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Published in:
Journal of Cell Science

Publication date:
1998

Journal of Cell Science 111, 1137-1145 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
JCS1566

Cyclic AMP stimulates sorting of the canalicular organic anion transporter (Mrp2/cMoat) to the apical domain in hepatocyte couplets

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Accepted 28 January; published on WWW 25 March 1998

SUMMARY

The canalicular membrane of rat hepatocytes contains an ATP-dependent multispecific organic anion transporter, also named multidrug resistance protein 2, that is responsible for the biliary secretion of several amphiphilic organic anions. This transport function is markedly diminished in mutant rats that lack the transport protein. To assess the role of vesicle traffic in the regulation of canalicular organic anion transport, we have examined the redistribution of the transporter to the canalicular membrane and the effect of cAMP on this process in isolated hepatocyte couplets, which retain secretory polarity. The partial disruption of cell-cell contact, due to the isolation procedure, leaves the couplet with both remnant apical membranes, as a source of apical proteins, and an intact apical domain and lumen, to which these proteins are targeted. The changes in distribution of the transporter were correlated to the apical excretion of a fluorescent substrate, glutathione-methylfluorescein. The data obtained in this study show that the transport protein, endocytosed from apical membrane remnants, first is redistributed along the basolateral plasma membrane. Then it is transcytosed to the remaining apical pole in a microtubule-dependent fashion, followed by the fusion of transporter-containing vesicles with the apical membrane. The cAMP analog dibutyrylcAMP stimulates all three steps, resulting in increased apically located transport protein, glutathione-methylfluorescein transport activity and apical membrane circumference. These findings indicate that the organic anion transport capacity of the apical membrane in hepatocyte couplets is regulated by cAMP-stimulated sorting of the multidrug resistance protein 2 to the apical membrane. The relevance of this phenomenon for the intact liver is discussed.

Key words: Intracellular sorting, cAMP, Multidrug resistance protein, Canalicular multispecific organic anion transporter, Glutathione S-conjugate, Hepatocyte

INTRODUCTION

The canalicular membrane of rat hepatocytes contains a transport system, named canalicular multispecific organic anion transporter (cMOAT) which is responsible for the secretion into bile of a wide variety of amphiphilic organic anions, including bilirubin glucuronides, glutathione S-conjugates and oxidized glutathione (Oude Elferink et al., 1989, 1991; Jansen et al., 1985, 1987; Ishikawa et al., 1990). This transporter has recently been cloned and is an isoform of the multidrug resistance protein, MRP (Büchler et al., 1996; Paulusma et al., 1996). It is now called mrp2 or cmoat (for reviews see Keppler and Kartenbeck, 1996; Müller and Jansen, 1997; Müller et al., 1996)). Transport via mrp2 is ATP-dependent (Oude Elferink et al., 1990; Kitamura et al., 1990; Ishikawa et al., 1990). The substrate specificity for this transporter is indicated by the diminished secretion of a variety of amphiphilic organic anions into bile of the GY/TR$^-$ (Groningen yellow/transport deficient rat) rat (Kitamura et al., 1990; Oude Elferink et al., 1989, 1991; Jansen et al., 1985, 1987; Ishikawa et al., 1990). This mutant rat is characterized by a hereditary conjugated hyperbilirubinemia due to the lack of transport activity caused by the absence of the mrp2 protein (Büchler et al., 1996; Paulusma et al., 1996).

However, in cultured hepatocytes mrp2-mediated organic anion transport activity is identified in intracellular vesicular structures as evidenced by the uptake of the fluorescent organic anion glutathione-bimane (GS-B), a mrp2 substrate (Oude Elferink et al., 1993). These fluorescent vesicles are not observed in GY/TR$^-$ hepatocytes or in freshly isolated hepatocytes from normal liver but they are formed concomitantly with a disappearance of conjugate transport activity from the plasma membrane (Roelofsen et al., 1995). These findings suggest that the intracellular mrp2 activity is derived from endocytosed remnant apical membrane after disruption of cell-cell contacts induced by the hepatocyte isolation procedure.

These previous studies used cultures of single hepatocytes. In the present study we have utilized isolated rat hepatocyte couplets where cell polarity is retained in short-term culture because cell-cell contact remains between the two adjacent hepatocytes and thus are only partially disrupted upon isolation (Gautam et al., 1987, 1989; Boyer et al., 1988, 1990; Boyer, 1993). Hepatocyte couplets consist of two cells connected to each other by tight-junctions which demarcate the limits of a canalicular space and define the remaining apical domain (Gautam et al., 1987; Boyer et al., 1988, 1990; Boyer, 1993). Following isolation, remnant apical membrane containing canalicular proteins, such as mrp2, are endocytosed and translocate to the remaining apical domain. Previously, we have found that dibutyryl cyclicAMP (DBcAMP) stimulates the targeting of vesicles destined for the apical membrane in hepatocyte couplets. The activity of both the canalicular membrane Cl^-/HCO_3^- exchanger (Benedetti et al., 1994) and bile acid transporter (Boyer and Soroka, 1995) are augmented by this maneuver which is also associated with translocation of an ecto-ATPase from the pericanalicular cytoplasm to the canalicular membrane. Furthermore, in the perfused rat liver, DBcAMP significantly stimulates bile flow and transcytosis of the fluid phase marker, horseradish peroxidase (HRP) (Hayakawa et al., 1990). These observations suggest that cAMP is able to regulate transport capacity at the canalicular membrane by stimulating exocytosis and/or fusion of vesicles containing canalicular transport protein activity. In the present study we examine the effect of cAMP on translocation of mrp2 to the apical domain in detail. This includes localization of this transport protein by immunofluorescence and determining the associated changes in apical transport activity by visualizing the transport of the fluorescent organic anion glutathione-methylfluorescein (GS-MF) across the canalicular membrane into the bile lumen.

The data obtained in cultured hepatocyte couplets in this study show that the redistribution of mrp2 to the apical domain takes place in three steps. First, mrp2 is endocytosed from apical membrane remnants forming clusters of vesicles, which break up and relocate along the basolateral plasma membrane. Secondly, mrp2 is transcytosed to the remaining apical pole in a microtubule-dependent fashion and thirdly, mrp2-containing vesicles fuse with the apical membrane. DBcAMP appears to stimulate all three steps resulting in a highly increased transport of the fluorescent mrp2 substrate GS-MF over the apical membrane.

MATERIALS AND METHODS

Materials

Collagenase (type I) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Leibovitz L-15 medium was obtained from Gibco (Grand Island, NY). 5-Chloromethylfluorescein diacetate (CMFDA) was from Molecular Probes (Eugene, OR). Cy3-conjugated anti-rabbit IgG was from Amersham (Buckinghamshire, UK). DibutyrylcAMP, isobutylmethylxanthine, nocodazole, esterase (EC 3.1.1.1., from rabbit liver) and glutathione S-transferase (EC 2.5.1.18, from equine liver) were from Sigma (St Louis, MO). All other chemicals were of the highest purity commercially available.

Animals

Male Sprague-Dawley rats were obtained from Camm Research Lab Animals, Wayne, NJ. Mutant GY/TR$^-$ rats were provided by Dr R. P. J. Oude Elferink of the Academic Medical Center of the University of Amsterdam, The Netherlands, and were bred in our own breeding facility. The GY/TR$^-$ strain has been characterized previously (Oude Elferink et al., 1989, 1990, 1991; Kitamura et al., 1990; Jansen et al., 1985, 1987; Ishikawa et al., 1990). Both mutant and normal rat strains were maintained on Purina rodent chow under a constant 12-hour light cycle. All experiments were carried out with fed male rats of 200-300 ***g***.

Isolation and culture of hepatocyte couplets

Hepatocyte couplets were isolated as described previously (Boyer et al., 1990). In brief, rat livers were perfused for 10 minutes with Hanks' A medium (in mmol/l: NaCl, 120; KCI, 5; KH_2PO_4, 0.4; Na_2HPO_4, 0.2; $NaHCO_3$, 25; EGTA, 0.5; D-glucose, 5.5; pH 7.4) then for 10 minutes with Hanks' B medium (in mmol/l: NaCl, 120; KCI, 5; KH_2PO_4, 0.2; $NaHCO_3$, 25; $MgSO_4$, 0.4; $MgCl_2$, 0.5; $CaCl_2$, 3; D-glucose, 5.5; pH 7.4) containing 0.05% collagenase and 0.8 units trypsin inhibitor per unit tryptic activity in the collagenase. The liver was removed and placed in Leibovitz L-15 medium, minced, and passed through serial nylon mesh filters, and the resultant cells were washed at 4ºC. Cells were kept on ice until use. Mean viability by Trypan blue exclusion was 84%. Hepatocytes were suspended at a concentration of 0.33×10^6 cells/ml in L-15 medium containing 50 i.u./ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum, plated on coverslips in polystyrene Petri dishes (0.1×10^6 cells/cm^2) and cultured for 3 hours at 37ºC.

Isolated rat liver perfusions

Isolated rat liver perfusions were performed as described previously from this laboratory (Corasanti et al., 1989). For perfusions with CMFDA, normal and mutant GY/TR$^-$ livers were perfused with 200 ml KRB supplemented with 1% BSA in a recirculating fashion. After 15 minutes, 2 μmol of CMFDA dissolved in 200 μl DMSO was added to the perfusate and the perfusion was continued for an additional 45 minutes. Bile samples were collected every 5 minutes for the first 15 minutes, every 2 minutes for the next 30 minutes and every 5 minutes for the last 15 minutes of the perfusion. Perfusate samples (200 μl) were collected every 5 minutes. Samples were diluted in phosphate-buffered saline (PBS, in mmol/l: NaCl, 140; Na_2HPO_4 9.2; NaH_2PO_4 1.3) and GS-MF fluorescence was measured with a Perkin-Elmer LS-5B luminescence spectrometer (Ex: 490 nm; Em: 520 nm).

To quantify the fluorescence, a 25 μM GS-MF standard was prepared as follows: 5 units of esterase (from rabbit liver), 5 units of glutathione S-transferase (from equine liver), 250 μM GSH and 25 μM CMFDA were dissolved in 1 ml 10 mM Hepes-HCl, pH 7.4, and incubated at 37ºC until no increase in fluorescence was observed. A calibration curve (1-25 nM GS-MF) was constructed in the linear range to which the fluorescence of the samples was correlated.

Immunocytochemistry

For studies on the intracellular distribution of mrp2, cells were cultured at 37°C on coverslips for 1, 1.5, 2, 2.5 and 3 hours in the presence or absence of 100 μM DBcAMP/500 μM isobutylmethylxanthine (IBMX). For studies on the effect of microtubule disruption on mrp2 distribution couplets were cultured for 3 hours. in the presence of 20 μM nocadazole. Nocodazole (20 μM) and/or DBcAMP (100 μM) and IBMX (500 μM) were added at the time of plating. The addition of DBcAMP/IBMX for the last 2 hours of culture gave similar results. The cells were fixed in acetone at –20ºC for 10 minutes and rehydrated in PBS (20 minutes). The polyclonal antibody (EAG15), directed against the carboxyl terminus of mrp2, was prepared as described (Büchler et al., 1996). The antibody was diluted 1:400 in 1% BSA in PBS and incubated with the cells for 2 hours at room temperature. After washing in PBS, the cells were incubated with Cy3-conjugated anti-rabbit IgG for 30 minutes in 1% BSA/PBS at room temperature. Immunolabeling was viewed on a Leitz DM IRB inverted microscope coupled to a Leica TCS 4D confocal microscope (both from Leica,

Heidelberg, Germany) equipped with an argon/krypton laser (excitation: 568 nm; emission >590). No fluorescence was detectable when cells were incubated with the secondary antibody only, using the confocal settings established for preparations where the first antibody was not omitted.

Semi-quantitative measurements of the increase in mrp2 protein on the canalicular membrane after DBcAMP treatment were obtained by determining the total intensity of the mrp2-related fluorescence on the canalicular membrane of the couplet. Control and DBcAMP treated couplets were cultured for 3 hours and stained for mrp2, as described above. Confocal images of couplets with expanded canaliculi were taken at the focal plane where the diameter of the vacuole appeared the largest, using identical settings (gain, laser power and zoom factor). The total fluorescence intensity on the canalicular membrane was determined by subtracting the total pixel intensity within the area defined by tracing the inner boundary of the vacuolar membrane, from the value obtained by tracing the outer boundary.

Incubations of hepatocytes with the fluorescent glutathione conjugate

For the visualization of glutathione-methylfluorescein (GS-MF) excretion into apical canalicular lumens, hepatocytes were cultured in the absence or presence of 100 μM DBcAMP/500 μM IBMX for 3 hours. For the last 15 minutes 2.5 μM of CMFDA was added. Then the cells were washed once with ice-cold Hepes-buffered medium (in mmol/l; NaCl, 130; KCl, 5; $MgSO_4$, 1; $CaCl_2$, 1.3; KH_2PO_4, 1.2; Hepes, 19.7; D-glucose, 5; pH 7.4) and placed on ice prior to microscopic examination.

For the quantification of the canalicular excretion of GS-MF a slightly different protocol was followed. Hepatocytes were cultured for a total time of 3 hours at 37ºC, either for 2 hours in L-15 medium alone or for 1 hour in L-15 and 2 hours in L-15 +100 μM DBcAMP/500 μM IBMX. In addition couplets were treated with 20 μM nocodazole for 2 hours alone or for 1 hour in 20 μM nocodazole and 1 hour in 20 μM nocodazole +100 μM DBcAMP/500 μM IBMX. Subsequently the coverslips were placed for 15 minutes in Hepes buffer containing 5 μM CMFDA and the above indicated compounds. They were then transferred to a CMFDA-free Hepes buffer supplemented with the indicated compounds and incubated for 45 minutes to let the cells secrete the excess fluorescent conjugate. The dishes were then placed on ice before microscopic examination. For both protocols, differences in canalicular fluorescence between control and DBcAMP-stimulated couplets were similar.

To visualize GS-MF accumulation, coverslips were transferred from the dishes to a chamber on the stage of a Zeiss Axiovert microscope (Oberkochen, Germany) and were observed using a Bio-Rad MRC-600 confocal imaging system (Cambridge, MA) and SOM software. GS-MF was excited at 488 nm and detected at >515 nm using a krypton/argon laser. Images were collected with the same confocal settings (gain, aperture, black level and neutral density filter) for all culture conditions in a particular set of experiments. With these settings no autofluorescence was detected and was unaffected by any of the treatment protocols. Couplets were selected for study if they had expanded canaliculi using normal light microscopy and were subsequently scanned with the laser and the data stored on disk for later analysis. Experiments in which the GS-MF fluorescence was quantified were performed blind.

Total canalicular fluorescence was determined by tracing the lumen and calculating the total fluorescence in that area. The canalicular circumference was calculated by tracing the lumen.

RESULTS

Redistribution of mrp2 to the remaining apical domain

We examined the redistribution of mrp2 from remnant apical membranes, which are generated due to the disruption of cell-cell contact during hepatocyte isolation, to the remaining apical pole in hepatocyte couplets. Couplets were cultured on coverslips for 1 to 3 hours. The cells were fixed and stained for mrp2 at 30 minute intervals. Fig. 1 shows images of optical sections taken by confocal scanning laser microscopy of the mid and top sections of representative couplets. After 1 hour in culture mrp2 is mostly confined to clusters of vesicles just beneath the plasma membrane, probably representing endocytosed remnants of canalicular domains which were disrupted upon isolation of the couplets. Furthermore, mrp2 is localized to the contact site between the cells, representing the apical domain. 30 minutes later (90 minutes) most mrp2 clusters have broken up and mrp2 is distributed along the whole plasma membrane and the apical domain. Also intracellular localized mrp2 can be observed. At 2 hours of culture, the clusters largely have broken up and numerous small mrp2-positive vesicular structures can be observed throughout the cytoplasm. Note that the staining at the apical membrane has mostly disappeared at this point. Over the next 30 minutes a significant portion of the vesicles has reached the apical membrane and a small canalicular vacuole is formed between the two hepatocytes. Frequently in other parts of the cell, small clusters of vesicles can be observed localized just underneath the plasma membrane, which resemble those in 1 hour cultured hepatocyte couplets. At 3 hours of culture most of the mrp2 fluorescence is confined to vesicular structures at the apical domain. Still vesicles can be observed peripheral to the apical membrane indicating that not all of the mrp2 has been incorporated into the apical membrane. The apical vacuole has become larger in size. Furthermore, in other parts of the cell small clusters of mrp2-containing vesicles persist.

Effect of DBcAMP on the redistribution of mrp2 to the apical domain

To study the effect of cAMP on the redistribution of mrp2 to the remaining apical domain, hepatocyte couplets were cultured for 1 to 3 hours in the presence of 100 μM DBcAMP and 500 μM IBMX. At 30 minute time intervals cells were fixed and stained for mrp2. Again optical sections through the middle and top part of couplets were taken by confocal microscopy. Representative couplets for the different time points are shown in Fig. 2. At 60 minutes mrp2 is localized throughout the cytosol with a preference for the region close to the plasma membrane and the remaining apical region. In contrast to couplets incubated without DBcAMP at this time point (Fig. 1, 60 minutes), no staining of apical remnants or clusters of mrp2-containing vesicles were observed. Surprisingly at 90 minutes of culture the cytoplasm is completely devoid of mrp2 staining. Almost all mrp2 is present on or close to the plasma membrane. The top section reveals a heterogeneous vesicular staining pattern suggesting that mrp2 is mostly present in vesicles. Note that the mrp2 localized at the contact site between the two cells has disappeared completely. At 2 hours the cytoplasm contains lots of small mrp2-positive vesicular structures and a large concentration of probably vesicular mrp2 at the cell-cell contact site, representing the apical pole. In other parts of the cell small clusters of vesicles can be found. In the next 30 minutes more mrp2-containing vesicles are recruited to the apical domain and a small apical vacuole is forming. At 3 hours of culture this

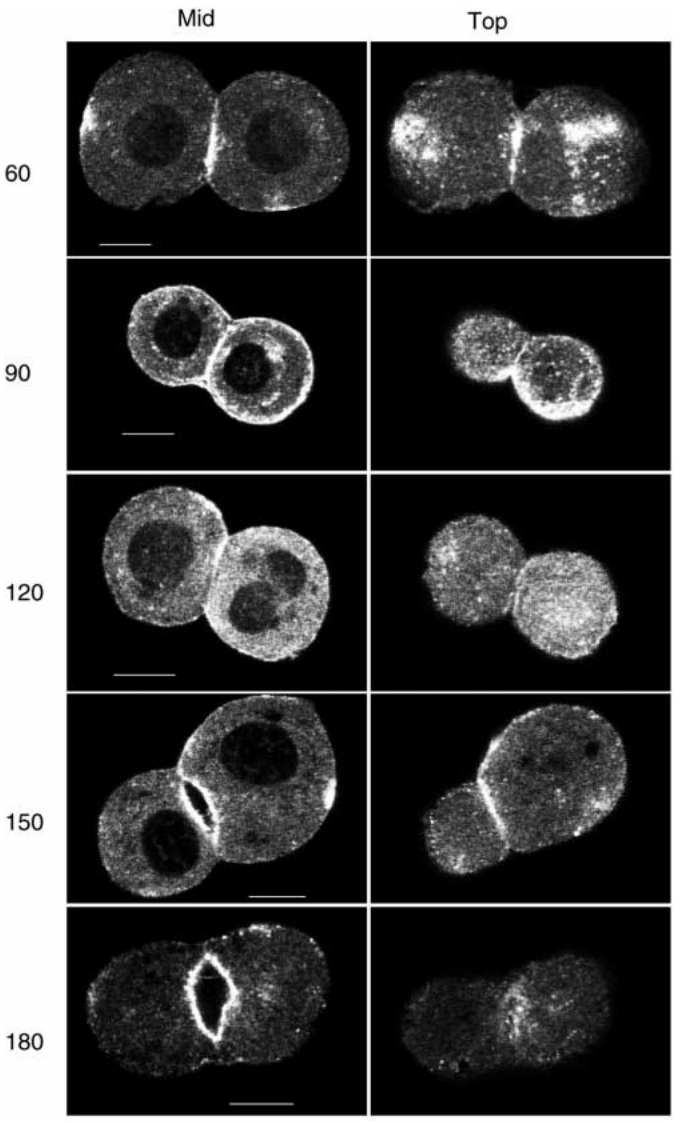

Fig. 1. Redistribution of mrp2 to the apical domain. Hepatocyte couplets were cultured for 60, 90, 120, 150 and 180 minutes in normal L-15 medium. The cells were fixed and labeled with a specific antibody against mrp2 (Büchler et al., 1996) followed by a secondary fluorescently labeled antibody. Images were taken from representative couplets by confocal laser microscopy. Optical sections taken from the upper part of the couplet, just beneath the plasma membrane (top), and the middle part (mid) are shown. Redistribution of mrp2 from distinct clusters of vesicles (60, 90 minutes) to the remaining apical vacuole forming between the two cells (150, 180 minutes) can be observed. Bars, 10 μm.

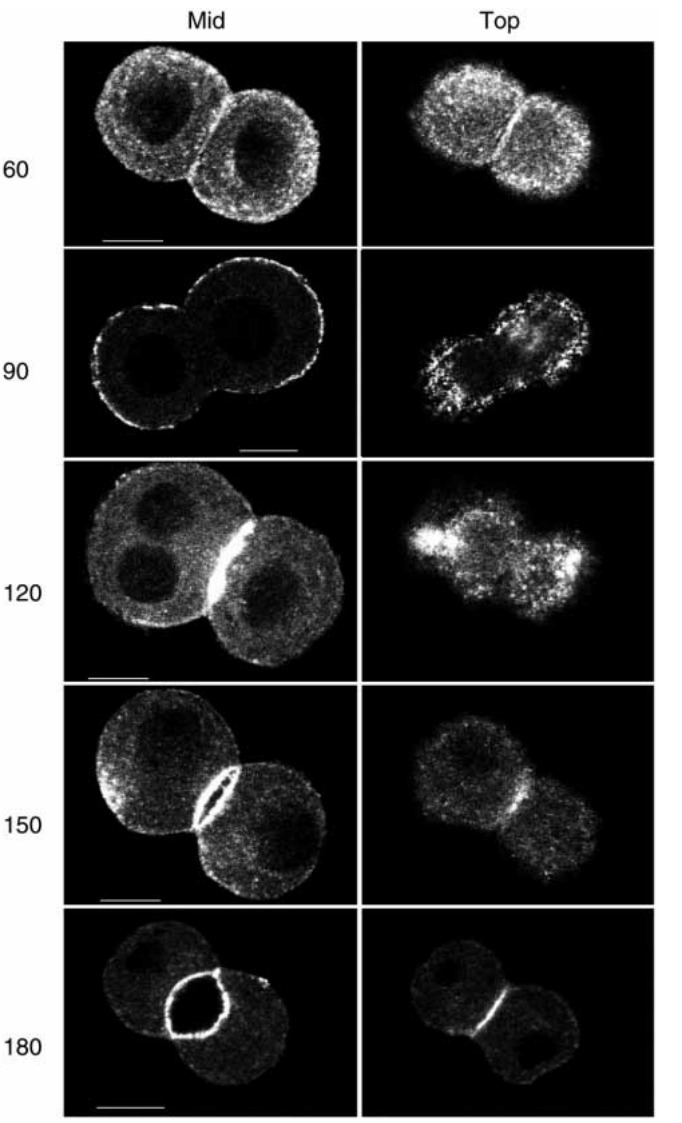

Fig. 2. Redistribution of mrp2 in the presence of DBcAMP/IBMX. Hepatocyte couplets were cultured for 60, 90, 120, 150 and 180 minutes in L-15 medium supplemented with 100 μM DBcAMP/500 μM IBMX. The cells were fixed and labeled with a specific antibody against mrp2 followed by a secondary fluorescently labeled antibody. Images were taken from representative couplets by confocal laser microscopy. Optical sections taken from the upper part of the couplet, just beneath the plasma membrane (top), and the middle part (mid) are shown. Redistribution of mrp2 from the basolateral domain (60, 90 minutes) to the remaining apical vacuole forming between the two cells (150, 180 minutes) can be observed. Bars, 10 μm.

vacuole becomes much larger and almost all mrp2 staining appears to be confined to the apical membrane.

To determine whether DBcAMP treatment results in an increased amount of mrp2 on the apical membrane, the total fluorescence intensity of the canalicular membrane was measured. Confocal images were taken of 25 couplets cultured for 3 hours with or without DBcAMP. The fluorescence was quantified by determining the pixel intensity of the apical membrane, as described in the Materials and Methods. The average pixel intensity determined in this way was 390,935±153,683 for the control group and 533,605±154,756 for the DBcAMP-treated group. This is a significant difference ($P<0.01$; two-sided paired Student's *t*-test). These data indicate that DBcAMP-treatment increases the amount of mrp2 on the apical membrane by 37% compared to untreated couplets.

Characterization of a fluorescent substrate for mrp2

To study whether the redistribution of mrp2 to the remaining apical domain is associated with changes in transport of amphiphilic organic anions into the canalicular vacuole, we used a fluorescent organic anion as a substrate for mrp2. Previous studies, which have examined the intracellular

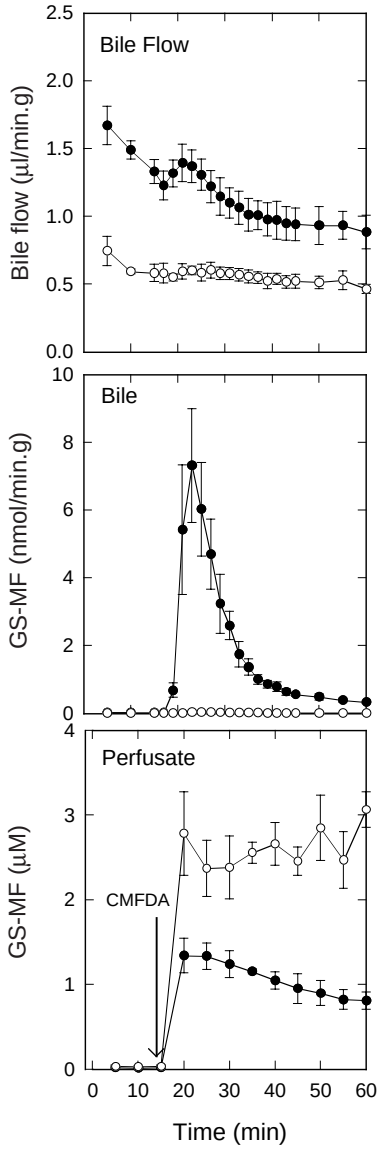

Fig. 3. Excretion of glutathione-methylfluorescein into bile. Livers from normal Spraque Dawley (closed circles) and mutant GY/TR$^-$ rats (open circles) were perfused in a recirculating fashion. After a 15 minutes preperfusion period, 2 µmole of 5-chloromethylfluorescein (CMFDA) (see arrow in the bottom panel) was added to the perfusate (200 ml). Bile was collected every 5 minutes for the first 15 minutes, every 2 minutes for the next 30 minutes, and every 5 minutes for the last 15 minutes. The perfusate was sampled every 5 minutes. Fluorescence was analyzed as described in Materials and Methods. The top panel shows bile flow; the middle panel shows excretion of GS-MF into bile; and the bottom panel shows the concentration of GS-MF in the perfusate. Data are expressed as means ± s.d. of 4 perfusions for the Sprague Dawley and 3 perfusions for the GY/TR$^-$ strain.

location of the transport mediated by mrp2, have used glutathione-bimane (GS-B) as a fluorescent substrate (Roelofsen et al., 1995; Oude Elferink et al., 1993). Since UV light is necessary to excite GS-B, we were unable to use this substrate with our confocal laser microscope, which uses visible laser excitation. However, the glutathione conjugate of 5-chloromethylfluorescein diacetate (CMFDA) is excited at higher wavelengths and is a suitable substrate for the mrp2 transporter. CMFDA is not fluorescent and is taken up into cells by diffusion across the plasma membrane where it is acted upon by intracellular esterases and glutathione S-transferases to form the fluorescent organic anion, glutathione-methylfluorescein (GS-MF) (Barhoumi et al., 1993; Poot et al., 1991). This compound is very poorly excreted into bile of the perfused GY/TR$^-$ liver which lacks mrp2, while it is rapidly excreted into bile of a normal rat liver as illustrated in Fig. 3. Excretion is maximal 6 minutes after the addition of the precursor, CMFDA, to the perfusate. Bile flow in the GY/TR$^-$ liver is reduced by ~50% compared to the normal liver as previously reported (Oude Elferink et al., 1990; Jansen et al., 1985) and probably reflects diminished biliary secretion of conjugates and reduced glutathione (GSH), which are determinants of bile salt-independent bile flow. In the normal liver, 40% of the administered dose of 2 µmole CMFDA is secreted into bile over a period of 45 minutes, while 13% accumulates in the perfusate. In contrast, only 0.3% was excreted in bile by the mutant GY/TR$^-$ liver while 31% was found in the perfusate. Excretion in the perfusate may be mediated by an Mrp isoform, which is expressed in the lateral membrane of the hepatocyte (Roelofsen et al., 1997; Keppler and Kartenbeck, 1996; Mayer et al., 1995). These results indicate that transport of GS-MF across the canalicular membrane and into bile of normal rat hepatocytes is mediated primarily by mrp2.

To assess the effects of pH on GS-MF fluorescence, the fluorescence intensity of 12.5 nM GS-MF solutions dissolved in phosphate buffer (pH 5.4 to 8.5) was determined. GS-MF fluorescence intensity decreased with decreasing pH, but was relatively stable in the physiologic range, pH 7 to 8 (data not shown).

Stimulation of canalicular excretion of GS-MF by DBcAMP

To assess the ability of DBcAMP to stimulate the excretion of GS-MF in hepatocyte couplets, cells were cultured in the presence or absence of DBcAMP/IBMX for 3 hours. CMFDA was added for the last 15 minutes. Images were taken with a confocal microscope with the same settings for both conditions. As shown in Fig. 4, the luminal accumulation of GS-MF fluorescence in cAMP-stimulated hepatocyte couplets is much higher than in unstimulated couplets. This indicates that much more mrp2 transport activity is present on the apical membrane of cAMP-stimulated couplets. These qualitative observations of GS-MF transport were confirmed by quantitative measurements shown in Table 1. Total GS-MF fluorescence intensity in the canalicular lumen was taken as a measure of canalicular excretion and was more than 7 times higher in couplets cultured in the presence of DBcAMP when compared to cells cultured in medium without DBcAMP. Furthermore, the circumference of the canalicular membrane also significantly increased by 36% in DBcAMP treated couplets as compared to controls. These results are similar to our previous published findings (Boyer and Soroka, 1995) regarding the canalicular transport of bile acids and suggest that the stimulation of luminal organic anion secretion is related to the insertion of mrp2-containing vesicles into the apical membrane. Luminal excretion and circumference were also quantified in mutant GY/TR$^-$ couplets cultured with or without DBcAMP/IBMX using the same

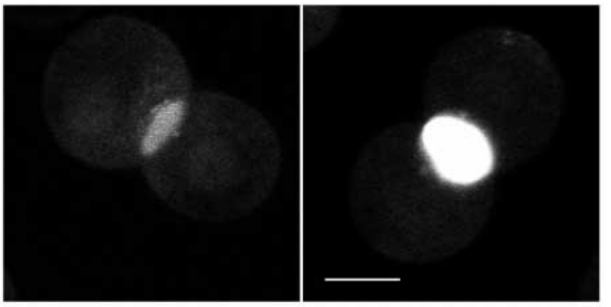

Fig. 4. Apical secretion of the fluorescent mrp2 substrate GS-MF is stimulated by DBcAMP. Hepatocyte couplets were cultured for 3 hours in the absence (left panel) or presence (right panel) of 100 μM DBcAMP/500 μM IBMX. For the last 15 minutes of culture 2.5 μM CMFDA was added. The accumulated fluorescence was visualized by confocal laser microscopy using identical settings for both conditions. A clear stimulation of canalicular secretion is observed in DBcAMP treated couplets. Bar, 10 μm.

Table 1. Measurements of GS-MF fluorescence intensity and circumference of the canaliculus of normal and GY/TR⁻ hepatocyte couplets cultured with or without DBcAMP/IBMX

Rat	Incubations	Lumen (total int.)	Circumference (μm)
Normal	Control	642±689	16.8±1.6
	DBcAMP/IBMX	4,615±1,174*	22.8±2.6*
GY/TR⁻	Control	25.6±15.9	21.0±2.2
	DBcAMP/IBMX	195±103*	23.2±1.8†

Hepatocyte couplets isolated from normal and mutant GY/TR⁻ rats were cultured with DBcAMP/IBMX or without (control), incubated with 5 μM CMFDA and examined by confocal fluorescence microscopy as described in the methods. Total luminal fluorescence intensity and the circumference of the canalicular vacuole were determined. Results are the mean ± s.d. of 5 and 7 separate isolations for normal and GY/TR⁻ rats, respectively, and 5 couplets per culture condition. Significance of differences were determined using a two-sided paired Student *t*-test.

*P<0.01 compared to control; †P<0.05 compared to control.

conditions and confocal settings as with normal cells. In isolated couplets from the mutant GY/TR⁻ rats, only small amounts of GS-MF could be detected within the canalicular lumen (Table 1) consistent with the absence of the transporter in these cells. Still a smaller but significant difference between the control and DBcAMP treated couplets was detected which may be due to diffusion across the canalicular membrane or a very low level of functional transport. As in control cells, DBcAMP treatment resulted in an increase in canalicular circumference in GY/TR⁻ cells. This finding is consistent with a stimulatory effect of cAMP on the sorting of lipid vesicles to the canalicular domain rather than on sorting of the transport protein.

Effect of nocodazole on redistribution of mrp2 to the apical membrane

To determine if the translocation of mrp2 depends on microtubules, the effect of nocodazole, an inhibitor of microtubule polymerization, on this process was studied. Depolymerization of microtubules with nocodazole was confirmed by immunocytochemistry as previously described

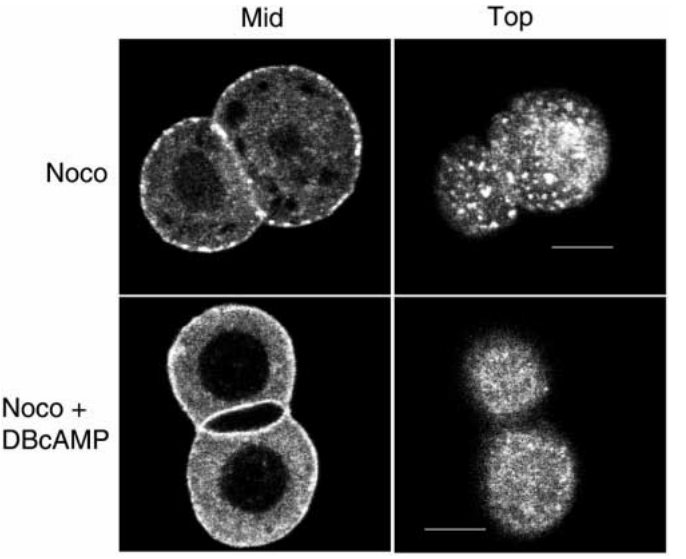

Fig. 5. Nocodazole treatment inhibits the redistribution of mrp2. Hepatocyte couplets were cultured for 3 hours in the presence of 20 μM nocodazole, an inhibitor of microtubule polymerization, in the absence (upper panels) or presence of 100 μM DBcAMP/500 μM IBMX (lower panels). Cells were fixed and stained for mrp2. Optical sections taken by confocal laser microscopy from the upper part of the couplet, just beneath the plasma membrane (top), and the middle part (mid) are shown. Translocation of mrp2 from the basolateral to the apical domain in nocodazole treated cells is inhibited (upper panels). Under these conditions the addition of DBcAMP could partially overcome the nocodazole block (lower panels). Bars, 10 μm.

(Boyer and Soroka, 1995) using an antibody to β-tubulin (data not shown). When nocodazole is present in the medium for the entire 3 hour culture period, translocation of mrp2 to the apical domain is significantly diminished as shown in Fig. 5. Under this condition, mrp2 is predominantly found in large structures present just beneath the plasma membrane. With a higher magnification large vacuoles with clear lumen containing mrp2 in their membrane can be observed (Fig. 6). Only a few couplets showed small expanded canalicular lumina. When the experiment was repeated with both nocodazole and DBcAMP/IBMX present in the medium a different picture was obtained as shown in Fig. 5. A significant portion of the mrp2 was retained on the canalicular membrane with many couplets showing expanded canalicular lumens. This was accompanied by a 98±27% increase in luminal GS-MF excretion, compared to control (P<0.01; paired Student's *t*-test). Furthermore, mrp2 was distributed throughout the cytoplasm on small punctuate structures and in somewhat larger structures at the basolateral membrane, reminiscent of cells treated with nocodazole alone. When compared to incubations with DBcAMP alone (Fig. 2, 180 minutes), a larger portion of the mrp2 is present in the cytoplasm or at the basolateral membrane in the nocodazole treated couplets. These results suggest that the redistribution of mrp2 to the apical domain depends on microtubules and that DBcAMP treatment can at least partially overcome the nocodazole block.

DISCUSSION

Previous studies on the sorting of ATP-dependent transporters in intact hepatocytes have assessed changes in transport

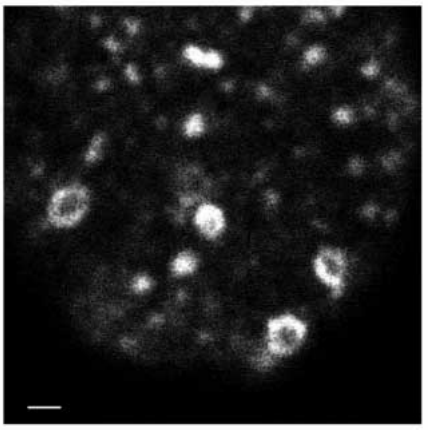

Fig. 6. Mrp2 is present in large vacuoles in nocodazole-treated cells. Hepatocyte couplets were cultured for 3 hours in the presence of 20 μM nocodazole. Cells were fixed and stained for mrp2. A high magnification image of the top section, taken just beneath the plasma membrane, of one half of a couplet is shown. Mrp2 is localized to large vacuoles, some with diameters of 1 μm, close to the basolateral membrane. Bar, 1 μm.

activity. These studies show that intracellular mrp2 transport activity in isolated hepatocytes is derived from endocytosed apical membrane, induced by the disruption of cell-cell contact due to the hepatocyte isolation procedure (Roelofsen et al., 1995; Oude Elferink et al., 1993). The hepatocyte couplet model used in the present study is unique in that the partial disruption of cell-cell contact leaves the couplet polarized with both remnant apical membranes, as a source of apical proteins, and an intact apical domain, to which these proteins are now targeted. The recent availability of antibodies against mrp2 (Büchler et al., 1996) makes it possible to study the sorting of this transport protein to the remaining apical domain and to correlate this to apical transport activity.

When hepatocyte couplets are cultured for up to 3 hours (Fig. 1) four steps can be distinguished as mrp2 redistributes to the apical domain: (1) initially (1 hour of culture), mrp2 is present at focal points in the plasma membrane, which presumably represent canalicular remnants, as well as distinct clusters of vesicular structures just beneath the plasma membrane and at the apical pole. These submembranous clusters may represent endocytosed mrp2 present in subapical compartments (SAC) as postulated by Ihrke and Hubbard (1995). (2) The clusters then break up and mrp2 is distributed along the whole plasma membrane in vesicular structures (90 minutes). (3) In turn these structures form smaller mrp2-containing vesicles which are then translocated through the cytosol to the remaining apical domain (120 minutes). (4) By 180 minutes, mrp2 is integrated into the apical membrane concomitantly with an enlargement of the apical vacuole, indicating recruitment of lipid as well as protein. Integration of functional mrp2 into the apical membrane is confirmed by the measurement of transport of the fluorescent substrate GS-MF into the lumen of the apical vacuole (Fig. 4; Table 1). GS-MF proved to be a good substrate for mrp2 since it is readily excreted into bile in normal rats while there is hardly any excretion into bile of the mutant GY/TR$^-$ rat which lacks mrp2. These findings suggest that at least some portion of the mrp2-containing vesicles fuse with the apical membrane under these conditions.

The redistribution of mrp2 in the presence of DBcAMP (Fig. 2) shows a slightly different picture. At 1 hour in culture, staining of apical remnant mrp2 is infrequent and is found on vesicular structures throughout the cytosol with a preference to the periphery of the plasma membrane. Mrp2 then appears to relocate to the basolateral plasma membrane, and even the apical domain, where the two cells make contact, is now devoid of mrp2 staining. This is in contrast to the situation in couplets incubated in the absence of DBcAMP where the redistribution of mrp2 along the basolateral membrane appears to be less pronounced (Fig. 1, 60 and 90 minutes). Thereafter, mrp2-containing punctuate structures translocate to the remaining apical domain where they then fuse, resulting in an expansion of the apical lumen, similar to control cells. Quantification of the mrp2-fluorescence on the canalicular membrane at 180 minutes of culture shows a 37% increase in mrp2 protein after DBcAMP treatment. At this point transport of the fluorescent organic anion GS-MF into the apical lumen was more than 7-fold higher in the presence of DBcAMP than in its absence. This increase in GS-MF secretion after DBcAMP treatment is not due to a direct modification (phosphorylation) of preexisting carriers in the canalicular membrane since short-time stimulation (10 minutes) of freshly isolated hepatocytes with DBcAMP did not show any change in the cmoat/mrp2 transport acitvity, as has been published previously (Roelofsen et al., 1991). Also the circumference of the apical vacuole was significantly increased (36%), indicating that redistribution of mrp2 has become more efficient in the presence of DBcAMP, although the over-time scale of the redistribution appears similar.

In a parallel set of experiments in which microtubules were disrupted by nocodazole treatment, the redistribution of mrp2 appears to be arrested at a point prior to the translocation to the apical domain. At 3 hours of culture mrp2 accumulates in the membrane of large vacuoles some with diameters of 1 μm, which remain confined to the area just beneath the peripheral plasma membrane (Figs 5 and 6). Only a few couplets had small expanded apical lumina. These data indicate that the translocation of mrp2 to the apical domain is dependent on microtubules. When the same experiment was performed in the presence of DBcAMP and nocodazole, many couplets had expanded lumina and apical membranes labeled for mrp2, while luminal GS-MF excretion had doubled. Furthermore, mrp2 was present on vesicular structures throughout the cytosol but remained predominantly in the area of the basolateral plasma membrane. These findings indicate that DBcAMP treatment can partially overcome the nocodazole block. However, the translocation process is considerably delayed by the disruption of microtubules since a considerable amount of the mrp2 remains localized to other parts of the cell membrane after 3 hours of culture.

On the basis of these sequential observations, three distinct steps can be defined: (1) endocytosis of apical membrane remnants forming clusters of vesicles which break up and relocate along the basolateral plasma membrane. (2) The microtubule dependent translocation of vesicular structures to the remaining apical pole. (3) Fusion of mrp2-containing vesicles with the apical membrane. DBcAMP appears to stimulate all three steps since: (1) relocation of mrp2 along the

basolateral membrane is enhanced; (2) translocation to the apical membrane is still taking place in the absence of intact microtubules; and (3) fusion of mrp2-containing vesicles with the apical membrane is enhanced, resulting in increased membrane circumference and transport activity of the mrp2 substrates.

The effects of cAMP appear not to be directed towards the mrp2 protein itself but rather influence the sorting of apically directed vesicles, which contain this transport protein. This conclusion is supported by the finding that DBcAMP also stimulates vesicle fusion and an increase in circumference of the apical vacuole in GY/TR$^-$ couplets, which lack mrp2. Furthermore, we have previously observed that DBcAMP stimulates the apical targeting of the (canalicular) Cl^-/HCO_3^- exchanger (Benedetti et al., 1994) and the canalicular bile acid transporter (Boyer and Soroka, 1995) in hepatocyte couplets, as well as the excretion of the fluid phase marker horseradish peroxidase (HRP) into bile of the perfused rat liver by microtubule dependent mechanisms (Hayakawa et al., 1990). These findings indicate that the stimulatory process is not specific for the mrp2 transporter but may be a more general phenomenon for apically targeted vesicles. Interestingly, Zegers and Hoekstra (1997) have also demonstrated that DBcAMP stimulates transport of fluorescent sphingolipids to the apical membrane in HepG2 hepatoma cells. Apically directed sphingolipid transport occurs via two routes, a direct pathway from the Golgi to the apical membrane and an indirect pathway involving transcytosis from the basolateral to the apical membrane. They show that both pathways are stimulated by protein kinase A (PKA) agonists. The transcytotic pathway for apically directed sphingolipids is probably a similar pathway followed by the mrp2-positive vesicles in hepatocyte couplets since the latter involves the transcytosis of apical remnant lipids and proteins from the newly formed basolateral membrane to the remaining apical canalicular domain. Furthermore, transcytosis is known to be microtubule dependent (Hayakawa et al., 1990) as is the translocation of mrp2-positive vesicles to the apical domain in couplets, shown in this study. These data suggest that PKA activated by cAMP primarily stimulates apically directed lipid transport and as a secondary effect directs the transport of apical proteins like mrp2 associated with these lipids. The cellular mechanisms by which cAMP stimulates this apically directed transport remains unclear but may involve heteromeric G proteins (Gsα) as suggested by previous studies in MDCK cells (Barroso and Sztul, 1994; Hansen and Casanova, 1994).

Since DBcAMP also stimulates biliary transport of HRP in the perfused rat liver (Hayakawa et al., 1990), the phenomenon is not restricted to the hepatocyte couplet system but is a significant function of the intact liver. The main route of delivery of newly synthesized canalicular proteins in hepatocytes appears to be indirect, involving transport from the Golgi compartment to the basolateral membrane followed by transcytosis to the apical membrane (Scott and Hubbard, 1992; Mostov et al., 1992; Maurice et al., 1994; Bartles et al., 1987). Therefore, based on the results of this study, activation of PKA may enhance the delivery of newly synthesized apical proteins to the canalicular domain by stimulating both transcytosis and apical exocytosis. Possibly, an apical sorting compartment, which has been postulated to explain the turnover of canalicular proteins (Ihrke and Hubbard, 1995), is involved in the sorting of apical proteins. Recent reports indicate the presence of subapical sorting compartments in MDCK cells (Barroso and Sztul, 1994; Apodaca et al., 1994). Cyclic AMP stimulates apical exocytosis from these compartments (Pimplikar and Simons, 1994; Hansen and Casanova, 1994). Therefore, cAMP may stimulate both the delivery of newly synthesized proteins (and lipids) to the apical domain (sorting compartment) and apical exocytosis from such a compartment as suggested by the initial loss of mrp2 from the apical domain at 90 minutes (Fig. 2). On the other hand, endocytosis of canalicular membrane, under normal conditions or as a result of cholestasis, may lead to an intracellular pool of transporters which may be reintegrated into the canalicular membrane upon activation of PKA. In this way, canalicular transport capacity may be regulated by endocytosis and/or exocytosis of vesicles from a subapical sorting compartment. The effect of DBcAMP on the intracellular distribution of mrp2 in the present study, as well as the ability of DBcAMP to stimulate fluorescent labeled bile acid excretion into the canalicular lumen (Boyer and Soroka, 1995) are both consistent with this hypothesis.

This work was supported by USPHS grants DK 25636 and DK 34989 to Dr J. L. Boyer, and grants from the Netherlands Organization for Scientific Research and the Netherlands Digestive Diseases Foundation to Dr H. Roelofsen. We thank John W. McGrath and Emma Bednarik for expert technical assistance, Philippe Male for his help with the photography, Dr Michael H. Nathanson for valuable advice concerning the use of the confocal laser scanning microscope and Dr Ronald P. J. Oude Elferink of the Academic Medical Center of the University of Amsterdam who provided us with mutant GY/TR$^-$ rats.

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