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Vectorial Transport by Double-Transfected Cells Expressing the Human Uptake Transporter SLC21A8 and the Apical Export Pump ABCC2

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ABSTRACT

Vectorial transport of endogenous substances, drugs, and toxins is an important function of polarized cells. We have constructed a double-transfected Madin-Darby canine kidney (MDCK) cell line permanently expressing a recombinant uptake transporter for organic anions in the basolateral membrane and an ATP-dependent export pump for anionic conjugates in the apical membrane. Basolateral uptake was mediated by the human organic anion transporter 8 (OATP8; symbol SLC21A8) and subsequent apical export by the multidrug resistance protein 2 (MRP2; symbol ABCC2). Under physiological conditions, both transport proteins are strongly expressed in hepatocytes and contribute to the hepatobiliary elimination of organic anions. Expression and localization of OATP8 and MRP2 in MDCK cells growing on Transwell membrane inserts was demonstrated by immunoblotting and confocal laser scanning microscopy. ^{3}H-Labeled sulfobromophthalein (BSP) was a substrate for both transport proteins and was transferred from the basolateral to the apical compartment at a rate at least six times faster by double-transfected MDCK-MRP2/OATP8 cells than by single-transfected MDCK-OATP8 or MDCK-MRP2 cells. Vectorial transport at a much higher rate by double-transfected than by single-transfected cells was also observed for the ^{3}H-labeled substrates leukotriene C_4, 17β-glucuronosyl estradiol, and dehydroepiandrosterone sulfate, for the fluorescent anionic substrate fluo-3, and for the antibiotic rifampicin. Inhibition studies indicated that intracellular formation of *S*-(2,4-dinitrophenyl)-glutathione from 2,4-chlorodinitrobenzene selectively inhibits the transcellular transport of [^{3}H]BSP at the site of MRP2-mediated export. The double-transfected cells provide a useful system for the identification of transport substrates and transport inhibitors including drug candidates.

Vectorial transport is an important function of all polarized cells contributing to detoxification and to the prevention of entry of toxins into organs. This is exemplified by kidney proximal tubule epithelia, by cells of the blood-brain barrier, by intestinal epithelia, and, last but not least, by hepatocytes. One of the major hepatocellular functions is the removal of endogenous and exogenous substances from the blood circulation and their secretion into the bile. Two transport processes play a decisive role in this vectorial transport by hepatocytes: the sinusoidal (basolateral) uptake from blood and the canalicular (apical) secretion into bile. In human hepatocytes, the sodium-independent uptake of amphiphilic organic anions is mediated by at least three transport proteins: the human organic anion transporters OATP2 (also known as OATP-C or LST1, symbol SLC21A6) (Abe et al., 1999; Hsiang et al., 1999; König et al., 2000a; Cui et al., 2001), human OATP8 (SLC21A8) (König et al., 2000b; Cui et al., 2001), and human OATP-B (SLC21A9) (Kullak-Ublick et al., 2001). All three transporters belong to the subgroup 21A of the solute carrier (SLC) superfamily. Whereas OATP-B is expressed also in a number of other tissues, OATP2 and OATP8 are expressed exclusively in human hepatocytes. The substrate spectrum of OATPs includes bile salts, conjugates of steroid hormones, thyroid hormones, and many other amphiphilic organic anions (Abe et al., 1999; König et al., 2000a,b; Kullak-Ublick et al., 2000, 2001; Cui et al., 2001). Unlike these basolateral uptake transporters which are thought to be of exchanger type (Li et al., 1998), the apical export transporters identified in human hepatocytes so far are members of the ATP-binding cassette (ABC) superfamily (Keppler and Arias, 1997; Jansen, 2000). The export of or-

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ABBREVIATIONS: OATP, human organic anion-transporting polypeptide; SLC, solute carrier superfamily; ABC, ATP-binding cassette superfamily; BSP, sulfobromophthalein; BSP-SG, sulfobromophthalein glutathione *S*-conjugate; MRP2, multidrug resistance protein 2; MDCKII, Madin-Darby canine kidney cells, strain II; LTC_4, leukotriene C_4; CDNB, 1-chloro-2,4-dinitrobenzene; Fluo-3, 1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxo-3H-xanthen-9-yl)]-2-(2′-amino-5′-methyl-phenoxyl)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid penta-ammonium salt; HEK, human embryonic kidney; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; $E_2$17βG, 17β-glucuronosyl estradiol; DHEAS, dehydroepiandrosterone 3-sulfate; HSA, human serum albumin.

ganic anions is predominantly mediated by the bile salt export pump (ABCB11) belonging to the MDR (ABCB) subgroup of the ABC superfamily (Strautnieks et al., 1998; Gerloff et al., 1998; Wang et al., 2001) and by the multidrug resistance protein 2 (MRP2, ABCC2) belonging to the MRP (ABCC) subgroup of the ABC superfamily (Büchler et al., 1996; Suzuki and Sugiyama, 1998; König et al., 1999; Borst et al., 2000). While the major substrates of the bile salt export pump are bile salts such as cholyl taurine and cholate (Gerloff et al., 1998), the organic anions transported by MRP2 are mainly conjugates of lipophilic substances with glutathione, glucuronate, or sulfate (Evers et al., 1998; Cui et al., 1999, König et al., 1999).

The transhepatic transport of amphiphilic organic anions has been frequently studied by use of model compounds like sulfobromophthalein (BSP) and indocyanine green (Scharschmidt et al., 1975). Functional characterization of the three human OATPs identified in the hepatocyte basolateral membrane demonstrated that all three are able to mediate the uptake of BSP, with the highest affinity for OATP2 (K_m = 140 nM) and the lowest affinity (K_m = 3.3 μM) for OATP8 (König et al., 2000b; Cui et al., 2001; Kullak-Ublick et al., 2001). In the hepatocyte, BSP is predominantly conjugated with glutathione to yield the BSP glutathione *S*-conjugate (BSP-SG) (Whelan et al., 1970; Snel et al., 1995). Studies with transport-deficient mutant rats (Jansen et al., 1987), which lack the canalicular export pump Mrp2 (Büchler et al., 1996; Paulusma et al., 1996; Ito et al., 1997), suggested that this export pump mediates the secretion of BSP-SG into bile. However, it was not established that BSP itself is a substrate for human MRP2.

So far, the transport proteins like OATPs and MRPs were studied mostly by use of transfected mammalian cells or by use of *Xenopus laevis* oocyte systems expressing only one exogenous recombinant transport protein (Madon et al., 1997; Evers et al., 1998; Ito et al., 1998; Abe et al., 1999; Cui et al., 1999, 2001; Hsiang et al., 1999; König et al., 2000a,b; Kullak-Ublick et al., 2001). To understand the sequential and concerted action of defined uptake transporters and export pumps during the vectorial transport across hepatocytes and other polarized cells, it has been of interest to express both transport systems in the same polarized cell. Several cell lines have been used for studies on the vectorial transport of organic anions. The OK (American opossum kidney) cells, for example, showed a significant transcellular transport of the organic anion *p*-aminohippurate (Hori et al., 1993). However, in such a cell system, the molecular identity of most endogenous transport proteins has not yet been characterized. In addition, the opossum transporters may differ significantly from the recombinant human transporters (Ishizuka et al., 1999). The aim of the work described here was to establish a cell system with defined human uptake and export transporters. In this study, we transfected both the human uptake transporter OATP8 and the human export pump MRP2 into the polarized Madin-Darby canine kidney cells (MDCK strain II). The organic anion BSP was used as a model substrate to characterize the transcellular transport mediated by recombinant human OATP8 and MRP2 in the double-transfected cells. Anionic drugs, such as rifampicin or rifamycin SV, were studied as potent inhibitors for the transcellular transport of BSP.

Materials and Methods

Chemicals. [^{3}H]BSP (0.5 TBq/mmol) was obtained from Hartmann Analytic (Köln, Germany) by custom synthesis (Cui et al., 2001). [14,15,19,20-^{3}H]LTC_4F (5.37 Bq/mmol), [1,2,6,7-^{3}H]dehydroepiandrosterone sulfate (0.6 TBq/mmol), [^{3}H]cholyl taurine (73 GBq/mmol), and 17β-D-glucuronosyl [6,7-^{3}H]estradiol (1.6 TBq/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). [^{14}C]Inulin carboxylic acid (82 MBq/g) was obtained from Biotrend Chemicals (Köln, Germany). 1-[2-Amino-5-(2,7-dichloro-6-hydroxy-3-oxo-3H-xanthen-9-yl)]-2-(2′-amino-5′-methyl-phenoxyl)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid penta-ammonium salt (Fluo-3) was from Calbiochem (Bad Soden, Germany). Rifampicin, rifamycin SV, acivicin, and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma (Deisenhofen, Germany). G418 (Geneticin) sulfate was from Invitrogen (Carlsbad, CA). Hygromycin was from Invitrogen (Groningen, Netherlands). Additional nonradioactive chemicals of analytical purity were obtained from Sigma.

Cell Culture and Transfection. Human embryonic kidney (HEK) 293 and MDCKII cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C, 95% humidity, and 5% CO_2. HEK-MRP2 and HEK-Co are HEK293 cells transfected with human MRP2 cDNA and control vector, respectively; MDCK-MRP2 and MDCK-Co are MDCKII cells transfected with human MRP2 cDNA and control vector, respectively, as described previously (Cui et al., 1999).

The human *OATP8* cDNA (König et al., 2000b) was subcloned into the mammalian expression vector pcDNA3.1/Hygro(−) (Invitrogen) and transfected into MDCKII cells using the polybrene method (König et al., 2000b). Transfectants expressing recombinant OATP8 were selected with hygromycin (950 μM). The clone with the highest OATP8 expression (MDCK-OATP8) was further transfected with the vector construct pcDNA3.1-MRP2 with the full-length human MRP2 cDNA (Cui et al., 1999). After selection with 950 μM hygromycin and 800 μM G418 disulfate for 3 weeks, the transfectants were screened for both MRP2 and OATP8 expression by immunoblot analyses. A clone with the highest MRP2 expression and a expression level of OATP8 similar to that of MDCK-OATP8 cells was designated as MDCK-MRP2/OATP8 and chosen for further studies.

Immunoblot Analysis. Crude membrane fractions were prepared from cultured MDCKII cells as described earlier (Cui et al., 1999). Proteins were separated by SDS-polyacrylamide gel electrophoresis (7.5% gels). OATP8 was detected by the polyclonal antibody SKT (König et al., 2000b) at a dilution of 1:5000 in Tris-buffered saline/Tween 20 (20 mM Tris, 145 mM NaCl, 0.05% Tween 20, pH 7.6). MRP2 was detected by the polyclonal antibody EAG5 (Büchler et al., 1996; Keppler and Kartenbeck, 1996) at a dilution of 1:10,000 in Tris-buffered saline/Tween 20.

Confocal Laser Scanning Immunofluorescence Microscopy. MDCKII cells were grown on Transwell membrane inserts (6.5-mm diameter, 0.4-μm pore size; Corning Costar, Bodenheim, Germany) for 3 days at confluence and induced with 10 mM sodium butyrate for 24 h (Cui et al., 1999). Cells were fixed with 2.5% paraformaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 8.0 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) for 20 min, permeabilized with 1% Triton X-100 in PBS for 20 min, and incubated for 1.5 h with primary antibodies at room temperature. The polyclonal antibody SKT (König et al., 2000b) at a dilution of 1:25 in PBS and the monoclonal mouse antibody M_2III-6 (Alexis Biochemicals, Grünberg, Germany) at a dilution of 1:20 in PBS were used to stain OATP8 and MRP2, respectively. Cells were then washed three times with PBS and incubated with secondary antibodies. Both Cy2-conjugated goat anti-rabbit IgG and Cy3-conjugated goat anti-mouse IgG were obtained from Jackson Laboratories (West Grove, PA) and used at a dilution of 1:200 in PBS. The Transwell membranes were then cut from the membrane inserts and mounted onto the slides using 50%

glycerol in PBS. Confocal laser scanning microscopy was performed with a LSM 510 apparatus from Zeiss (Oberkochen, Germany).

Transport Assays. For transport assays, MDCKII cells were grown on Transwell membrane inserts (24-mm diameter, 0.4-μm pore size, Corning Costar) at confluence for 3 days and induced with 10 mM sodium butyrate for 24 h. Cells were first washed with transport buffer (142 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM glucose, and 12.5 mM HEPES, pH 7.3); subsequently, ^{3}H-labeled substrates were added in transport buffer either to the apical compartments (1 ml) or to the basolateral compartments (1.5 ml). After the times indicated, the radioactivity in the opposite compartments was measured. The intracellular accumulation of the radioactivity was determined by lysing the cells with 2 ml of 0.2% SDS in water and measuring the radioactivity in cell lysates.

To study the transcellular transport of Fluo-3, cells were incubated with 2 μM Fluo-3 in the basolateral compartments at 37°C for 30 min. The fluorescence of Fluo-3 in the apical compartment was measured with a RF-510 fluorescence spectrometer (Shimadzu, Duisburg, Germany) at an excitation wavelength of 506 nm (5-nm bandwidth) and an emission wavelength of 526 nm (10-nm bandwidth) in the presence of 1.5 mM Ca^{2+}(Nies et al., 1998).

To study the transcellular transport of rifampicin, cells were incubated with 50 μM rifampicin in the basolateral compartments at 37°C for 30 min. To determine the concentration of rifampicin in the apical compartments its absorption at 475 nm was measured with a spectrophotometer (Ultrospec III; Amersham Pharmacia Biotech, Freiburg, Germany). A calibration curve for the calculation of the concentrations of rifampicin was determined in the concentration range between 1 and 50 μM.

For inhibition studies, the inhibitors were added simultaneously with [^{3}H]BSP into the basolateral compartments, with the exception of CDNB. In this case, MDCKII cells grown on Transwell membrane inserts were preincubated with CDNB in transport puffer in both apical and basolateral compartments for 20 min at room temperature. The transcellular transport was then started by replacing the buffer in the basolateral compartments by fresh buffer containing CDNB and [^{3}H]BSP.

The transcellular leakage was determined by incubating cells with 50 μM [^{14}C]inulin carboxylic acid in the basolateral compartments for 30 min and measuring the radioactivity in the apical compartments. For all four MDCKII cell lines used in this work, the transcellular leakage was lower than 1%.

HPLC Analysis of [^{3}H]BSP. MDCK-MRP2/OATP8 cells grown on Transwell membrane inserts were incubated with 1 μM [^{3}H]BSP in the basolateral compartment for 30 min at 37°C after a preincubation with 5 mM acivicin, an inhibitor of γ-glutamyltransferase (Allen et al., 1980), in both compartments for 30 min at 37°C. The radioactivity in the apical compartment and in the cell lysate was analyzed by HPLC. Reversed-phase HPLC analyses on a C_{18} Hypersil column (5-μm particles; Shandon, Runcorn, UK) were performed as described (Cui et al., 2001) using a linear gradient elution from 100% buffer A (45% methanol/55% water containing 2 mM tetrabutylammonium hydroxide at pH 6.0) to 100% buffer B (90% methanol/10% water containing 2 mM tetrabutylammonium hydroxide at pH 6.0) at a flow rate of 1 ml/min. [^{3}H]BSP-SG was synthesized for the HPLC analyses by incubating 1 mM GSH, 0.4 mM [^{3}H]BSP (1 μCi), and 5 mM acivicin with 350 μl of mouse liver cytosol in a final volume of 500 μl for 1 h at 37°C. After deproteinization with 3 volumes of methanol, 200 μl of supernatant was analyzed by HPLC as described above.

Vesicle Transport Studies. Transport of [^{3}H]BSP into membrane vesicles was measured by the rapid filtration method (Keppler et al., 1998). Briefly, membrane vesicles (30 μg of protein) were incubated in the presence of 4 mM ATP, 10 mM creatine phosphate, 100 μg/ml creatine kinase, and [^{3}H]BSP in an incubation buffer (250 mM sucrose, 10 mM Tris/HCl, pH 7.4) in a final volume of 55 μl at 37°C. Aliquots (15 μl) were taken at the indicated time points, diluted in 1 ml of ice-cold incubation buffer, and immediately filtered through presoaked nitrocellulose membrane (0.2-μm pore size; Schleicher & Schüll, Dassel, Germany). Filters were rinsed twice with 5 ml of incubation buffer, dissolved in liquid scintillation fluid, and counted for radioactivity. In control experiments, ATP was replaced by an equal concentration of 5′-AMP. ATP-dependent transport was calculated by subtracting values obtained in the presence of 5′-AMP from those obtained in the presence of ATP.

Results

Expression and Localization of Recombinant Human OATP8 and MRP2 in MDCKII cells. The expression of human OATP8 and MRP2 in the transfected MDCKII cells was first analyzed by immunoblotting (Fig. 1). As shown in Fig. 1A, MRP2 expression was detected by the antibody EAG5 in MDCKII cells transfected with MRP2 cDNA alone (MDCK-MRP2) or with both OATP8 and MRP2 cDNA (MDCK-MRP2/OATP8). The expression of OATP8 was detected by the antibody SKT in MDCKII cells transfected with OATP8 cDNA alone (MDCK-OATP8) and in MDCK-MRP2/OATP8 cells (Fig. 1B). Consistent with our earlier report (König et al., 2000b), the fully glycosylated form of human OATP8 has an apparent molecular mass of about 120 kDa; the bands with lower apparent molecular mass detected by the antibody SKT resulted from the underglycosylated forms of OATP8. In the MDCKII cells transfected with the control vector (MDCK-Co), expression of neither human OATP8 nor MRP2 could be detected (Fig. 1).

The cellular localization of the recombinant transporters in the transfectants was studied by means of immunofluorescence and confocal laser scanning microscopy. In MDCK-OATP8 cells, OATP8 could be stained with the antibody SKT in the basolateral membrane (Fig. 2, A and D). Some intracellular staining of OATP8 was observed as well in these cells. Double-labeling experiments using the antibody SKT and 3,3′-dihexyloxacarbocyanine iodide, which stains specifically the endoplasmic reticulum (Terasaki et al., 1984), indicated that the intracellular OATP8 fraction was localized to the ER (not shown). The partial ER localization of OATP8 corresponds to its biogenesis and is consistent with the existence of underglycosylated OATP8 observed in immunoblot analyses (Fig. 1B). In MDCK-MRP2/OATP8 cells, MRP2 was

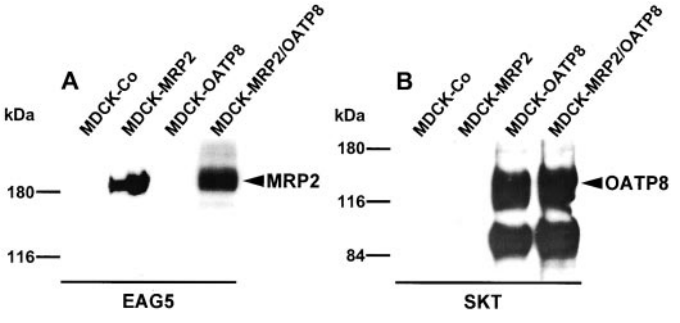

Fig. 1. Immunoblot analysis of MRP2 and OATP8 in transfected MDCK cells. Crude membrane fractions from MDCK cells permanently transfected with control vector (MDCK-Co), with human MRP2 (MDCK-MRP2), with human OATP8 (MDCK-OATP8), or with both MRP2 and OATP8 cDNA (MDCK-MRP2/OATP8) were separated by SDS-polyacrylamide gel electrophoresis. A, human MRP2 was detected by the polyclonal antibody EAG5 (Keppler and Kartenbeck, 1996; Schaub et al., 1999). B, human OATP8 was detected by the polyclonal antibody SKT (König et al., 2000b). In case of OATP8, only the fully glycosylated form is indicated by an arrowhead, whereas the band at about 90 kDa represents an under-glycosylated form of the protein (König et al., 2000b).

localized to the apical membrane, in addition to the lateral appearance of OATP8 (Fig. 2, B, C, E, and F). Figure 2B shows an image focused in the plane of the nuclei; only OATP8 was visible in this plane. Figure 2C shows an image focused at the top of the cells, where the MRP2 staining could be seen. In vertical sections, both OATP8 and MRP2 were visible (Fig. 2, E and F). As demonstrated in Fig. 2F, OATP8 is present in the lateral membrane and on intracellular, in part subapical membranes. An apical localization of OATP8, as Fig. 2D may wrongly suggest, is not detectable by confocal laser scanning microscopy because of the lack of colocalization of MRP2 and OATP8 in Fig. 2, E and F. Moreover, when MDCK cells grow on Transwell membranes, OATP8 is not detectable in the basal membrane (Fig. 2, D–F) but predominantly in the lateral membrane. Functionally, however, this represents a basolateral transport protein and corresponds to the truly basolateral localization of OATP8 in the human liver (König et al., 2000b). Figure 3 shows schematically the role of both recombinant transporters in the vectorial transport by the respective transfected cell line.

Transcellular Transport of [^{3}H]BSP Mediated by OATP8 and MRP2. The function of human OATP8 and MRP2 in the double-transfected cells was studied by measurement of the transcellular transport of the organic anion [^{3}H]BSP, a substrate of human OATP8 (König et al., 2000b, Cui et al., 2001). Thus, polarized MDCKII cells grown on Transwell membrane inserts were incubated with [^{3}H]BSP at a concentration of 1 μM in the basolateral compartments. At different time points, the radioactivity accumulated in the apical compartment and in the cells was measured. As shown in Fig. 4A, the intracellular accumulation of [^{3}H]BSP was significantly higher in MDCKII cells transfected either with *OATP8* cDNA alone (MDCK-OATP8) or with both OATP8 and MRP2 cDNA (MDCK-MRP2/OATP8) than in the control-transfected (MDCK-Co) and in the MRP2-transfected (MDCK-MRP2) cells, demonstrating that OATP8 is sufficient for the intracellular accumulation of [^{3}H]BSP. However, when the radioactivity in the apical compartment was measured, no significant transfer of [^{3}H]BSP from the basolateral compartments into the apical compartments could be observed for MDCK-Co, MDCK-MRP2, and MDCK-OATP8 cells (Fig. 4B), whereas a marked transcellular transport of [^{3}H]BSP could be observed with MDCK-MRP2/OATP8 cells (Fig. 4B). Figure 4C demonstrates a higher total uptake of [^{3}H]BSP (intracellular accumulation plus transcellular transport) by the MDCK-MRP2/OATP8 cells than by the MDCK-OATP8 cells.

The transcellular transport of [^{3}H]BSP by the MDCK-MRP2/OATP8 cells was a vectorial process, as shown in Fig. 5A. Only a basolateral to apical transport of [^{3}H]BSP was observed, whereas the apical to basolateral transport was negligible. This was expected from the cellular localization of OATP8 and MRP2 in the double transfectants and from the unidirectionality of MRP2-mediated transport (Fig. 3).

The efflux of [^{3}H]BSP was determined after incubation of MDCK-OATP8 and MDCK-MRP2/OATP8 cells with 1 μM [^{3}H]BSP for 30 min in the basolateral compartment. As shown in Fig. 5B, [^{3}H]BSP was released from the MDCK-OATP8 cells mainly across the basolateral membrane, whereas it was exported from the MDCK-MRP2/OATP8 cells mainly across the apical membrane. When the radioactivity remaining in the cells after the 30-min efflux experiments was compared, the MDCK-MRP2/OATP8 cells showed a significantly lower intracellular [^{3}H]BSP level than the MDCK-OATP8 cells, indicating the efficient export via MRP2 in the apical membrane.

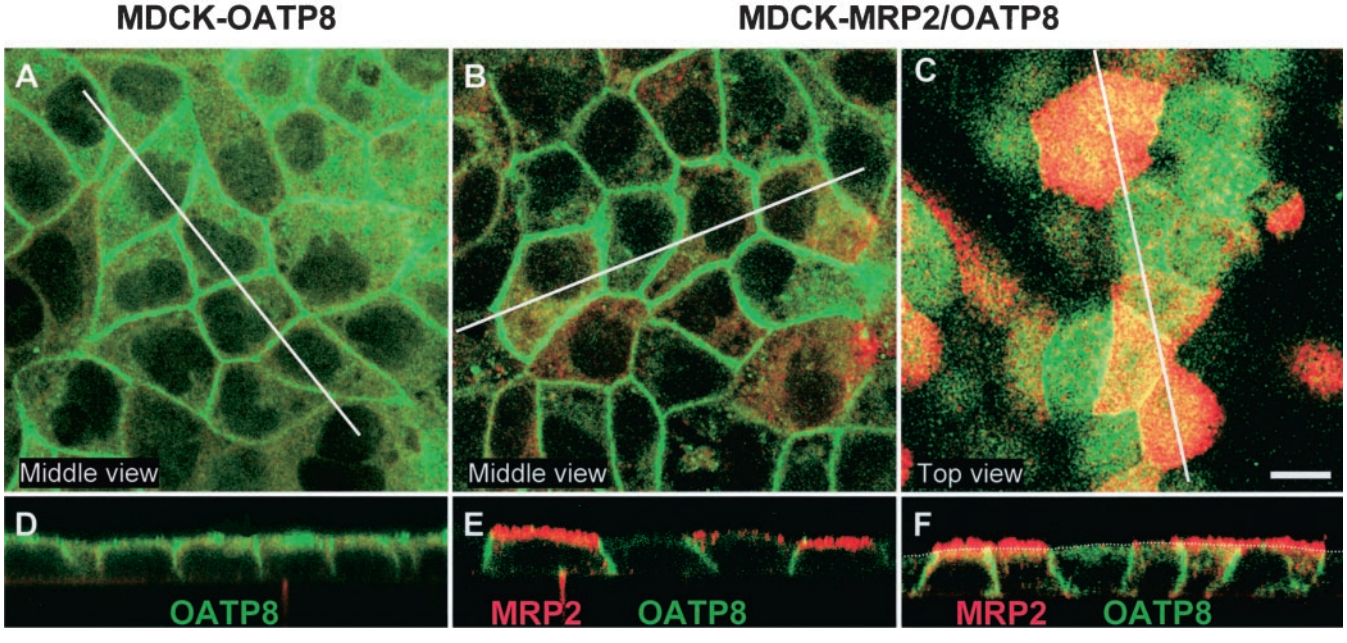

Fig. 2. Immunolocalization of recombinant MRP2 and OATP8 in MDCK cells. MDCK cells expressing OATP8 alone (A and D) or both MRP2 and OATP8 (B, C, E, and F) were grown on Transwell membrane inserts and examined by confocal laser scanning microscopy. OATP8 (green fluorescence) and MRP2 (red fluorescence) were stained using the polyclonal antibody SKT (König et al., 2000b) and the monoclonal antibody M_2III6 (Evers et al., 1998), respectively. A and B are en face images focused at the middle of the cell monolayer; C is an en face image focused at the top of the cell monolayer. D, E, and F are vertical sections at the positions indicated by the white lines in A, B, and C. Besides the lateral localization of OATP8, some additional staining of this protein can be seen in the vertical sections (D, E, and F). In F, the horizontal interrupted line indicates the level of the apical membrane, with MRP2-containing microvilli above and OATP8-containing intracellular structures below this level. No colocalization of MRP2 (red) and OATP8 (green) is seen in the vertical sections shown in E and F. Bar in C, 10 μm; same magnification for all panels.

BSP Itself and Not Its Glutathione *S*-Conjugate Is Transported by MRP2 in the Double-Transfected MDCK Cells. Studies in rat liver showed that BSP is secreted into bile mainly as glutathione conjugate (Combes, 1965; Klaassen and Plaa, 1967). To investigate whether this was also true for the double transfectants, we checked the radioactivity in the apical compartment of the MDCK-MRP2/OATP8 cells by radio-HPLC after incubation of the cells with 1 μM [^{3}H]BSP in the basolateral compartment for 30 min. The majority of the radioactivity (>98%) that was accumu-

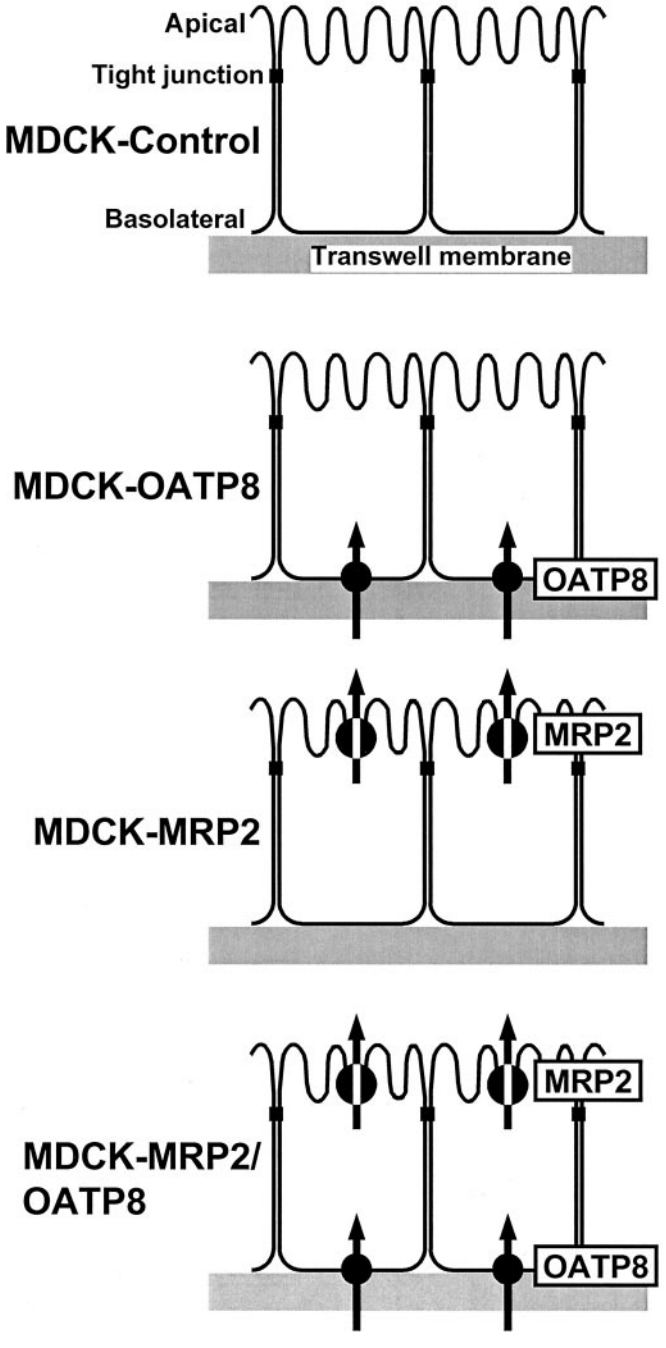

Fig. 3. Scheme of the MDCK transfectants growing on the Transwell membrane inserts as a polarized monolayer. The tight junctions separate the apical membrane facing the culture medium from the basolateral membrane facing the membrane support. The export pump MRP2 is localized to the apical membrane, whereas the uptake transporter OATP8 is localized to the basolateral membrane.

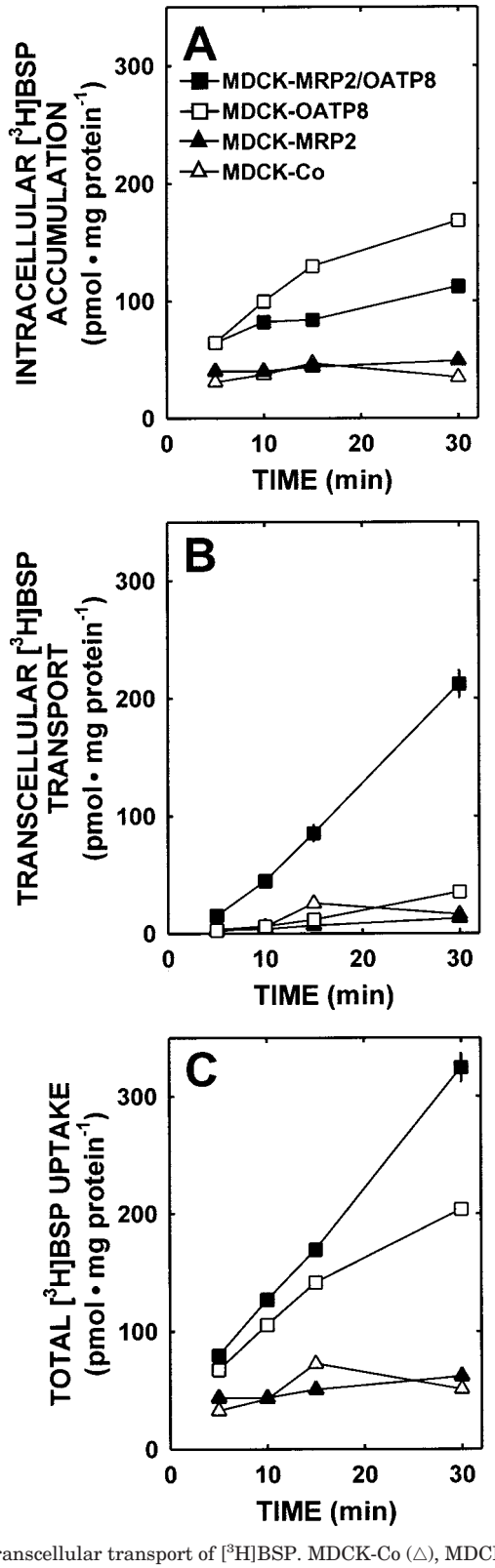

Fig. 4. Transcellular transport of [^{3}H]BSP. MDCK-Co (△), MDCK-MRP2 (▲), MDCK-OATP8 (□), and MDCK-MRP2/OATP8 (■) cells were grown on Transwell membrane inserts. [^{3}H]BSP (1 μM) was given to the basolateral compartments. At the time points indicated, radioactivity in the apical compartments (Transcellular [^{3}H]BSP transport) and inside the cells (Intracellular [^{3}H]BSP accumulation) was determined. Total uptake of [^{3}H]BSP was calculated as the sum of intracellular and apical radioactivity. Data represent means ± S.D. (n = 4). For most measurements, the standard deviation was within the size of the symbols.

lated in the apical compartment (Fig. 6B) and in the cells (Fig. 6C) showed the same retention time (17 min) as the unconjugated [^{3}H]BSP (Fig. 6A). Only a small peak of [^{3}H]BSP-SG (retention time, 15 min) was observed in the apical compartment (Fig. 6B). These results suggest that BSP is taken up by human OATP8 and is itself a substrate for MRP2, rather than BSP-SG, during transcellular transport. To confirm this hypothesis, we investigated the transport of [^{3}H]BSP into inside-out membrane vesicles prepared from HEK-MRP2 cells (HEK293 cells transfected with human MRP2; Cui et al., 1999). As shown in Fig. 7A, [^{3}H]BSP was transported ATP-dependently into membrane vesicles from HEK-MRP2 cells. The accumulation of [^{3}H]BSP in the membrane vesicles from HEK-MRP2 cells was significantly higher than in the membrane vesicles from control-transfected HEK-Co cells (Fig. 7B), demonstrating that [^{3}H]BSP is a substrate for human MRP2. A K_m value of 12 μM for BSP was determined for MRP2 (Fig. 7C).

Transcellular Transport of Other Organic Anions. Transcellular transport of other organic anions that are substrates for both OATP8 and MRP2 was also studied. [^{3}H]Leukotriene C_4 (LTC_4), 17β-glucuronosyl [^{3}H]estradiol ($E_2$17βG), and [^{3}H]dehydroepiandrosterone sulfate (DHEAS) have already been identified as OATP8 substrates (König et al., 2000b; Cui et al., 2001; Kullak-Ublick et al., 2001). [^{3}H]LTC_4 and [^{3}H]$E_2$17βG have been shown to be high-affinity substrates for MRP2 (Cui et al., 1999). Our studies also identified [^{3}H]DHEAS as a substrate of MRP2 (R. Gologan, I. Leier, and D. K. Keppler,

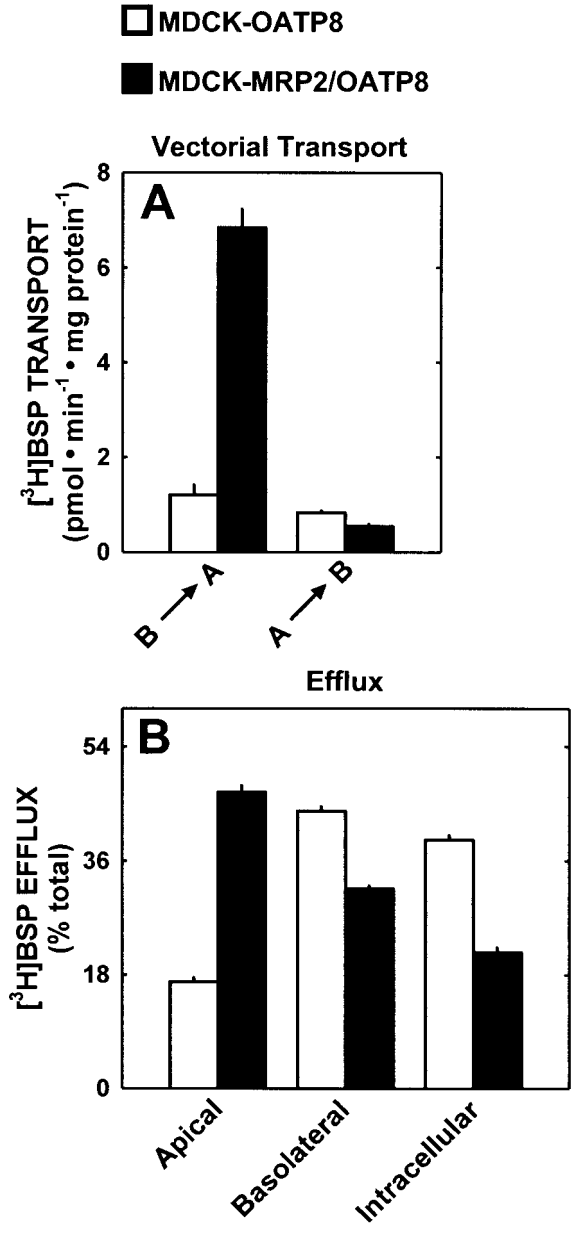

Fig. 5. Vectorial transport and efflux of [^{3}H]BSP. MDCK-OATP8 and MDCK-MRP2/OATP8 cells were grown on Transwell membrane inserts. A, vectorial transport of [^{3}H]BSP. [^{3}H]BSP (1 μM) was given either to the basolateral compartments (B $\rightarrow$ A) or to the apical compartments (A $\rightarrow$ B). After 15 min at 37°C, radioactivity in the opposite compartments was measured. B, efflux of [^{3}H]BSP. MDCK-OATP8 and MDCK-MRP2/OATP8 cells were incubated with [^{3}H]BSP (1 μM) in the basolateral compartments at 37°C for 30 min. The cells were then washed with cold buffer and incubated with buffer without [^{3}H]BSP at 37°C for 30 min. The radioactivity subsequently released into the basolateral and the apical compartment and inside the cells was measured. Data represent means ± S.D. (n = 4).

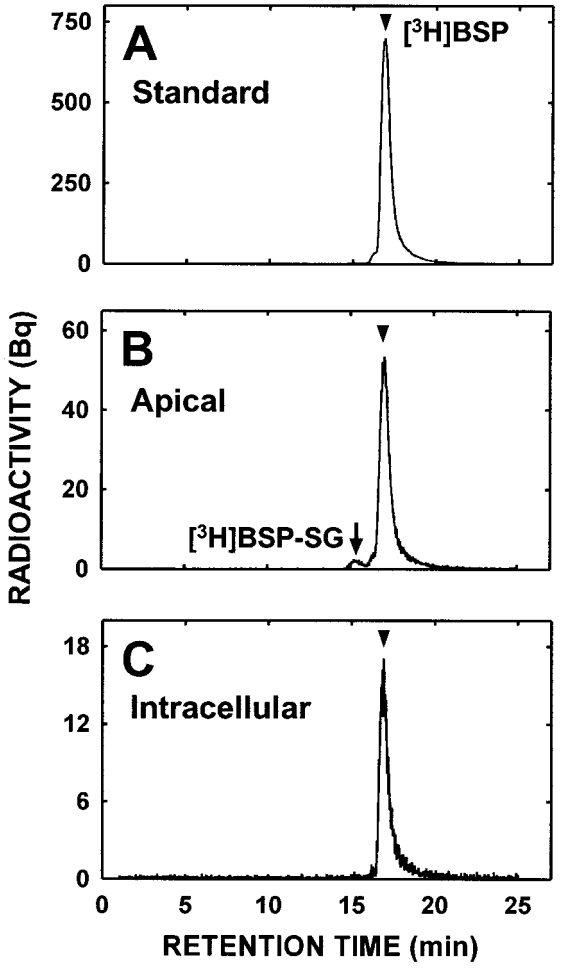

Fig. 6. HPLC analyses of [^{3}H]BSP. MDCK-MRP2/OATP8 cells grown on Transwell membrane inserts were incubated with 1 μM [^{3}H]BSP in the basolateral compartment at 37°C for 30 min. The medium in the apical compartment and the cell lysate were analyzed by radio-HPLC as described under *Materials and Methods*. A, authentic [^{3}H]BSP (Cui et al., 2001). B, radioactivity collected in the apical compartment. C, radioactivity accumulated in the cells. [^{3}H]BSP (arrowhead) and the glutathione *S*-conjugate of [^{3}H]BSP ([^{3}H]BSP-SG, arrow) are indicated. Acivicin, an inhibitor of the degradation of the glutathione moiety of [^{3}H]BSP-SG, was added to the incubation at a concentration of 5 mM.

unpublished observations). Similar to [^{3}H]BSP (Fig. 8A), all three compounds were transported with much higher velocities across MDCK-MRP2/OATP8 cells than across MDCK-OATP8 or MDCK-Co cells (Fig. 8, B–D). Fluo-3 is a fluorescent compound (Minta et al., 1989) and a good substrate for MRP2 (Nies et al., 1998). Like the other compounds mentioned above, Fluo-3 was transported across the MDCK-MRP2/OATP8 cell monolayer with a higher transport rate compared with the MDCK-OATP8 cell monolayer (Fig. 8E), suggesting that Fluo-3 is also a substrate for human OATP8.

The transcellular transport of [^{3}H]cholyl taurine, which is a substrate for neither human OATP8 (König et al., 2000b, Cui et al., 2001) nor MRP2, (Madon et al., 1997), was studied (Fig. 8F). Unlike the other compounds tested, [^{3}H]cholyl taurine was transported across all three MDCKII cell lines with quite a high velocity, but no difference in transport rates was observed between MDCK-Co, MDCK-OATP8, and MDCK-MRP2/OATP8 cells. The high transport rate for [^{3}H]cholyl taurine by the MDCK cells is probably a result of the expression of endogenous transport systems for bile salts.

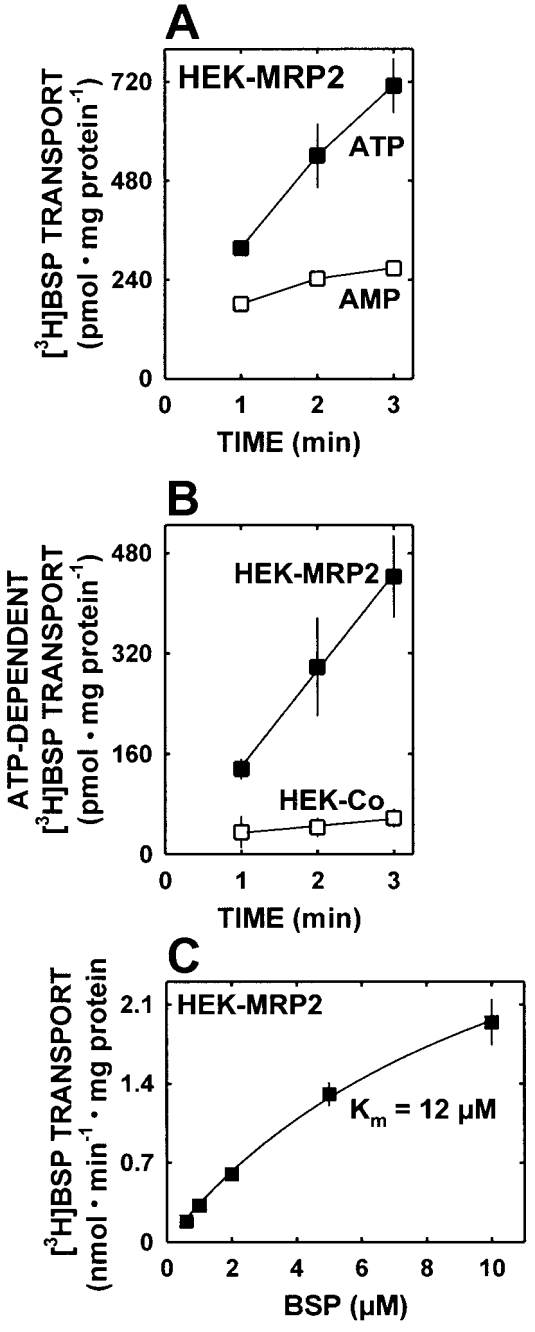

Fig. 7. ATP-dependent transport of [^{3}H]BSP by human MRP2. A, inside-out membrane vesicles from HEK293 cells transfected with human MRP2 (HEK-MRP2) were incubated with 1 μM [^{3}H]BSP in the presence of ATP (■) or 5′-AMP (□). B, net ATP-dependent transport of [^{3}H]BSP into the vesicles from HEK-MRP2 cells (■) or HEK-Co cells (□) was calculated by subtracting the values determined in the presence of 5′-AMP from those in the presence of ATP. C, the K_m value of human MRP2 for BSP was determined at BSP concentrations between 1 and 10 μM. Data represent means ± S.D. (n = 4).

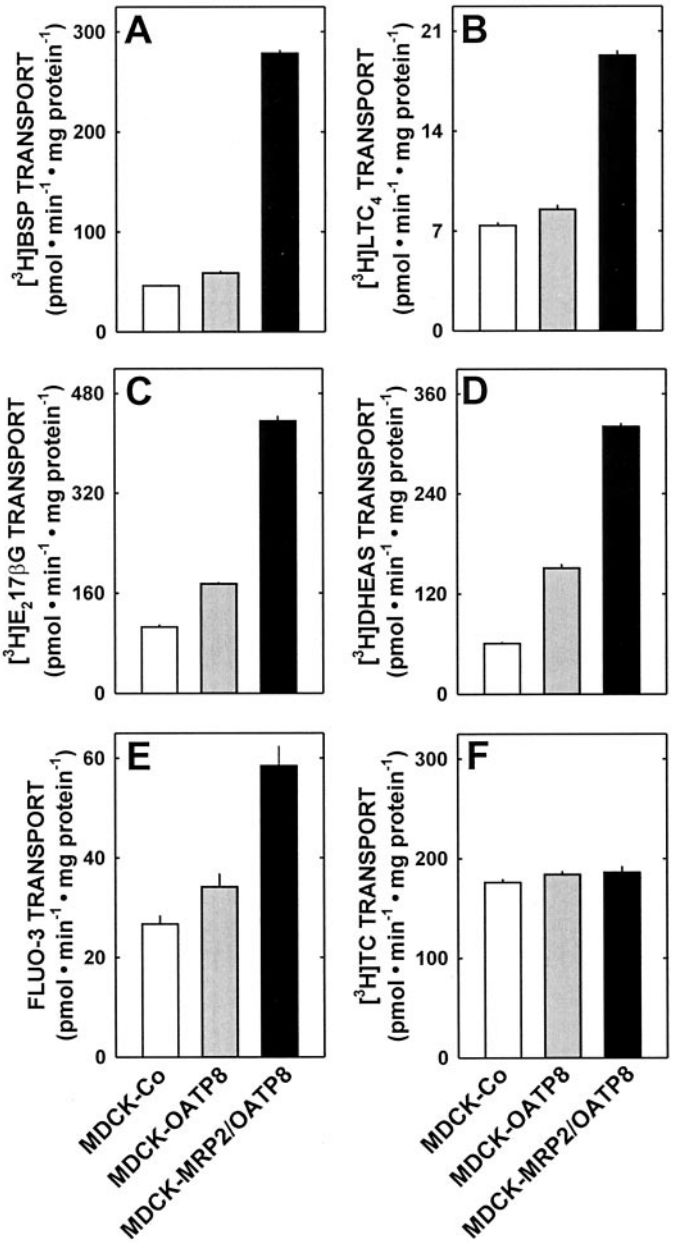

Fig. 8. Transcellular transport of organic anions. MDCK-Co, MDCK-OATP8, and MDCK-MRP2/OATP8 cells grown on Transwell membrane inserts were incubated with [^{3}H]BSP (1 μM), [^{3}H]LTC$_4$ (0.5 μM), [^{3}H]E$_2$17βG (5 μM), [^{3}H]DHEAS (5 μM), Fluo-3 (2 μM), or [^{3}H]cholyl taurine (CT, 5 μM) in the basolateral compartments at 37°C. The radioactivity (labeled substrates) or fluorescence (Fluo-3) in the apical compartments was then measured after 30 min. Data represent means ± S.D. (n = 4).

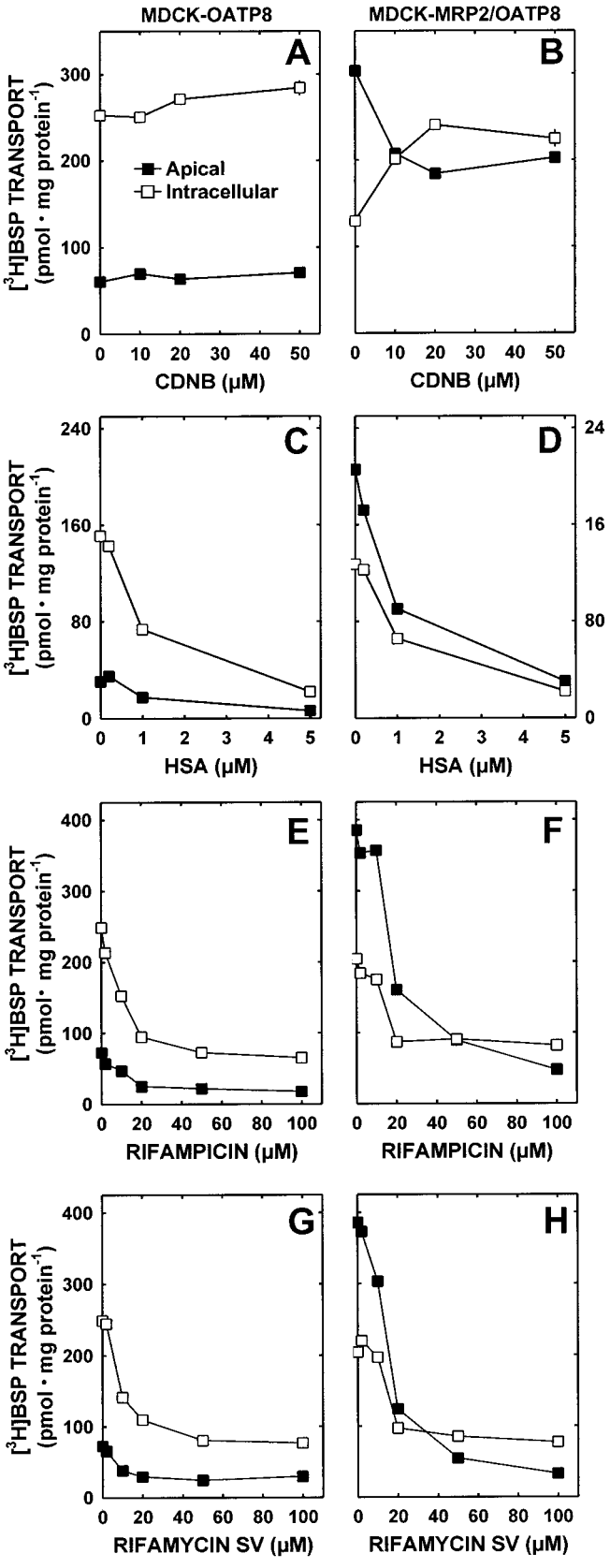

Fig. 9. Inhibition of the transcellular transport of [^{3}H]BSP. MDCK-OATP8 (A, C, E, and G), and MDCK-MRP2/OATP8 (B, D, F, and H) cells grown on Transwell membrane inserts were incubated with [^{3}H]BSP (1 μM) in the basolateral compartments in the presence of different concentrations of HSA (C, D), rifampicin (E, F), or rifamycin SV (G, H). For CDNB (A, B), cells were preincubated with CDNB at room temperature for 20 min, transport of [^{3}H]BSP was started by replacing the buffer in the basolateral compartments by fresh buffer containing [^{3}H]BSP and CDNB. After incubation for 30 min at 37°C, the radioactivity in the apical compartments and inside the cells was measured. Data represent means ± S.D. (n = 4).

Inhibition of Transcellular [^{3}H]BSP Transport. The hydrophobic compound CDNB, which is thought to enter cells via diffusion, is conjugated with glutathione inside the cell and then pumped out via MRP2 (Evers et al., 1998). These properties of CDNB allowed us to differentiate between the uptake mediated by OATP8 and the export mediated by MRP2. Transfected MDCK cells were preincubated with different concentrations of CDNB at room temperature for 20 min before measurement of the transcellular [^{3}H]BSP transport. As shown in Fig. 9A, the intracellular accumulation and the transcellular transport of [^{3}H]BSP in MDCK-OATP8 cells was not inhibited by CDNB up to a concentration of 50 μM. However, CDNB exerted a completely different effect on MDCK-MRP2/OATP8 cells. The intracellular accumulation of [^{3}H]BSP was enhanced, whereas the transcellular transport of [^{3}H]BSP was markedly inhibited by the preincubation with CDNB (Fig. 9B). These results indicate that CDNB does not affect the OATP8-mediated uptake but inhibits the MRP2-mediated export of [^{3}H]BSP after formation of dinitrophenylglutathione inside the cells.

We have recently reported that human serum albumin (HSA) potently inhibits OATP8-mediated uptake of [^{3}H]BSP, probably by binding [^{3}H]BSP with very high affinity (Cui et al., 2001). Thus, we studied here the effect of HSA on the transcellular transport of [^{3}H]BSP across the MDCKII transfectants. In the presence of 5 μM HSA the OATP8-mediated accumulation of [^{3}H]BSP in MDCK-OATP8 cells was suppressed almost completely (Fig. 9C). Also in the MDCK-MRP2/OATP8 cells, the [^{3}H]BSP accumulation was strongly inhibited by HSA (Fig. 9D). Consequently, the transcellular transport of [^{3}H]BSP was also diminished in the MDCK-MRP2/OATP8 cells (Fig. 9D).

OATP8-mediated uptake of [^{3}H]$E_2$17βG into OATP8-transfected HEK293 cells can be inhibited by the antibiotics rifampicin and rifamycin SV (Cui et al., 2001). Figure 9, E-H, demonstrates that both antibiotics interfere strongly with both OATP8 and MRP2. Consistent with our earlier studies (Cui et al., 2001), both antibiotics inhibited the intracellular accumulation of [^{3}H]BSP in the MDCK-OATP8 cells (Fig. 9, E and G). In the MDCK-MRP2/OATP8 cells, however, the intracellular accumulation of [^{3}H]BSP was suppressed to only about 50% of control, whereas the transcellular transport of [^{3}H]BSP was inhibited more strongly by rifampicin and rifamycin SV (Fig. 9, G and H). These results suggest that both compounds inhibit MRP2-mediated export as well. This is confirmed by transport studies using membrane vesicles from HEK-MRP2 cells. Rifampicin at a concentration of 50 μM inhibited [^{3}H]LTC_4 transport into HEK-MRP2 membrane vesicles to 50% of control, and 50 μM rifamycin SV inhibited LTC_4 transport into HEK-MRP2 membrane vesicles to 38% of control.

Transcellular Transport of Rifampicin. Because rifampicin strongly inhibits both human OATP8 and MRP2, we were interested in whether the transcellular transport of rifampicin itself can be measured using our double transfectants. We took advantage of the intensive absorption of ri-

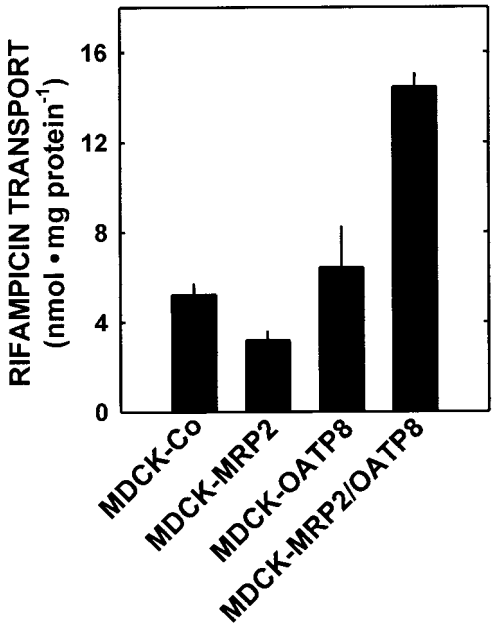

Fig. 10. Transcellular transport of rifampicin. MDCK-Co, MDCK-MRP2, MDCK-OATP8, and MDCK-MRP2/OATP8 cells grown on Transwell membrane inserts were incubated with 50 μM rifampicin in the basolateral compartments at 37°C for 30 min. The concentration of rifampicin in the apical compartments was determined by the specific absorption of rifampicin at 475 nm. Data represent means ± S.D. (n = 4).

fampicin at 475 nm to determine its concentration. A rifampicin concentration as low as 1 μM could be measured by this method. As shown in Fig. 10, a significantly higher transcellular transport of rifampicin could be observed with MDCK-MRP2/OATP8 cells in comparison with the other three MDCKII transfectant cell lines. A rifampicin concentration of about 50 μM in these experiments was the lowest one that we could use to obtain rifampicin transport into the apical compartments in an amount detectable by the photometric method.

Discussion

In this work, we established a cell system (MDCK-MRP2/OATP8) expressing a recombinant human uptake transporter (OATP8) in the basolateral membrane and a recombinant human export pump (MRP2) in the apical membrane (Figs. 1–3). Both transport proteins were functionally active in the transfected MDCKII cells, as demonstrated by the transcellular transport of [^{3}H]BSP (Figs. 4 and 5), a model compound frequently used for the studies on hepatic transport. A number of other compounds that are substrates for both OATP8 and MRP2 were also transported across the monolayer of MDCK-MRP2/OATP8 cells (Fig. 8). Our studies also show that the use of an appropriate inhibitor enables a differential interference with either transporter. CDNB, for example, which is conjugated with glutathione inside the cell, caused a selective inhibition of MRP2 without affecting OATP8 (Fig. 9, A and B). Using these double-transfectants, we identified new substrates transported sequentially by OATP8 and MRP2, namely [^{3}H]BSP, the fluorescent anion Fluo-3, and the antibiotic rifampicin. [^{3}H]BSP was transported across the monolayer of MDCK-MRP2/OATP8 cells predominantly in its unconjugated form (Fig. 6). With inside-out membrane vesicles prepared from MRP2-transfected HEK293 cells, we demonstrate that unconjugated [^{3}H]BSP itself, with a K_m value of 12 μM, is a good substrate for human MRP2 (Fig. 7). Fluo-3 is also a useful substrate for MRP2 (Nies et al., 1998). However, Fluo-3 itself cannot be used in whole-cell assays because it is negatively charged and thus can not penetrate the cell membrane at a sufficient rate in the absence of an uptake transporter. The transcellular transport of Fluo-3 by the double-transfected cells indicates that Fluo-3 is a substrate of OATP8. This makes Fluo-3 an interesting alternative to the labeled substrates for the characterization of inhibitors for OATP8. Rifampicin has been reported to interfere with the hepatic clearance of BSP in human liver, but it was not known whether the uptake or the secretion of BSP or both transport steps are inhibited by rifampicin (reviewed by Acocella, 1978). Our present work indicates that both the uptake of [^{3}H]BSP by OATP8 and the export of [^{3}H]BSP by MRP2 is inhibited by rifampicin (Fig. 9). The experiments with the double-transfected cells suggest, in addition, that because of rifampicin's higher transcellular transport across the MDCK-MRP2/OATP8 cells relative to MDCK-OATP8 and MDCK-MRP2 cells, it is a substrate for OATP8 and MRP2 (Fig. 10).

The identification of the new substrates for MRP2 and OATP8 demonstrates that the double-transfected cell line is useful for the characterization of these transporters. Compared with MDCK cells transfected separately with either OATP8 or MRP2, the double-transfectants have several advantages. It remains difficult to study MRP2 function in whole cells because most substrates for MRP2 are negatively charged under physiological conditions and thus can not penetrate the plasma membrane without an uptake transporter. Therefore, MRP2 has been mostly studied using inside-out membrane vesicles prepared from MRP2-expressing cells. With the double-transfected MDCK cells expressing OATP8 and MRP2 and with compounds such as [^{3}H]BSP and Fluo-3, which are substrates for both transporters, we may now screen more easily for MRP2 inhibitors with intact cells. An inhibitor for MRP2 alone will inhibit the transcellular transport and enhance the intracellular accumulation of [^{3}H]BSP. An inhibitor for both MRP2 and OATP8 will reduce the transcellular transport more strongly than the intracellular accumulation of [^{3}H]BSP. Because of the easier handling of double-transfected cells grown on Transwell membrane inserts compared with the preparation and handling of membrane vesicles, it is possible to develop high-throughput screening systems for MRP2 inhibitors by the use of the double-transfected cells. The use of the fluorescent pentaanion Fluo-3 as a substrate for both OATP8 and MRP2 may further facilitate the screening.

The double-transfected cells are also a suitable system to study the interference of drugs and drug metabolites with the hepatic or renal transport of organic anions, as exemplified by the interference of rifampicin, rifamycin SV, or CDNB with the transcellular transport of [^{3}H]BSP. The MDCKII cells possess relatively low levels of endogenous transporters and a different repertoire of metabolic enzymes than the human hepatocytes and other polarized cells of interest. This may limit the use of the double transfectants, particularly for studies on the effect of the intracellular drug metabolism on transport. In this case, one may further modify this model system by transfecting a third component (e.g., a drug-me-

tabolizing enzyme) into the double transfectants. This third component may vary according to the drug to be studied. Moreover, the combination of OATP8 with MRP2 may be changed for certain purposes. A combination, for example, of OAT1 (SLC22A6) (Hosoyamada et al., 1999) with MRP2, both of which are expressed in human kidney proximal tubule cells (Schaub et al., 1999; Tojo et al., 1999), may serve to study the renal clearance of organic anions. It is clear, however, that such cellular models may provide valuable information on partial functions but never serve as a complete human tissue model.

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