GASTROENTEROLOGY 1997;112:511–521

Increased Levels of the Multidrug Resistance Protein in Lateral Membranes of Proliferating Hepatocyte-Derived Cells

HAN ROELOFSEN,* THERA A. VOS,* INGRID J. SCHIPPERS,†,§ FOLKERT KUIPERS,§ HANS KONING,* HAN MOSHAGE,* PETER L. M. JANSEN,* and MICHAEL MÜLLER*

*Division of Gastroenterology and Hepatology, Department of Internal Medicine, Groningen Institute for Drug Studies, Groningen; †Bio-Medical Technology Center, Groningen; and §Department of Pediatrics, Laboratory of Nutrition and Metabolism, University Hospital, Groningen, The Netherlands

Background & Aims: The multidrug resistance protein (MRP) functions as an organic anion efflux carrier. Recent studies suggest that hepatocytes contain two mrp homologues, named mrp1 and mrp2, localized on the lateral and canalicular membrane, respectively. The aim of this study was to evaluate the role of MRP1. Protein levels and localization of MRP1 in human hepatocytes, HepG2 hepatoma cells, and SV40 large T antigen–immortalized human hepatocytes were studied. _Methods:_ Using specific antibodies, MRP1 protein levels and cellular localization were examined by Western blotting and fluorescence confocal microscopy, respectively. In addition, a fluorescent substrate, glutathione-methylfluorescein, was used to measure plasma membrane transport activity and to observe intracellular transport activity. _Results:_ The level of MRP1 in normal hepatocytes is very low. In contrast, MRP1 is highly increased in both HepG2 and immortalized hepatocytes. In these cells MRP1 is localized in lateral membranes of adjacent cells. Plasma membrane staining is absent in separate cells. Glutathione-methylfluorescein is transported in the medium and intracellular vesicles. _Conclusions:_ MRP1 protein level is greatly increased in the lateral membrane of proliferating hepatocyte-derived cells. The presence of a lateral domain seems necessary for plasma membrane localization. These results suggest that MRP1-mediated organic anion transport is important in proliferating hepatocytes, but not in quiescent cells.

The multidrug resistance protein (MRP) is an efflux pump involved in multidrug resistance (MDR). Like MDR1 P-glycoprotein (Pgp), MRP is a member of the adenosine triphosphate (ATP)-binding cassette superfamily.[1] However, homology between MDR1/Pgp and MRP is very low. The *MRP* gene has been isolated first by Cole et al.[2] from the doxorubicin-resistant small cell lung cancer cell line that shows non–Pgp-mediated multidrug resistance. MRP is a 1531–amino acid, 190-kilodalton, N-glycosylated integral membrane protein, encoded by a 6.5-kilobase messenger RNA.[2,3] Recently, it has been shown that MRP transports anionic glutathione S–conjugates in an ATP-dependent fashion.[4–6] Substrates include leukotriene C_4 and glutathione-dinitrophenyl. Immunocytochemical studies performed in *MRP* complementary DNA–transfected HeLa cells and SW1573 lung carcinoma cells, and in drug-induced MRP-overexpressing cells such as the human small cell lung cancer cell line GLC4/ADR,[7–9] show that MRP is localized mainly in the plasma membrane. However, these are all nonpolarized cells. A recent study by Mayer et al.[10] indicates that localization of MRP may be different in polarized cells. The investigators show that MRP/mrp is present in both human and rat hepatocytes, and it is localized in the canalicular as well as the lateral membrane. The canalicular membrane of the hepatocyte contains an ATP-dependent organic anion efflux pump named canalicular multispecific organic anion transporter (cMOAT).[11] This carrier is responsible for the transport of a wide variety of organic anions into bile, such as glutathione S–conjugates and bilirubin diglucuronides. Until recently, this carrier was only functionally characterized by the absence of its transport activity in the TR^- (transport deficient) rat. This mutant rat strain is deficient in canalicular organic anion transport.[12–16] The substrate specificity of cMOAT is very similar to that of MRP.[4–6] This suggests that cMOAT may be similar or identical to rat mrp. However, organic anion transporters on both the canalicular and lateral membrane of the hepatocyte seem incompatible with vectorial transport of its substrates into bile. A recent study by Paulusma et al.[17] reports that a homologue of mrp, called mrp2, is absent in the TR^- rat. This suggests that mrp2 may

Abbreviations used in this paper: BCECF, 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein; CMFDA, chloromethylfluorescein diacetate; cMOAT, canalicular multispecific organic anion transporter; FITC, fluorescein isothiocyanate; GS-MF, glutathione-methylfluorescein; MDR, multidrug resistance; MRP, multidrug resistance protein; Pgp, P-glycoprotein; TRITC, tetramethylrhodamine isothiocyanate.

© 1997 by the American Gastroenterological Association
0016-5085/97/$3.00

be identical to the canalicular organic anion transporter cMOAT. Therefore, probably two mrps are present in the rat hepatocyte, one lateral form (mrp1) and one apical form (mrp2 or cMOAT).

If it can be confirmed that mrp2 is identical to cMOAT, its function clearly is the transport of organic anions from liver to bile. On the other hand, the function of mrp1 needs to be defined. Abundance, localization, and function of MRP1 in HepG2 hepatoma cells, human hepatocytes immortalized by transfection with the SV40 large T antigen, and normal human hepatocytes were studied to examine this in human hepatocytes. The HepG2 cell line is well differentiated and has retained many hepatocyte-specific functions such as albumin and bile acid synthesis.[18,19] Furthermore, in a monolayer, a certain fraction of HepG2 cells is polarized, with microvilli-lined vacuoles between adjacent cells resembling bile canaliculi. These vacuoles contain apical markers, stain for actin, fodrin, and villin, and are sealed by tight junctions and desmosomes.[20,21] Furthermore, it has been shown that both rhodamine-labeled phosphatidylethanolamine and 7-nitrobenz-2-oxa-1,3-diazole–labeled ceramide accumulate in these microvilli-lined vacuoles.[22] To study expression and localization of MRP protein in these cells, a polyclonal antibody was developed that proved specific for both rat and human MRP1. For comparison, the localization of Pgp protein was determined in these cells. Pgp is known to be localized in the canalicular membrane of the hepatocyte.[23] Also the localization of MRP transport activity was studied by using the fluorescent glutathione conjugate glutathione-methylfluorescein (GS-MF), which is formed intracellularly from chloromethylfluorescein diacetate (CMFDA) by the action of esterase and glutathione S–transferase. A previous study indicates that GS-MF is a substrate for MRP/mrp (Roelofsen et al., unpublished data).

The MRP1 level is significantly increased in HepG2 and immortalized human hepatocytes compared with normal human hepatocytes. MRP1 is confined mainly to the lateral membranes of adjacent cells in both cell lines and is not detectable in the plasma membrane of separate cells.

Materials and Methods

Materials

The rabbit polyclonal antibody against MRP1 (MRPk5) was raised against a 15–amino acid peptide of the carboxyl end of human MRP1.[24] Monoclonal antibodies MRPr1, MRPm6,[8] and JSB-1 were kindly provided by Dr. R. J. Scheper (Free University, Amsterdam, The Netherlands). The monoclonal antibody against mrp2 was obtained from Dr. Oude Elferink (Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands). The mouse monoclonal antibody C219 against Pgps was obtained from Centocor (Antwerp, Belgium). Tetramethylrhodamine isothiocyanate (TRITC)- and fluorescein isothiocyanate (FITC)-labeled phalloidin, FITC-labeled goat anti–mouse immunoglobulin (Ig) G, and TRITC-labeled goat anti–rabbit and rabbit anti–rat IgG were from Sigma (St. Louis, MO). TRITC-labeled rabbit anti–mouse IgG, polyclonals rabbit anti–human albumin, and rabbit anti–human fibrinogen were from Dako (Glostrup, Denmark). Polyclonal anti–human apolipoprotein B was from Calbiochem (San Diego, CA). Monoclonal anti–human apolipoprotein B was from Pierce (Rockford, IL), and the monoclonal anti–human albumin and anti–human fibrinogen were from Cedarlane (Hornby, Ontario, Canada). Prolong Antifade mounting medium and CMFDA were from Molecular Probes Europe (Leiden, The Netherlands). Complete protease inhibitor set, containing antipain-hydrochloride, bestatin, chymostatin, E-64, leupeptin, pepstatin, phosphoramidon, pefabloc SC, ethylenediaminetetraacetic acid, and aprotinin, was from Boehringer (Almere, The Netherlands).

Cell Culture and Immortalization of Human Hepatocytes

The human hepatoma cell line HepG2[18] was cultured on glass coverslips or culture dishes in Williams' medium E with glutamax (Gibco, Breda, The Netherlands) supplemented with 5% fetal calf serum, 200 U/mL penicillin G, and 200 μg/mL streptomycin.

To obtain human immortalized hepatocytes, tumor-free human liver tissue was excised from tissue obtained after partial hepatectomy. The patient was a 59-year-old man, with a single colon cancer metastasis. Human hepatocytes were isolated from this tissue by collagenase perfusion as described previously.[25] Cells were seeded on six-well plates, precoated with collagen R, at a density of 2.5×10^6 cells/well in complete Williams' medium E containing 10% fetal calf serum, 2 mmol/L glutamine, 20 mU/mL insulin, 50 nmol/L dexamethasone, 100 U/mL penicillin, and 100 μg/mL streptomycin as described previously.[25] Twelve hours after seeding, cells were washed and transfected with a SV40 large T antigen–containing plasmid[26] (provided by B. Klein, Sylvius Laboratory, Leiden) by means of Lipofectin (Gibco) according to the method by Willems et al.[27] Transfected cells were subsequently cultured in a hormonally defined medium,[27] but without 10 mmol/L nicotinamide. After 2 months, cells were trypsinized (0.05% trypsin and 0.02% ethylenediaminetetraacetic acid) and growing cell lines were selected and subcultured in complete Williams' medium E with 10% fetal calf serum. Human-specific sandwich enzyme-linked immunosorbent assay techniques revealed that these cell lines produced albumin, fibrinogen, and apolipoprotein B. All procedures were performed in accordance with the regulations of the Medical Ethical Committee of the Groningen Institute for Drug Studies.

Preparation of Crude Membrane Fractions and Immunoblot Analysis

Cultured human cells were washed twice by phosphate-buffered saline (PBS) that contained 140 mmol/L sodium chlo-

ride, 9.0 mmol/L Na_2PO_4, and 1.3 mmol/L NaH_2PO_4. Then the cells were harvested from the culture flasks using a rubber policeman and subsequently centrifuged at 5000*g*. Cells were permeabilized in the presence of a cocktail of protease inhibitors (Complete, 1:10 wt/vol) in 5 mmol/L HEPES/KOH at pH 7.4 by freezing (−180°C) and rapid thawing. The cell homogenates were centrifuged at 10,000*g* and the resulting pellets were used as crude membrane fractions. Human hepatocytes were taken from the same cell preparation used for immortalization, as described previously. Membrane fractions of human hepatocytes were prepared as described for cultured cells. Mixed plasma membranes from liver homogenates of control (TR^+) and TR^- rats were prepared according to Meier et al.[28] Equal amounts of proteins were size fractionated on a 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. MRP was detected using the antibodies MRPm6, MRPr1, and MRPk5. Pgps were detected with the monoclonal antibody C219. For detection, the enhanced chemiluminescence kit from Amersham (Den Bosch, The Netherlands) was used.

Immunofluorescence Microscopy

HepG2 cells grown on glass coverslips and ∼75% confluent were used for immunolocalization of MRP and Pgp. Cells were fixed for 10 minutes in acetone at −20°C, washed with PBS for 15 minutes, incubated with the primary antibodies diluted in 1% bovine serum albumin in PBS for 20 hours at 10°C, and then incubated for 1 hour at 37°C in a humidified chamber. Cells were then washed three times for 10 minutes with PBS and incubated for 30 minutes with the secondary antibody diluted in 1% bovine serum albumin in PBS. For Pgp detection, FITC-labeled goat anti–mouse IgG was used. For MRP detection, TRITC-labeled goat anti–rabbit IgG (MRPk5), rabbit anti–rat IgG (MRPr1), or rabbit anti–mouse IgG (MRPm6) was used. After the second incubation, cells were washed three times for 10 minutes with PBS and mounted in Prolong Antifade mounting medium. Control stainings were performed by omitting the primary antibody.

For actin staining, cells were fixed for 20 minutes in 4% paraformaldehyde in a cytoskeleton-stabilizing buffer containing 100 mmol/L 1,4-piperazinebis(ethane sulfonic acid), 5 mmol/L ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, and 2 mmol/L $MgCl_2$ at pH 6.8. After washing with PBS, cells were permeabilized with 0.2% Triton X-100 (Sigma) in PBS for 5 minutes and washed three times for 5 minutes with PBS. Subsequently, cells were incubated with TRITC-labeled phalloidin (final concentration, 1 μg/mL) for 30 minutes and washed three times for 10 minutes and then mounted.

Images were taken with a confocal scanning laser microscope (True Confocal Scanner 4D; Leica, Heidelberg, Germany) equipped with an argon-krypton laser and coupled to a Leitz DM IRB inverted microscope (Leica). Double-label images were taken sequentially at 488 and 562 nm to avoid bleedthrough into the other channel.

Detection and Measurement of Transport Activity

For the detection of intracellular MRP activity, the fluorescent glutathione conjugate GS-MF was used. This is formed intracellularly by the action of esterase and glutathione S–transferase, from the nonfluorescent compound CMFDA. The latter compound is taken up by diffusion. Cells grown on coverslips were incubated with 2.5 μmol/L CMFDA for 15 minutes at 37°C. Coverslips were then transferred to HEPES-buffered medium containing 130 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgSO_4$, 1.3 mmol/L $CaCl_2$, 1.2 mmol/L KH_2PO_4, 19.7 mmol/L HEPES, and 5 mmol/L D-glucose at pH 7.4, and then the coverslips were put on ice. For examination, a small chamber was constructed by putting a coverslip on top of the coverslip to which the cells were attached. The two coverslips were separated by spacers and kept together with silicon grease. The intracellular accumulation of the substrate was observed by confocal microscopy.

For measurement of MRP-mediated transport out of the cells, cells cultured in six-well plates were loaded with 10 μmol/L CMFDA for 60 minutes at 10°C. Thereafter, cells were washed once with ice-cold HEPES-buffered medium and stored on ice in HEPES medium. GS-MF efflux from the cells was measured at 37°C or 10°C by adding media of 37°C or 10°C, respectively. At certain time intervals, 200-μL samples were removed and fluorescence was measured with a fluorescence enzyme-linked immunosorbent assay–plate reader (Titertek Fluoroskan II; ICN Biomedicals, Zoetermeer, The Netherlands) (excitation, 490 nm;emission, 520 nm).

Uptake of CMFDA by the cells was assessed by lysing the cells with 0.5% Triton X-100 in PBS after the 60-minute uptake period and subsequent washes. Fluorescence of the homogenate was determined.

To quantify the amount of fluorescence, a 25 μmol/L GS-MF standard was prepared as follows: 5 U of esterase, 5 U of glutathione S–transferase, 250 μmol/L reduced glutathione, and 25 μmol/L CMFDA were dissolved in 1 mL HEPES medium and incubated at 37°C until no increase in fluorescence was observed. A calibration curve of 0.1–1.0 μmol/L GS-MF was constructed in the linear range to which the fluorescence of the samples was correlated.

Results

Levels of MRP1 in Human Liver, HepG2, and Immortalized Human Hepatocytes

A polyclonal antibody was raised against a synthetic peptide consisting of the last 15 amino acids of the carboxyl terminal end of human MRP. Specificity of this antibody, MRPk5, for human and rat MRP1/mrp1 was tested by Western blot (Figure 1). For this reason, membrane fractions of the MRP1-overexpressing human lung cancer cell line GLC4/ADR[6] and the *MRP1*-transfected cell line S1/MRP1[8] were used and compared with their parental cell lines GLC4 and SW1573/S1, respec-

tively. Furthermore, mixed membranes of both normal (TR^+) and TR^- rat livers were compared with membrane fractions of HepG2 and human erythrocytes. MRPk5 recognizes a protein of ±190 kilodaltons in the parental cell lines SW1573/S1 and GLC4, which is much more abundant in the MRP1-overexpressing cell lines S1/MRP and GLC4/ADR, respectively. The bands of ±70 kilodaltons in S1/MRP and GLC4/ADR are caused by the breakdown of MRP1. MRP1 is known to be very prone to breakdown.[8] The molecular mass of 190 kilodaltons corresponds to that previously reported for human MRP.[6,7,9] Furthermore, the antibody reacts with a band of ±190 kilodaltons in HepG2 and human erythrocytes and reacts with a slightly lower band of ±170 kilodaltons in both normal and TR^- rat liver membranes with equal intensity. The molecular mass of ~170 kilodaltons for rat liver mrp was also reported by Mayer et al.[10] It has been shown that mrp2 is not present in the TR^- rat liver.[10,17] Therefore, a greater mrp-related signal in membranes from normal livers would be expected if MRPk5 recognizes both mrp1 and mrp2. However, the mrp detection signal is similar in both normal and TR^- livers. This suggests that the MRPk5 antibody is specific for the mrp1 homologue.

With this antibody and the two monoclonal antibodies MRPm6 and MRPr1,[8] the level of MRP1 in human liver cells was studied. Crude membranes were isolated from human liver, isolated human hepatocytes, two sublines of human SV40 large T antigen–immortalized hepatocytes (IH1 and IH2), and the human hepatoma cell line HepG2. For comparison, membranes of erythrocytes were also investigated. All three antibodies recognized a band corresponding to 190 kilodaltons in both the immortalized hepatocyte sublines and in HepG2, similar to that found in the erythrocytes (Figure 2). In the particulate fraction of whole liver, no MRP1 is detectable, whereas in the membrane fraction prepared from isolated human hepatocytes, a weak signal is detected with MRPr1 (Figure 2). Both immortalized hepatocyte sublines and HepG2 cells show a similar level of MRP1, which is greater than that of erythrocytes. This indicates that MRP1 is present at a low level in quiescent hepatocytes and is up-regulated in immortalized hepatocytes and HepG2. No difference in Pgp level is observed, in these cell types.

Localization of MRP1 and Pgps

MRPk5 was used to localize the mrp1 protein in frozen sections of rat and human liver. However, no labeling of hepatocytes was detected (data not shown). On Western blot, MRP1 is detectable in a membrane fraction prepared from isolated hepatocytes (Figure 2), which must be caused by the relatively low expression of the protein. The localization of MRP1 was also studied in HepG2 cells. Part of the HepG2 cells form microvilli-

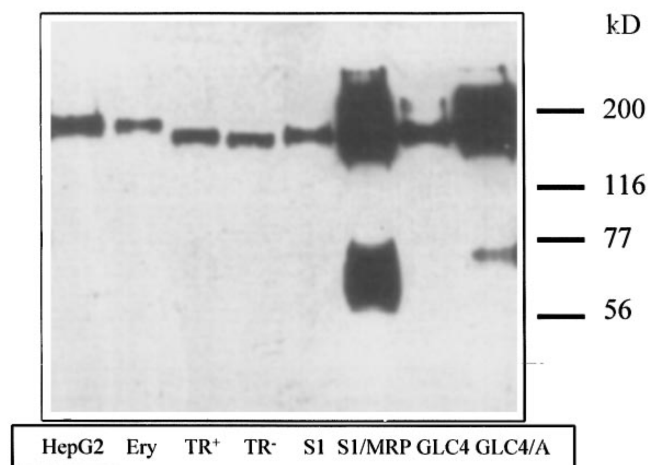


Figure 1. Detection of MRP1/mrp1 in membrane fractions of different human cell lines and rat hepatocytes using the MRPk5 antibody. Membranes were prepared from HepG2, human erythrocytes, MRP-overexpressing cell lines S1/MRP and GLC4/ADR, and their parental cell lines SW1573/S1 (S1) and GLC4, respectively. The mixed membrane fraction of normal (TR^+) and mutant TR^- rat livers were used. Equal amounts of protein (30 μg), S1/MRP, and GLC4/ADR (GLC4/A, 3 μg), were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose by electroblotting. The nitrocellulose blots were incubated with the polyclonal antibody MRPk5, and protein was observed using a chemiluminescence detection system.

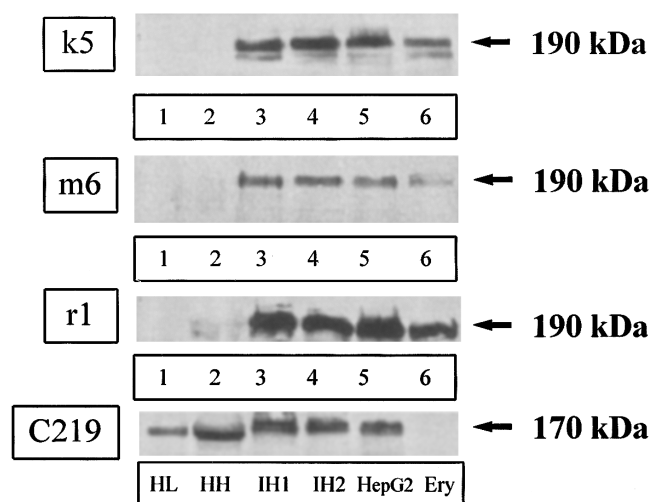


Figure 2. Immunoblot analysis of levels of MRP1 and Pgps in human hepatocytes, immortalized hepatocytes, and HepG2 cells. Crude membrane fractions were prepared from homogenates of human liver (HL), isolated human hepatocytes (HH), immortalized hepatocyte sublines (IH1 and IH2), and HepG2 cells. Membranes of human erythrocytes (Ery) were used for comparison. MRP1 was detected on immunoblot with the antibodies MRPk5, MRPm6, and MRPr1, respectively. The migration positions of MRP1 (190 kilodaltons) and Pgps (170 kilodaltons), detected by C219 antibody, are indicated.

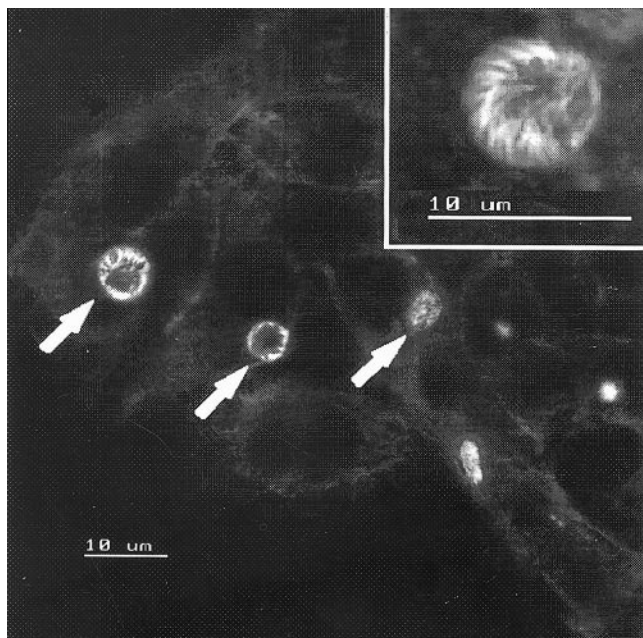


Figure 3. Detection of microvilli-lined apical vacuoles in HepG2 cells by actin labeling. HepG2 cells were fixed with 4% paraformaldehyde and processed for labeling with TRITC-labeled phalloidin. Images were taken with a confocal microscope. Next to the plasma membrane, several vacuolar structures (*arrows*) mostly located between two adjacent cells, were densely labeled. A higher magnification of such a vacuole (*inset*) shows that it contains numerous actin fibers representing microvilli.

lined apical vacuoles.[20,21] To study this, cells were stained for actin using TRITC-phalloidin. Some cells have formed large vacuolar structures, mostly asymmetrically situated in the cell membrane between two adjacent cells (Figure 3). Under greater magnification (Figure 3, insert), actin containing microvilli can be observed in these vacuoles. To determine whether Pgps, which are located apically in normal hepatocytes, are also present in these apical vacuoles, HepG2 cells were labeled for actin (Figure 4*A*) and Pgps (C219 antibody; Figure 4*B*). Colocalization of actin-positive vacuoles and Pgps is evident (Figure 4*A* and *B*). Also, a punctate staining in the cytoplasm was visible, probably representing Pgps located in the membranes of intracellular vesicles. No staining of the plasma membrane was apparent. This indicates that Pgps are almost exclusively present in apical vacuoles. To determine the localization of MRP1, HepG2 cells were labeled for MDR1-Pgp (JSB-1 antibody) and MRP1 (MRPk5 antibody) (Figure 4*C* and *D*). The results indicate that MRP1 is located in the lateral membrane of adjacent cells. Furthermore, 37% of MDR1-Pgp positive vacuoles (n = 150) also labeled for MRP1. No staining of the basal membrane, the membrane that is in contact with the medium, is apparent. Immortalized human hepatocytes show a similar labeling pattern for MDR1-Pgp and MRP1 (Figure 4*E* and *F*). MDR1-Pgp is mainly present in large (apical) vacuoles (Figure 4*E*). In contrast, MRP1 is located mainly in lateral membranes (Figure 4F). Similar results in both cell types were observed with the monoclonal anti–MRP antibodies MRPm6 and MRPr1 (data not shown).

Both lateral membranes of adjacent HepG2 cells contain MRP1 (Figure 5). Furthermore, punctate intracellular staining can be observed. This probably represents MRP1 located in vesicular membranes. Also, with this magnification, no labeling of the basal membrane is detected. This indicates that strong lateral staining cannot be explained by an additive effect of fluorescent signals from the lateral membranes of two adjacent cells. In contrast to Pgp, the occasionally observed MRP1 staining of apical vacuoles in HepG2 does not completely surround the entire circumference of the vacuole within the plane of focus, as is also apparent from Figure 4*D.* On a compiled three-dimensional z-series image of an apical vacuole stained for both MRP1 (MRPk5) and Pgps (C219), it seems that MRP is confined to a belt-like region around the vacuole (Figure 6). In contrast, the vacuole seems uniformly stained for Pgps. This staining pattern may represent a ring in which the lateral membrane makes contact with the apical vacuole. This shows that MRP1 is not located in the apical membrane. To determine whether MRP1 will only localize on the plasma membrane when a defined lateral domain is available, MRP1 localization in separate cells was studied. Therefore, HepG2 cells were double-labeled with acridine orange (to stain nuclei) and MRPk5. No staining of the plasma membrane of separate cells is detected while in the same preparation the lateral membranes of adjacent cells are strongly labeled (Figure 7). This suggests that defined lateral domains are a prerequisite for the appearance of MRP1 at the plasma membrane.

Localization of MRP Transport Activity

To compare the localization of MRP1 with MRP activity, the efflux rate of the fluorescent glutathione–conjugate GS-MF into the medium was determined. To determine efflux of GS-MF, cells were loaded with 10 μmol/L CMFDA at 10°C for 60 minutes. Under these conditions, 45% of CMFDA is taken up by the cells as measured in cell homogenates. The efflux of GS-MF from HepG2 cells at 37°C is ±5-fold greater than at 10°C (Figure 8). This temperature dependency of GS-MF efflux indicates involvement of a carrier-mediated process. The initial efflux rate of GS-MF was 289 ± 28 pmol $\cdot$ $min^{-1} \cdot$ mg $protein^{-1}$ (n = 5), as determined during the first 2.5 minutes.

GS-MF was also used to observe intracellular (vesicu-

lar) MRP transport activity. HepG2 cells grown on coverslips were incubated for 15 minutes with 2.5 μmol/L CMFDA. After washing, the cells were put on ice to reduce transport activity. Vesicular accumulation of the substrate was observed using confocal microscopy. GS-MF accumulates in randomly distributed intracellular vesicles and in large vacuolar structures, probably representing apical vacuoles (Figure 9*A* and *B*). This indicates that MRP activity is present in these structures. Because no MRP1 staining was found on apical vacuoles, transport of GS-MF into apical vacuoles may be mediated by MRP2. This was confirmed with a monoclonal antibody raised against rat mrp2. With this antibody, clear staining of apical vacuoles in HepG2 can be observed (data not shown), suggesting that MRP2 is present in these vacuoles and thus may be responsible for the observed GS-MF accumulation. GS-MF–accumulating vesicles predominantly have a round appearance, although in some cells, tubular structures are found (Figure 9*B*). Furthermore, GS-MF–negative vesicles can be observed (Figure 9*B*), indicating that these vesicles do not contain MRP activity. GS-MF accumulating vesicles were also observed in immortalized hepatocytes (data not shown).

Discussion

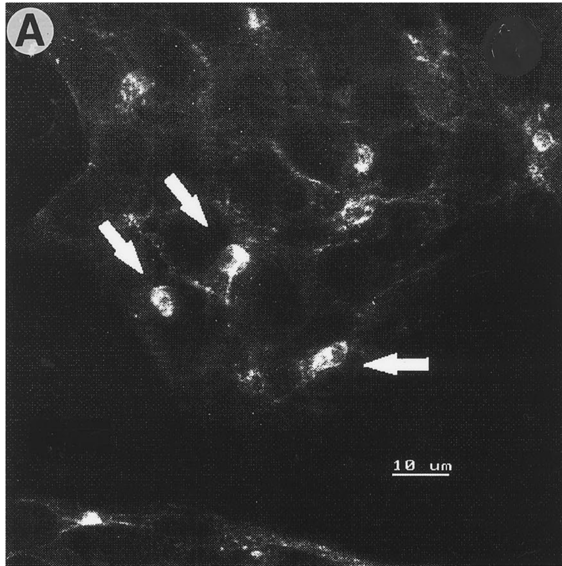
MRP was first described as a protein involved in multidrug resistance[2] and shown to function as an ATP-dependent glutathione S–conjugate efflux pump.[4–6] In a recent paper, Mayer et al.[10] presented data that indicate MRP/mrp is localized in the canalicular as well as the lateral membrane of human and rat hepatocytes. In the latter study, no apical staining was found in livers of the mutant TR^- rat, which lacks canalicular organic anion transport activity mediated by cMOAT.[11] Recently, Paulusma et al.[17] reported that in rats, apical mrp represents a homologue of mrp, named mrp2, which is mutated in the TR^- rat. This suggests that mrp2 is probably identical to cMOAT. The function of the mrp1/MRP1 is not known. Organic anion transporters on both the canalicular and lateral membranes of the hepatocyte seem incompatible with the vectorial transport of organic anions into bile, unless the level of mrp1/MRP1 is very low compared with mrp2/MRP2.

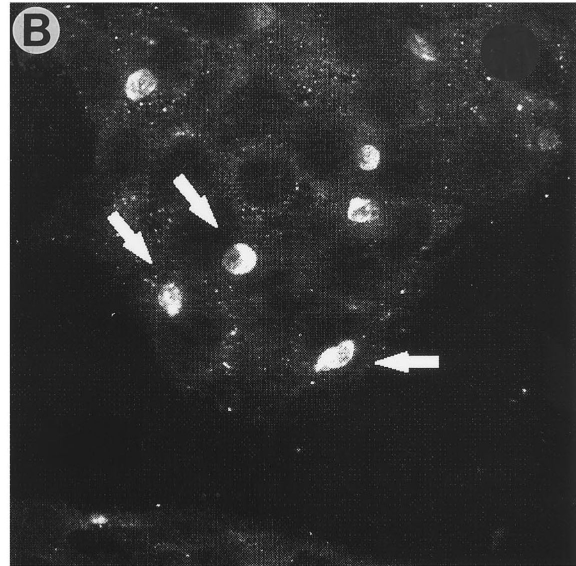
To study the function of human MRP1, the abundance of MRP1 in human hepatocytes was compared with that in proliferating liver cell lines, such as HepG2 hepatoma cells and human hepatocytes, immortalized by transfection with the SV40 large T antigen. To determine levels of MRP1 in these cells, a polyclonal antibody (MRPk5) against an MRP1-derived synthetic peptide was raised. On Western blot, MRPk5 recognizes a protein of ±190 kilodaltons, which is highly abundant in the human MRP1–overexpressing cell lines S1/MRP and GLC4/ADR compared with their parental cell lines. Furthermore, MRPk5 detects mrp1 with a molecular weight of ±170 kilodaltons in both normal and TR^- rat liver membranes. This slightly lower molecular mass in rat liver has previously been reported by Mayer et al.[10] and was attributed to a difference in glycosylation. The similar amounts of mrp detected in both normal and TR^- hepatocyte membranes indicate that MRPk5 does not significantly cross-react with mrp2. These results indicate that MRPk5 specifically recognizes MRP1/mrp1 in both human and rat.

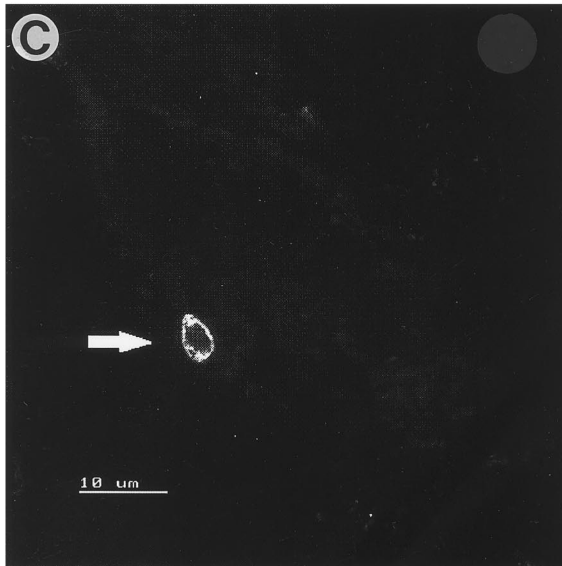
MRP1 is barely detectable in whole human liver or in isolated human hepatocytes. In contrast, relatively high levels of MRP1 were found in HepG2 cells. Also, when proliferation of human hepatocytes is induced by transfection with SV40 large T antigen, the level of MRP1 is highly increased to a similar level as in HepG2. Levels of Pgps did not differ between these three cell types. This indicates that MRP1 appears up-regulated in proliferating hepatocytes.

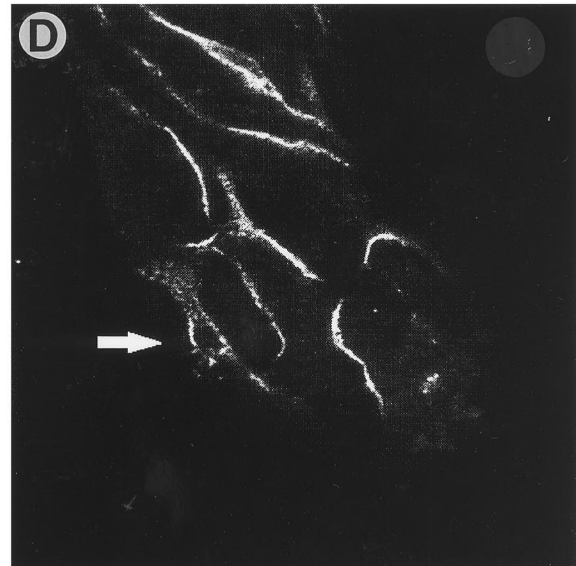
The localization of MRP1 and Pgps was compared in HepG2 cells. HepG2 cells form apical vacuoles lined by microvilli according to previous studies.[20,21] This study shows that Pgps are localized in these apical vacuoles as well as in intracellular vesicles. This indicates that Pgps, which in normal hepatocytes are located apically,[23] are also sorted apically in HepG2 cells. In contrast, MRP1 is localized mainly in the lateral membrane, i.e., the domain where the two membranes of adjacent cells meet. Although 37% of the Pgp-positive apical vacuoles also label for MRP1, labeling never uniformly surrounded the entire circumference of the vacuole in the plane of focus. When several focal planes of a MRP1-stained apical vacuole were merged with a single focal plane of the Pgp label in a composite three-dimensional image, MRP1 appears to be confined to a belt-like region around the Pgp-positive vacuole. This distribution pattern is

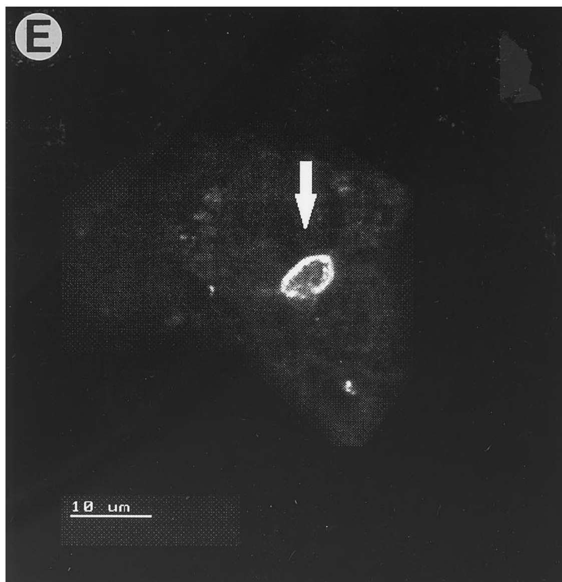
Figure 4. Localization of MRP1 and Pgps in HepG2 cells and immortalized human hepatocytes. To determine localization of Pgps in HepG2, cells were labeled for (*A*) actin (FITC-phalloidin) and (*B*) Pgps (C219 monoclonal antibody). Pgps (*arrows*) are predominantly present in actin-positive apical vacuoles and in intracellular punctate structures. Double labeling of HepG2 cells for (*C*) MDR1-Pgp (JSB-1 monoclonal antibody) and (*D*) MRP1 (MRPk5 polyclonal antibody) revealed that MRP1 is present mainly on the membrane between adjacent cells. Thirty-seven percent of Pgp-positive apical vacuoles also labeled for MRP1 (*arrow*). Double labeling of immortalized hepatocytes for (*E*) MDR1-Pgp (JSB-1 monoclonal antibody) and (*F*) MRP1 (MRPk5 polyclonal antibody) also showed predominant staining for MRP1 of membranes between adjacent cells, whereas Pgp was mainly present in apical vacuoles (*arrow*).

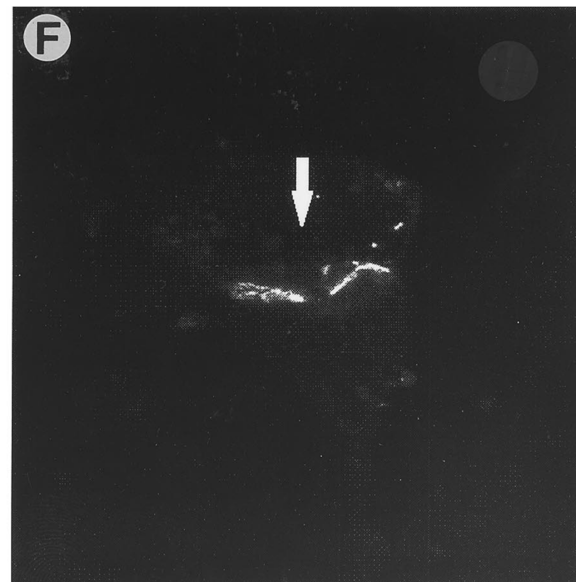

A
10 um


B


C
10 um


D


E
10 um


F

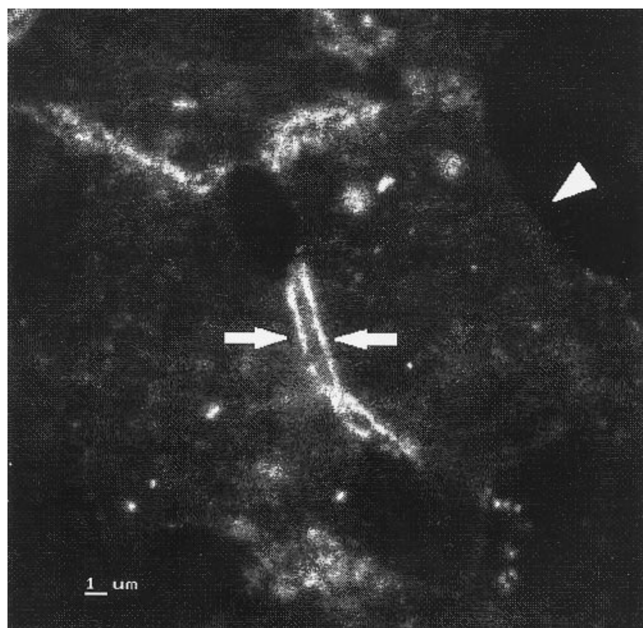


Figure 5. MRP1 is localized on both lateral membranes of adjacent cells. HepG2 cells were labeled for MRP1 with the polyclonal antibody MRPk5. A high magnification image was taken using confocal microscopy. Both membranes of adjacent cells (*arrows*) that border the lateral intercellular space contain MRP1. In contrast, no MRP1 is detectable on membranes directed to the medium (*arrowhead*).

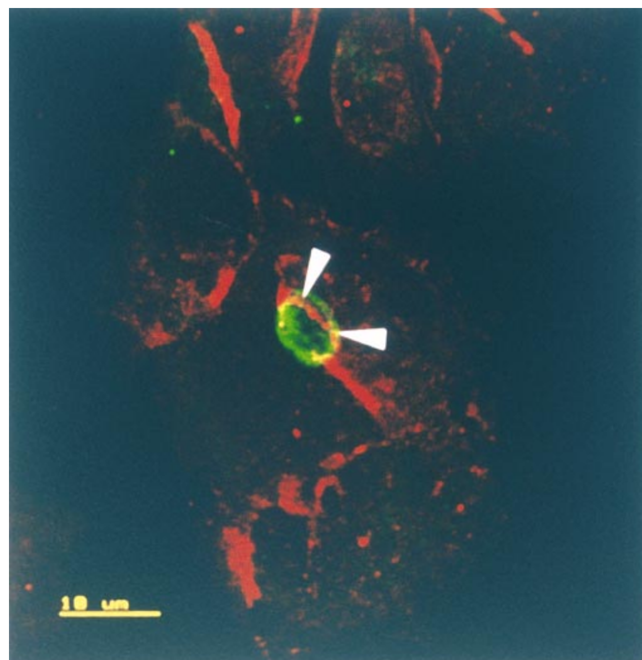


Figure 6. MRP1 localized on apical vacuoles is confined to a belt-like region. To study the occasionally observed MRP1 labeling of apical vacuoles in more detail, HepG2 cells were double labeled for Pgps (C219 monoclonal antibody) and MRP1 (MRPk5 polyclonal antibody). The image of a Pgp-stained apical vacuole (*green*) was merged with a compilation of six z-series images of MRP1 labeling (*red*). Arrows indicate that MRP1 is confined to a belt-like region running around the Pgp-stained vacuole. This belt-like distribution of MRP1 on apical vacuoles probably represents a ring of contact sites with the lateral membrane.

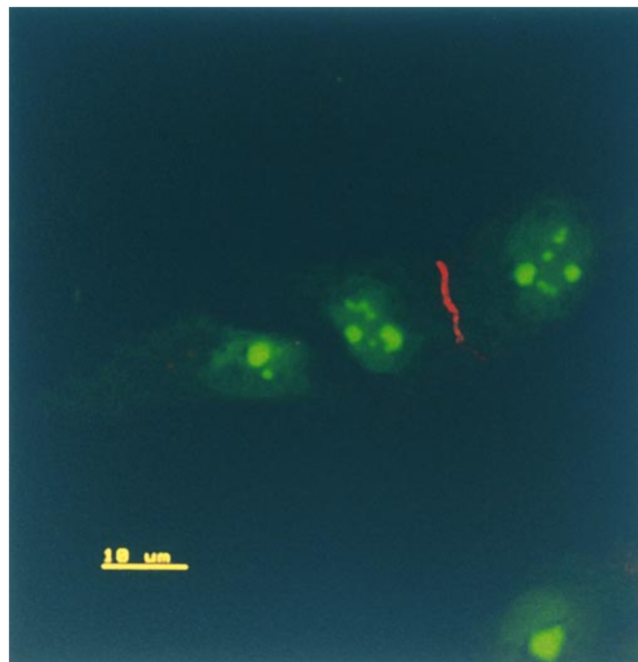


Figure 7. MRP1 is not detectable on the plasma membrane of single cells. HepG2 cells were labeled with acridine orange (*green*) to stain nuclei, and MRPk5 to stain MRP1 (*red*). No MRP1 is detectable in the plasma membrane of single cells (*left cell*), whereas a strong labeling is observed between two adjacent cells (*right cluster*). This suggests that cell–cell contact sites are a prerequisite for MRP1 localization in the plasma membrane.

similar to the distribution of the tight-junctional protein ZO-1 on apical vacuoles, as described for WIF-B hepatoma cells,[29] and may thus represent the sites where the lateral membrane makes contact with the apical vacuole. These results indicate that MRP1 is exclusively localized on the lateral membrane. No labeling of the basal membrane (the membrane directed to the medium) is detectable. Furthermore, no MRP1 is detected on the plasma membrane of separate HepG2 cells. This suggests that the presence of a lateral domain is necessary for localization of MRP1 on the plasma membrane of these cells. Like HepG2 cells, the immortalized hepatocytes also form apical vacuoles, which are positive for Pgps, with MRP1 specifically located at the lateral membrane. MRP also seems to be localized laterally in normal liver as reported recently for frozen sections of both rat and human livers.[10] However, with the antibodies used in this study, no mrp/MRP staining of hepatocytes in frozen rat and human liver sections is detected. This is probably caused by the low amount of MRP1 in normal liver.

The fluorescent glutathione S–conjugate GS-MF was used to study the function of MRP1. GS-MF is formed intracellularly from the nonfluorescent CMFDA by the action of esterase and glutathione S–transferase. It was previously shown that GS-MF is not excreted into the

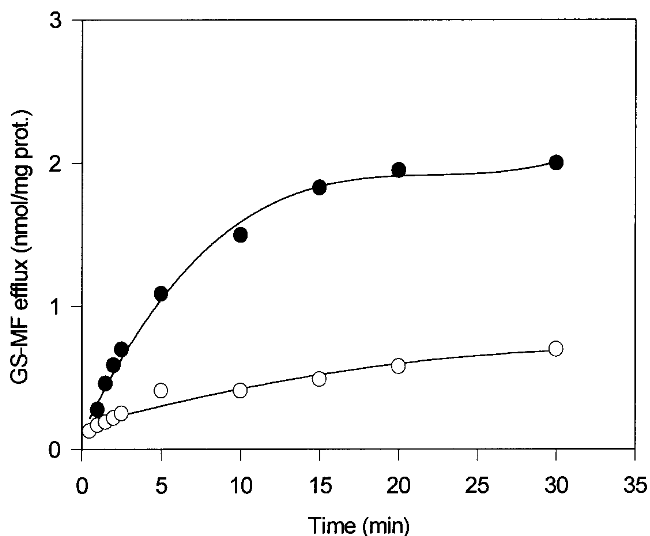


Figure 8. Excretion of the glutathione S–conjugate GS-MF by HepG2 cells. To determine whether MRP1 functions as an organic anion transporter in HepG2, cells were loaded at 10°C with CMFDA. Subsequently, efflux of the intracellularly formed MRP-substrate GS-MF was measured at 10°C and 37°C. ●, GS-MF efflux at 37°C; ○, efflux at 10°C. The temperature dependency of the GS-MF excretion indicates carrier-mediated transport.

bile of the TR^- rat, indicating that GS-MF is a substrate for cMOAT/mrp2 (Roelofsen et al., unpublished data). Substrates tested for MRP are mainly glutathione S–conjugates[4–6,10] and the substrate specificity is similar to that of cMOAT.[11] HepG2 cells, loaded with CMFDA, excrete GS-MF in a temperature-dependent fashion. This indicates the involvement of a carrier-mediated process. We were not able to directly show excretion of GS-MF into the lateral intercellular space. However, the high abundance of MRP1 on lateral membranes makes it likely that a large part of the GS-MF efflux into the medium is mediated by MRP1. Furthermore, evidence from other studies indicate that, in other cell types, there is organic anion transport in the lateral intercellular space. In large dome-shaped polarized renal MDCK and $LLC\text{-}PK_1$ cells, the organic anion 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF), a fluorescein derivative used as a pH indicator, is secreted into the lateral space.[30,31] Evers et al.[32] showed that MRP1 is located predominantly on lateral membranes in $LLC\text{-}PK_1$, making MRP1 a likely candidate for mediating BCECF excretion in the intercellular space of these cells. In addition to excretion of GS-MF into the medium, GS-MF was shown accumulating in small intracellular vesicles and large apical vacuoles in living HepG2 cells. This indicates that MRP-mediated transport activity is present in these vesicles. As discussed previously, we did not find specific staining of apical vacuoles for MRP1. However, an antibody against mrp2 clearly stains apical vacuoles in HepG2, suggesting that MRP2 is present in these vacuoles and probably is responsible for the observed

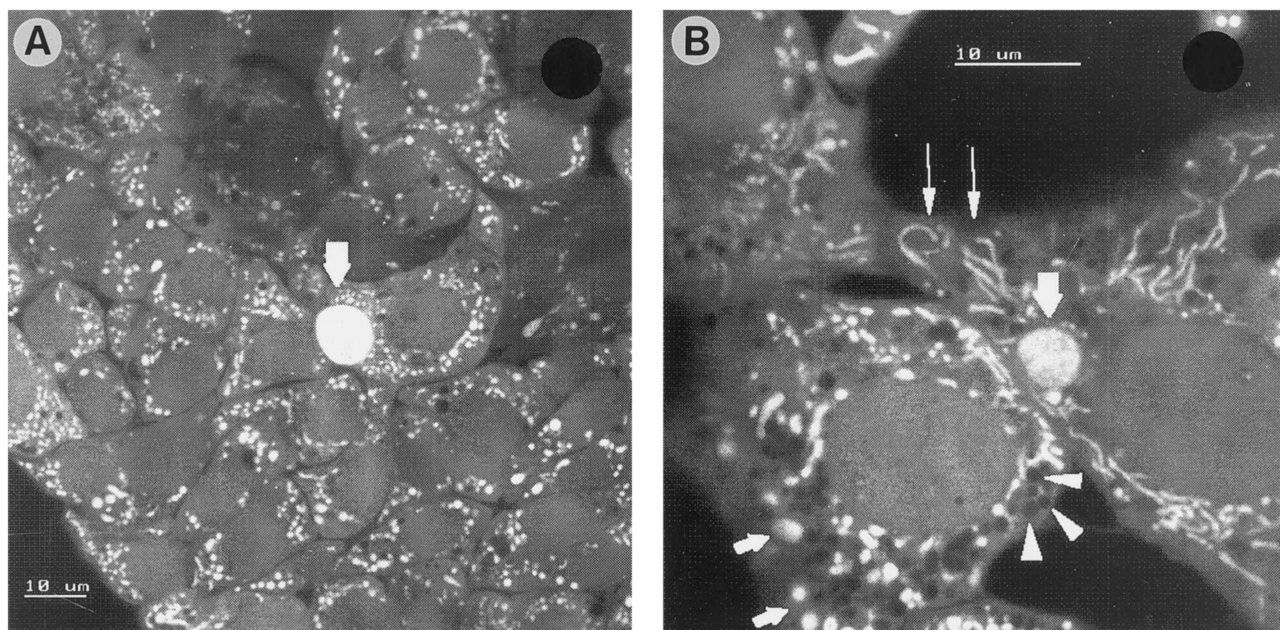


Figure 9. Organic anion transport into intracellular vesicles and apical vacuoles. HepG2 cells, grown on coverslips, were loaded with 2.5 μmol/L CMFDA for 15 minutes at 37°C. Cover slips were transferred to fresh medium and stored on ice. Intracellular accumulation of the MRP-substrate GS-MF was observed by confocal microscopy. (*A*) GS-MF accumulates in small intracellular vesicles and large (apical) vacuoles, indicating MRP transport activity is present there. (*B*) At higher magnification, differently shaped GS-MF accumulating vesicles can be observed: large vacuoles (*thick arrow*), smaller vesicles (*medium arrow*), and tubular structures (*thin arrow*). There is also a population of vesicles that does not take up GS-MF (*arrowheads*).

accumulation of GS-MF. Thus, both MRP1 and MRP2 are present in HepG2 cells. Also in other polarized cell types, excretion of organic anions over both the apical and basolateral membrane has been observed. Collington et al.[33] reports that BCECF is excreted in an ATP-dependent way in MDCK and Caco-2 cells with a threefold higher excretion in the apical than in the basolateral medium. Oude Elferink et al.[34] reported an ATP-dependent secretion of glutathione-dinitrophenyl in polarized Caco-2 cells. In this Caco-2 subline, the secretion rate over the basolateral membrane was approximately twofold higher than that over the apical membrane. Although no data on *MRP* expression are available, it may well be that also in these cell lines, MRP1 and MRP2 are responsible for the respective (baso)lateral and apical excretion of organic anions.

The increased level of MRP1 and the localization on lateral membranes in proliferating hepatocytes suggest that MRP1 may play a role during cell proliferation. In previous studies, there is evidence for a correlation between *MRP1* expression and the expression of the *myc* proto-oncogene, whose gene product functions as a transcription factor.[35] Bordow et al.[36] reported a high correlation between *MRP* and N-*myc* gene expression in neuroblastoma tumors and cell lines. Treatment of the cells with retinoic acid, an inducer of cell differentiation, decreases both N-*myc* and *MRP* gene expression. In contrast, *MDR1* gene expression increased by retinoic acid treatment. Similar results were obtained by Ishikawa et al.[37] They report that glutathione-conjugate pump activity is reduced by 50% in HL-60 leukemia cells during treatment with retinoic acid, dimethylsulfoxide, or the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate, which are all differentiation-promoting agents. This reduction of transport activity is accompanied by a reduction of *c-myc* levels. Also, HepG2 cells have a relatively high constitutive expression of *c-myc*.[38] These results together with our data provide evidence for the hypothesis that the abundance of MRP1 is linked to the proliferation or differentiation state of epithelial cells. The question remains: what is the role of MRP1 in proliferating epithelial cells? Although speculative, the function of MRP1 as a glutathione-conjugate transporter may be important in the protection of proliferating hepatocytes against oxidative stress. In this respect, MRP1 may take over the function of MRP2 when hepatocytes lose their polarized phenotype during cell proliferation. For example, this may occur during liver regeneration. Additionally, the localization of MRP1 on the lateral membrane and its function as an organic anion pump may be important for the formation of a lateral space between the cells by providing an osmotic driving force so that water can enter and expand this intercellular space. At present, no data are available for a role of organic anion transport in the process of cell division.

Recapitulating, we found that MRP1 level is low in normal hepatocytes and highly increased in proliferating hepatocyte-derived cell lines. MRP1 is localized specifically in the lateral membrane of these cells where it is probably responsible for the active transport of glutathione S–conjugates into the medium. Furthermore, a well-defined lateral domain seems necessary for the localization of MRP1 on the plasma membrane. These results indicate that the level of MRP1 is related to the proliferation state of the cell and suggest that MRP1 is of minor importance in quiescent hepatocytes. The role of MRP1 in proliferating hepatocytes remains to be studied.

References

1. Higgins CF. ABC transporters: from microorganisms to man. Annu Rev Cell Biol 1992;8:67–113.
2. Cole SPC, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AMV, Deeley RG. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science 1992;258:1650–1654.
3. Almquist KC, Loe DW, Hipfner DR, Mackie JE. Characterization of the Mr 190,000 multidrug resistance protein (MRP) in drug-selected and transfected human tumor cells. Cancer Res 1995; 55:102–110.
4. Jedlitschky G, Leier I, Buchholz U, Center M, Keppler D. ATP-dependent transport of glutathione S–conjugates by the multidrug resistance–associated protein. Cancer Res 1994;54: 4833–4836.
5. Leier I, Jedlitschky G, Buchholz U, Cole SP, Deeley RG, Keppler D. The *MRP* gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates. J Biol Chem 1994;269:27807–27810.
6. Müller M, Meijer C, Zaman GJR, Borst P, Scheper RJ, Mulder NH, de Vries EGE, Jansen PLM. Overexpression of the gene encoding the multidrug resistance–associated protein results in increased ATP-dependent glutathione S–conjugate transport. Proc Natl Acad Sci USA 1994;91:13033–13037.
7. Hipfner DR, Gauldie SD, Deeley RG, Cole SP. Detection of the M(r) 190,000 multidrug resistance protein, MRP, with monoclonal antibodies. Cancer Res 1994;54:5788–5792.
8. Flens MJ, Izquierdo MA, Scheffer GL, Fritz JM, Meijer CJ, Scheper RJ, Zaman GJ. Immunochemical detection of the multidrug resistance-associated protein MRP in human multidrug-resistant tumor cells by monoclonal antibodies. Cancer Res 1994;54: 4557–4563.
9. Zaman GJ, Flens MJ, van Leusden MR, de Haas M, Mulder HS, Lankelma J, Pinedo HM, Scheper RJ, Baas F, Broxterman HJ, Borst P. The human multidrug resistance–associated protein MRP is a plasma membrane drug-efflux pump. Proc Natl Acad Sci USA 1994;91:8822–8826.
10. Mayer R, Kartenbeck J, Büchler M, Jedlitschky G, Leier I, Keppler D. Expression of the *MRP* gene–encoded conjugate export pump in liver and its selective absence from the canalicular membrane in transport-deficient mutant hepatocytes. J Cell Biol 1995;131: 137–150.
11. Oude Elferink RPJ, Jansen PLM. The role of the canalicular multispecific organic anion transporter in the disposal of endo- and xenobiotics. Pharmacol Ther 1994;64:77–97.
12. Jansen PLM, Peters WH, Lamers WH. Hereditary chronic conju-

gated hyperbilirubinemia in mutant rats caused by defective hepatic anion transport. Hepatology 1985;5:573–579.
13. Jansen PLM, Groothuis GM, Peters WH, Meijer DF. Selective hepatobiliary transport defect for organic anions and neutral steroids in mutant rats with hereditary-conjugated hyperbilirubinemia. Hepatology 1987;7:71–76.
14. Oude Elferink RPJ, Ottenhoff R, Liefting W, de Haan J, Jansen PLM. Hepatobiliary transport of glutathione and glutathione conjugate in rats with hereditary hyperbilirubinemia. J Clin Invest 1989;84:476–483.
15. Huber M, Guhlmann A, Jansen PLM, Keppler D. Hereditary defect of hepatobiliary cysteinyl leukotriene elimination in mutant rats with defective hepatic anion excretion. Hepatology 1987;7:224–228.
16. Kitamura T, Jansen P, Hardenbrook C, Kamimoto Y, Gatmaitan Z, Arias IM. Defective ATP-dependent bile canalicular transport of organic anions in mutant (TR^-) rats with conjugated hyperbilirubinemia. Proc Natl Acad Sci USA 1990;87:3557–3561.
17. Paulusma CC, Bosma PJ, Zaman GJR, Bakker CTM, Otter M, Scheffer GL, Scheper RJ, Borst P, Oude Elferink RPJ. Congenital jaundice in rats with a mutation in a multidrug resistance–associated protein gene. Science 1996;271:1126–1128.
18. Knowles BB, Howe CC, Aden DP. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science 1980;209:497–499.
19. Javitt NB. HepG2 cells as a source for metabolic studies: lipoprotein, cholesterol, and bile acids. FASEB J 1990;4:161–168.
20. Chiu JH, Hu CP, Lui WY, Lo SC, Chang CM. The formation of bile canaliculi in human hepatoma cell lines. Hepatology 1990;11:834–842.
21. Sormunen R, Eskelinen S, Lehto VP. Bile canaliculus formation in cultured HepG2 cells. Lab Invest 1993;68:652–662.
22. Zaal KJ, Kok JW, Sormunen R, Eskelinen S, Hoekstra D. Intracellular sites involved in the biogenesis of bile canaliculi in hepatic cells. Eur J Cell Biol 1994;63:10–19.
23. Buschman E, Arceci RJ, Croop JM, Che M, Arias IM, Housman DE, Gros P. mdr2 encodes P-glycoprotein expressed in the bile canalicular membrane as determined by isoform-specific antibodies. J Biol Chem 1992;267:18093–18099.
24. Krishnamachary N, Center MS. The MRP gene associated with a non–P-glycoprotein multidrug resistance encodes a 190-kDa membrane bound glycoprotein. Cancer Res 1993;53:3658–3661.
25. Lin Y, Havinga R, Verkade HJ, Moshage H, Slooff MJH, Vonk RJ, Kuipers F. Bile acids suppress the secretion of very-low-density lipoprotein by human hepatocytes in primary culture. Hepatology 1996;23:218–228.
26. Dinsart C, Cornelis JJ, Klein B, van der Eb AJ, Rommelaere J. Transfection with extracellular UV-damaged DNA induces human and rat cells to express a mutator phenotype towards parvovirus H-1. Mol Cell Biol 1984;4:324–328.
27. Willems M, Roskams T, Moshage H, Roels L, Desmet VJ, Yap SH. Immortalized human liver cell lines with phenotypical characteristics of hepatoblast and/or biliary epithelial cells: an in vitro model for the study of liver cell–specific functions. Transplantology 1996;5:151–159.
28. Meier PJ, Sztul ES, Reuben A, Boyer JL. Structural and functional polarity of canalicular and basolateral plasma membrane vesicles isolated in high yield from rat liver. J Cell Biol 1984;98:991–1000.
29. Ihrke G, Neufeld EB, Meads T, Shanks MR, Cassio D, Laurent M, Schroer TA, Pagano RE, Hubbard AL. WIF-B cells: an in vitro model for studies of hepatocyte polarity. J Cell Biol 1993;123:1761–1775.
30. Harris PJ, Chatton JY, Tran PH, Bungay PM, Spring KR. pH, morphology, and diffusion in lateral intercellular spaces of epithelial cell monolayers. Am J Physiol 1994;266:C73–C80.
31. Chatton JY, Spring KR. Acidic pH of the lateral intercellular spaces of MDCK cells cultured on permeable supports. J Membr Biol 1994;140:89–99.
32. Evers R, Zaman GJR, van Deemter L, Jansen H, Calafat J, Oomen CJM, Oude Elferink RPJ, Borst P, Schinkel AH. Basolateral localization and export activity of the human multidrug resistance–associated protein in polarized pig kidney cells. J Clin Invest 1996;97:1211–1218.
33. Collington GK, Hunter J, Allen CN, Simmons NL, Hirst BH. Polarized efflux of 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein from cultured epithelial cell monolayers. Biochem Pharmacol 1992;44:417–424.
34. Oude Elferink RPJ, Bakker CT, Jansen PLM. Glutathione-conjugate transport by human colon adenocarcinoma cells (Caco-2 cells). Biochem J 1993;290:759–764.
35. Marcu KB, Bossone SA, Patel AJ. *myc* function and regulation. Annu Rev Biochem 1992;61:809–860.
36. Bordow SB, Haber M, Madafiglio J, Cheung B, Marshall GM, Norris MD. Expression of the multidrug resistance-associated protein (MRP) gene correlates with amplification and overexpression of the N-myc oncogene in childhood neuroblastoma. Cancer Res 1994;54:5036–5040.
37. Ishikawa T, Wright CD, Ishizuka H. GS-x pump is functionally overexpressed in cis-diamminedichloroplatinum(II)-resistant human leukemia HL-60 cells and down-regulated by cell differentiation. J Biol Chem 1994;269:29085–29093.
38. Huber BE, Thorgeirsson SS. Analysis of c-myc expression in a human hepatoma cell line. Cancer Res 1987;47:3414–3420.

Received February 20, 1996. Accepted September 19, 1996.

Address requests for reprints to: Han Roelofsen, Ph.D., Division of Gastroenterology and Hepatology, University Hospital Department of Internal Medicine, P.O. Box 30001, 9700 RB Groningen, The Netherlands. Fax: 3150-3619306.

Supported by grant PL 931436 from the Biomed and Health Research Program and grant 902-23-208 from the Netherlands Foundation for Scientific Research.

The authors thank the Groningen Human Liver Cell Group for the isolation of human hepatocytes, Dr. Binie Klein for providing the SV40 large T antigen–containing plasmid, Dr. Elisabeth G. E. de Vries for providing GLC4/ADR cells, Dr. Guido J. R. Zaman for providing SW1573/S1 and S1/MRP cells, Dr. Rik Scheper for providing monoclonal antibodies against MRP1, and Dr. Ronald Oude Elferink for providing the antibody against mrp2.