0 cells was found in the total cellular membrane fraction with molecular mass  $\sim 190$  kDa; only trace amounts of Mrp2 were detected in cytosol. In day 4 cells, all the Mrp2 was found in the total cellular membrane fraction with molecular mass  $\sim 200$  kDa (Fig. 3). Disposition of the Mrp2 substrate CDF in sandwich-cultured rat hepatocytes over the same culture period confirmed the progressive localization of Mrp2 to the canalicular domain (Fig. 4). Because of Mrp2 internalization after hepatocyte isolation, CDF fluorescence was localized predominantly within the cell at day 0. As canalicular networks gradually formed over time in culture, CDF excretion into canalicular networks increased; fluorescence was localized primarily to the canalicular networks by day 4. The combined cellular + bile canalicular accumulation of CDF on day 1 was  $846.1 \pm 98.4$  pmol/mg of protein and was  $491.9 \pm 28.1$  pmol/mg of protein on day 4. In contrast, the cellular accumulation of CDF decreased from  $815.8 \pm 116.4$  pmol/mg of protein on day 1 to  $351.3 \pm 1.0$  pmol/mg of protein on day 4. Because of negligible biliary excretion of CDF on days 1 and 2, the biliary excretion index and  $Cl_{\text{bile}}$  of CDF in sandwich-cultured rat hepatocytes were

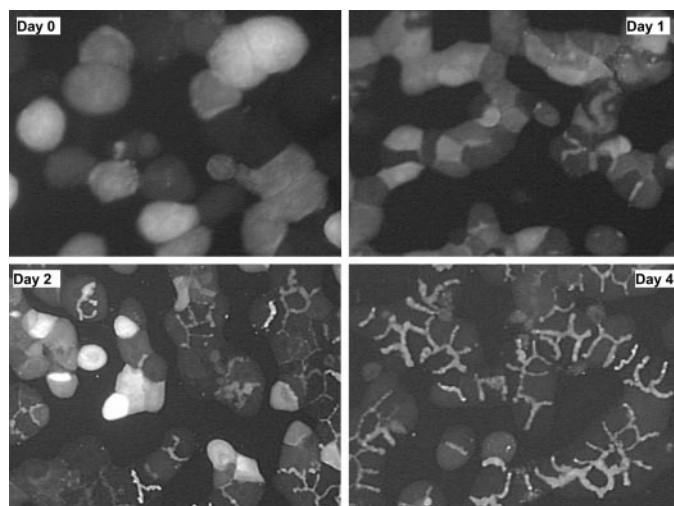
approximately zero, but these values increased to  $\sim 30\%$  and  $\sim 11$  ml/min/kg by day 4, respectively (Table 1).

**Glycosylation of Mrp2 Protein.** Treatment of sandwich-cultured rat hepatocytes with tunicamycin for 56 h (from day 1 to day 4) resulted in a reduction in the intensity of the 200-kDa Mrp2 protein band and an increase in the 180-kDa band (Fig. 5). As shown in Fig. 6, the shift in molecular mass from 200 to 180 kDa was proportional to treatment time with tunicamycin. Densitometric analysis of these data suggested that the half-life of the  $\sim 200$ -kDa Mrp2 protein was  $\sim 45$  h in sandwich-cultured rat hepatocytes.

Day 0 and day 4 sandwich-cultured rat hepatocytes were lysed with protease inhibitor cocktail and treated with or without PNGase F to deglycosylate Mrp2 protein. Samples from fresh rat liver were used as a control. The molecular mass of Mrp2 in day 0 hepatocytes and fresh liver was  $\sim 190$  kDa, but it decreased to 180 kDa after PNGase F treatment. Incubation of day 4 hepatocyte lysate with PNGase F caused the Mrp2 protein band at 200 kDa to split into two bands: a 190-kDa band that is less glycosylated and a 180-kDa band that is nonglycosylated (Fig. 7A). All Mrp2 protein became nonglycosylated ( $\sim 180$  kDa) after a 40-min incubation with PNGase F (Fig. 7B); the molecular mass of nonglycosylated Mrp2 was consistent with data collected from Sf9 cells (data not shown).



**Fig. 3.** Mrp2 protein in total cellular membranes and cytosol from day 0 and day 4 hepatocytes was detected by Western blot. Most of the Mrp2 in day 0 cells was detected in total cellular membranes with only a weak band in the cytosolic fraction from day 0 cells. Mrp2 protein was only detected in total cellular membranes from day 4 cells.



**Fig. 4.** CDF disposition in sandwich-cultured rat hepatocytes measured by fluorescence microscopy over time in culture. The duration of culture time before addition of CDF is indicated. CDF fluorescence was localized in the canalicular networks by day 4.

**Mrp2 Trafficking to the Canalicular Domain.** After treatment of sandwich-cultured rat hepatocytes with tunicamycin, immunohistochemical localization of Mrp2 protein was determined by confocal microscopy. On day 4, Mrp2 was localized predominantly to the canalicular domain. Treatment with tunicamycin for 24 h (from day 3 to day 4) resulted in partial relocation of Mrp2 to the perinuclear region (Fig. 8, top). Tunicamycin treatment for 48 h (from day 2 to day 4) resulted in almost complete internalization of Mrp2 compared with vehicle treatment (Fig. 8, bottom). Immunofluorescence analysis of the cells treated with tunicamycin for 48 h demonstrated colocalization of Mrp2 (green) with calreticulin, a standard endoplasmic reticulum marker (red) (overlap is shown in yellow; Fig. 9, top); minimal overlap in Mrp2 (green) and Golgi (red) staining was observed (Fig. 9, bottom). This study suggested that nonglycosylated Mrp2 most likely was retained inside the endoplasmic reticulum rather than the Golgi.

Discussion

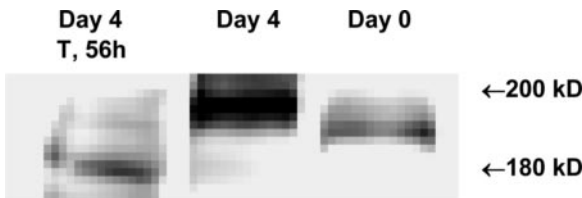
Sandwich-cultured rat hepatocytes maintain a more physiologically normal morphology with a higher level of transport protein expression and function compared with hepatocytes cultured in a conventional configuration (Liu et al., 1998). The sandwich configuration facilitates repolarization of the cells necessary for the formation of canalicular networks (LeCluyse et al., 1994). In the present study, Mrp2 protein levels, as determined by Western blot analysis, remained constant over 4 days in culture; however, the molecular mass of the Mrp2 band increased by ~10 kDa.

The constant level of Mrp2 protein over the 4 days in culture represents a balance between the degradation of existing Mrp2 and the synthesis of new Mrp2 in sandwich-cultured rat hepatocytes. After disruption of cell polarity by collagenase treatment during hepatocyte isolation, the apical

TABLE 1  
Accumulation of CDF in sandwich-cultured rat hepatocytes  
Values are presented as mean ± S.D.

Day	CDF Accumulation		BEI	Cl <sub>bile</sub>
	In Cells + Bile	In Cells		
	<i>pmol / mg of protein</i>			
1	846.1 ± 98.4	815.8 ± 116.4	1	5.2 ± 5.2
2	614.5 ± 152.9	643.4 ± 124.9	0	0
3	479.9 ± 16.9	458.1 ± 4.1	4.7	1.7 ± 1.7
4	491.9 ± 28.1	351.3 ± 1.0	28.1	11.3 ± 2.2

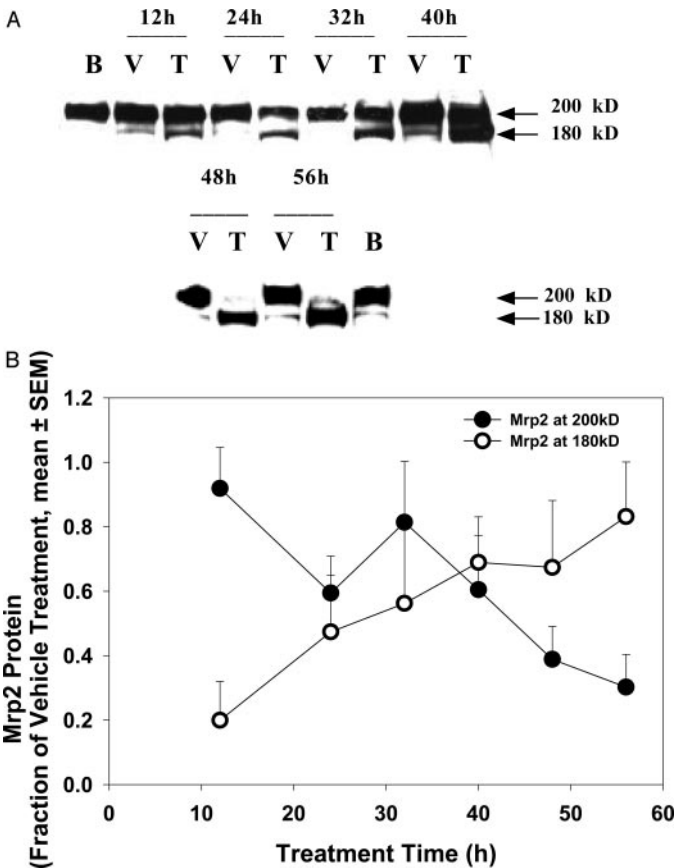
BEI, biliary excretion index.



**Fig. 5.** Representative Western blot of Mrp2 protein in day 0, day 4, and 0.1 μg/ml tunicamycin-treated day 4 sandwich-cultured rat hepatocytes. Lane 1 (~180 kDa), day 4 hepatocytes treated with 0.1 μg/ml tunicamycin (T) from day 1 to day 4; lane 2, day 4 hepatocytes (~200 kDa) treated with vehicle; and lane 3, day 0 hepatocytes (~190 kDa). Treatment with tunicamycin caused the Mrp2 protein band at ~200 kDa to disappear and the Mrp2 protein band at ~180 kDa to increase.

membrane containing Mrp2 is rapidly endocytosed (Røelofsen et al., 1995). Mrp2 in fresh liver and day 0 sandwich-cultured rat hepatocytes was partially glycosylated and was glycosylated further over days in culture, resulting in an increase in the molecular mass of Mrp2 by ~10 kDa. In day 0 or fresh liver, the molecular mass of Mrp2 decreased from ~190 to ~180 kDa after treatment with PNGase F, yielding nonglycosylated Mrp2. These results indicated that Mrp2 in fresh liver and day 0 hepatocytes was glycosylated. Mrp2 was glycosylated further over 4 days in culture, producing a band at ~200 kDa. Deglycosylation of Mrp2 from day 4 hepatocytes by PNGase F resulted in a band with the same ~180-kDa molecular mass as that of day 0 cells treated with PNGase F. The ~180-kDa Mrp2 protein was not glycosylated and was the same molecular mass as Mrp2 protein expressed in Sf9 cells, which do not glycosylate large membrane proteins (Germann et al., 1990).

Day 0 hepatocytes had low levels of glycosylation, similar to that of fresh liver. It seems that low levels of Mrp2 glycosylation are enough to traffic Mrp2 to the canalicular membrane under physiological conditions. Further glycosylation of Mrp2 in sandwich-cultured rat hepatocytes may be important in trafficking this protein to the canalicular membrane. Using immunofluorescence confocal microscopy, Mrp2 was internalized initially (day 1) and then gradually localized to



**Fig. 6.** A, representative Western blot of Mrp2 protein in vehicle and 0.1 μg/ml tunicamycin-treated day 4 sandwich-cultured rat hepatocytes (B, no vehicle; T, tunicamycin; V, vehicle). Treatment time is indicated in each lane. Tunicamycin blocked Mrp2 glycosylation causing the Mrp2 band at ~200 kDa to gradually disappear. B, time course of changes in Mrp2 protein in sandwich-cultured rat hepatocytes measured by densitometry in the presence of tunicamycin (●, ~200-kDa band; ○, ~180-kDa band) (mean ± S.E.M., n = 4).

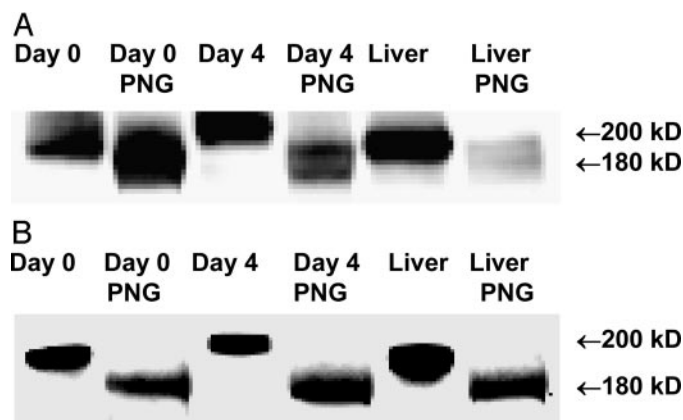
the canalicular domain (day 4). Western blot analysis suggested that after internalization, Mrp2 was still in the cellular membrane fraction (Fig. 3). The time required for localization may be associated with the time required to glycosylate the protein and translocate it through the cell. By day 4, canalicular networks were formed (Fig. 4), and Mrp2 had increased in molecular mass by ~10 kDa, consistent with glycosylation of the protein.

Subsequent experiments demonstrated that the localization of Mrp2 affects its transport function. CDF diacetate, which exhibits weak fluorescence, rapidly diffuses across the cell membrane of hepatocytes and is hydrolyzed by intracellular esterases in the cytoplasm to a highly fluorescent product, CDF (Zamek-Gliszczynski et al., 2003). CDF is a high-affinity substrate for Mrp2 (Liu et al., 1999b) and is transported efficiently into the canalicular lumen. Images of cells loaded with CDF obtained at different times in culture demonstrated that most of the fluorescence was retained inside the cell before day 2. By day 4, however, almost all of the fluorescence was concentrated in the sealed canalicular networks via transport by Mrp2. Cellular accumulation of CDF also was decreased on day 4 relative to day 1, most probably because of increased basolateral excretion of CDF by Mrp3, which was up-regulated over days in culture (Zhang et al., 2001). The biliary excretion index of CDF was measurable by day 3, indicating that at least a portion of the Mrp2 was localized to the canalicular domain and functional. Based on these results, sandwich-cultured rat hepatocytes should be cultured as specified for at least 3 days to ensure both proper Mrp2 localization and transport assessment.

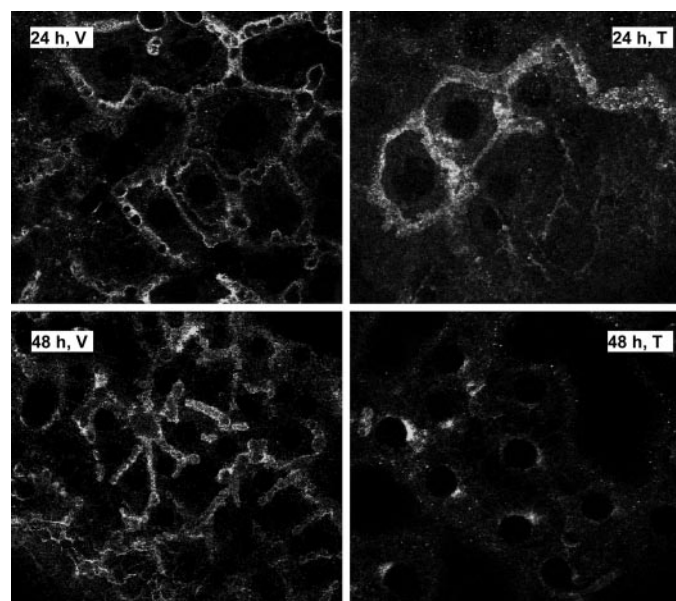
To further examine the relationship between glycosylation and localization of Mrp2, the time course of changes in Mrp2 localization after tunicamycin treatment was examined in sandwich-cultured rat hepatocytes. Mrp2 immunofluorescence images demonstrated that treatment with tunicamycin for 48 h caused Mrp2 to concentrate and localize around the nuclear membrane, with some fluorescence localized in a diffuse manner inside the hepatocytes. Based on Western blot analysis, the glycosylated Mrp2 at ~200 kDa gradually disappeared and an ~180-kDa nonglycosylated Mrp2 band gradually occurred. Immunofluorescence studies revealed

that Mrp2 colocalized with the endoplasmic reticulum, suggesting that nonglycosylated Mrp2 after tunicamycin treatment was trapped in the endoplasmic reticulum (Fig. 9), consistent with the hypothesis that the nonglycosylated form of Mrp2 could not traffic to the canalicular membrane. Parodi et al. (1999) observed that only correctly folded and glycosylated proteins exit the endoplasmic reticulum to the Golgi complex. Proteins retained within the endoplasmic reticulum are subject to degradation by proteases. Thus, if Mrp2 was not promptly glycosylated, the protein may be retained within the endoplasmic reticulum and subject to degradation by proteasomes (Keitel et al., 2000). Based on Western blot analysis, a very small band (~40 kDa; data not shown) did occur after 48 h of tunicamycin treatment, but not in vehicle-treated cells, supporting the hypothesis that Mrp2 was degraded when glycosylation was inhibited by tunicamycin. The immunofluorescent images of Mrp2 after 48 h of tunicamycin treatment were decreased in intensity compared with vehicle treatment, consistent with the observation that the total amount of Mrp2 protein after tunicamycin treatment was less than that in vehicle-treated cells.

Because tunicamycin does not hydrolyze the existing glycosylated groups on Mrp2 but rather prevents new protein from being glycosylated, the ~180-kDa band most probably represents newly synthesized protein. However, we cannot exclude the possibility that internalized ~190-kDa Mrp2 was deglycosylated and degraded to ~180 kDa, as a result of normal degradation processes in the cell. The protein synthesis inhibitor cycloheximide was used to inhibit Mrp2 synthesis, but the results were inconclusive because incubation for ≥48 h with 10 μg/ml cycloheximide caused toxicity in the hepatocytes. Lower concentrations of cycloheximide did not seem to inhibit Mrp2 protein synthesis completely. Because there was no newly glycosylated Mrp2 that could occur in this system because of the presence of tunicamycin in the culture medium, the half-life of the glycosylated Mrp2 protein was estimated to be ~45 h. However, because of the the

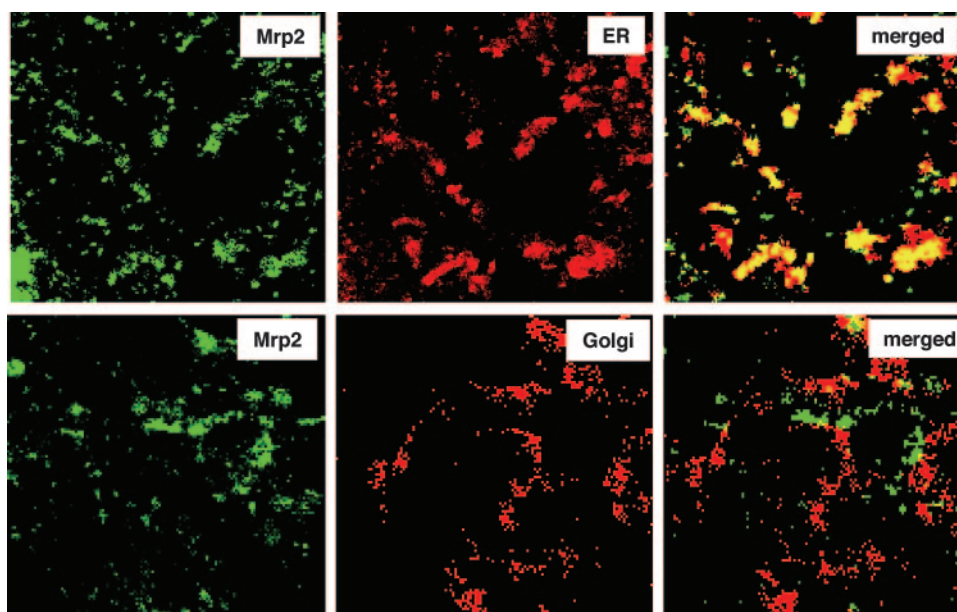


**Fig. 7.** Representative Western blot of Mrp2 protein in day 0 and day 4 sandwich-cultured rat hepatocytes, and fresh liver, with or without PNGase F (PNG) treatment for 20 (A) or 40 (B) min. Treatment with PNGase F for 20 min split the 200-kDa band in day 4 hepatocytes into ~190- and ~180-kDa bands. Treatment with PNGase F for 40 min totally shifted the ~200-kDa band from day 4 hepatocytes into an ~180-kDa band.



**Fig. 8.** Confocal microscopy images revealing immunofluorescent localization of Mrp2 in day 4 sandwich-cultured rat hepatocytes. The cells were treated with vehicle (V) or 0.1 μg/ml tunicamycin (T) for 24 or 48 h before they were fixed.





**Fig. 9.** Costaining of Mrp2 with endoplasmic reticulum marker (top) and costaining of Mrp2 with Golgi (bottom) in day 4 sandwich-cultured rat hepatocytes treated with 0.1  $\mu$ g/ml tunicamycin for 48 h. Top, mouse anti-Mrp2 (1:400) and rabbit anti-endoplasmic reticulum marker (1:600) were used to detect Mrp2 (green) and endoplasmic reticulum marker (red); colocalization of Mrp2 and the endoplasmic reticulum marker was observed (yellow). Bottom, rabbit anti-Mrp2 (1:600) and mouse anti-Golgi (1:600) were used to detect Mrp2 (green) and Golgi (red). No colocalization was observed.

poor separation of the 200- and 180-kDa bands in some blots (Fig. 6A), it is possible that the band at  $\sim$ 180 kDa might partially overlap with the band at  $\sim$ 200 kDa. Thus, this half-life of Mrp2 may be an overestimate of the true half-life.

Several factors influence the trafficking and localization of transport proteins. One of these factors, protein glycosylation, has been shown to be crucial for the correct trafficking of many membrane transporters (Lee et al., 2003). Mutations causing amino acid sequence changes in human MRP2 result in Dubin-Johnson syndrome, a condition characterized by impaired bilirubin secretion into bile (Keitel et al., 2000). This mutation results in defective folding of MRP2 within hepatocytes and thereby impairs MRP2 localization and function. In one patient with Dubin-Johnson syndrome, deletion of two amino acids in the sequence of MRP2 resulted in a mutant protein that was more sensitive to endoglycosidase H digestion, and this patient subsequently expressed a less glycosylated form of MRP2. In this case, MRP2 was trapped in the endoplasmic reticulum and was unable to traffic to the apical membrane. Several other mutations found in Dubin-Johnson syndrome patients result in both glycosylated and nonglycosylated forms of MRP2. The glycosylated MRP2 mutant could correctly traffic to the apical cell membrane of hepatocytes, whereas the nonglycosylated mutant exhibited deficient maturation and impaired sorting (Hashimoto et al., 2002). These studies suggest that MRP2 glycosylation is an important determinant of MRP2 trafficking and function in humans. Under sandwich-cultured conditions, Mrp2 seems to require more extensive glycosylation to traffic to the canalicular membrane. No clear evidence at this point has elucidated the physiological role of more extensive glycosylation of Mrp2, but it may play a role in certain pathological conditions, such as cholestasis. Despite the above-mentioned observations with MRP2, glycosylation is not necessarily required for all transport proteins to properly localize to their respective membranes and function optimally. Nonglycosylated rabbit Mrp2 functioned properly in membrane vesicles from Mrp2-transfected Sf9 cells (van Aubel et al., 1998), and studies performed with different glycosylated forms of P-glycoprotein and Mrp1 have demonstrated that glycosylation

has no effect on the transport activity of these proteins (Cai et al., 2001; Urbatsch et al., 2001). In addition to glycosylation of MRP2, radixin, a member of a protein family responsible for the cross linking of actin filaments and integral membrane proteins (Tsukita and Yonemura, 1999), recently was reported to be involved in Mrp2 trafficking. Radixin deficiency resulted in selective loss of Mrp2 from the bile canalicular membrane, suggesting that radixin also may be involved in correct Mrp2 localization (Kikuchi et al., 2002). The relationship between radixin and Mrp2 in sandwich-cultured hepatocytes is the subject of ongoing studies in our laboratory.

In summary, Mrp2 in rat hepatocytes is internalized after isolation at day 0. For the internalized  $\sim$ 190-kDa Mrp2 to be trafficked to the correct domain in sandwich-cultured rat hepatocytes, both reestablishment of the canalicular network and more extensive glycosylation of the Mrp2 protein seem to be required. The time course of glycosylation correlates with the time course of canalicular Mrp2 localization. Mrp2 transport function correlated with the localization of Mrp2 to the canalicular domain in sandwich-cultured rat hepatocytes. These data suggest that glycosylation plays an important role in the canalicular sorting of this protein in sandwich-cultured hepatocytes.

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