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Adenosine Triphosphate-Dependent Transport of Anionic Conjugates by the Rabbit Multidrug Resistance-Associated Protein Mrp2 Expressed in Insect Cells

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ABSTRACT

The multidrug resistance-associated protein Mrp2 is expressed in liver, kidney, and small intestine and mediates ATP-dependent transport of conjugated organic anions across the apical membrane of epithelial cells. We recently cloned a rabbit cDNA encoding a protein that on basis of highest amino acid homology and tissue distribution was considered to be the rabbit homolog of rat Mrp2. To investigate whether rabbit Mrp2 mediates ATP-dependent transport similar to rat Mrp2, we expressed rabbit Mrp2 in *Spodoptera frugiperda* (Sf9) cells using recombinant baculovirus. Mrp2 was expressed as an underglycosylated protein in Sf9 cells and to a higher level compared with rabbit liver and renal proximal tubules. Both 17β-estradiol-17-β-D-glucuronide ([^{3}H]$E_2$17βG, 50 nM) and [^{3}H]leukotriene C_4 (3 nM) were taken up by Sf9-Mrp2 membrane vesicles in an ATP-dependent fashion. Uptake of [^{3}H]$E_2$17βG was dependent on the osmolarity of the medium and saturable for ATP (K_m = 623 μM). Leukotriene C_4, MK571, phenolphthalein glucuronide, and fluorescein-methotrexate were good inhibitors of [^{3}H]$E_2$17βG transport. The inhibitory potency of cyclosporin A and methotrexate was moderate, whereas fluorescein, α-naphthyl-β-D-glucuronide, and p-nitrophenyl-β-D-glucuronide did not inhibit transport. In conclusion, we show direct ATP-dependent transport by recombinant rabbit Mrp2 and provide new data on Mrp2 inhibitor specificity.

Elimination of endogenous waste products and xenobiotics from the body is mediated by renal and hepatic transport pathways. Excretion of anionic conjugates across liver canalicular (apical) membranes into bile is mediated by the multidrug resistance-associated protein MRP2 (Müller and Jansen, 1997). Initially, this transporter was named cMOAT and characterized by using natural mutant strains of Wistar (TR^-) and Sprague-Dawley (EHBR) rats (Müller and Jansen, 1997). Recently, cloning of rat Mrp2 revealed that the impaired conjugate transport in canalicular membranes of these rats is caused by a premature termination of the *mrp2* gene product (Paulusma *et al*., 1996; Ito *et al*., 1997). Similarly, a mutation leading to a truncated MRP2 was identified in a patient with Dubin-Johnson syndrome, a disease that resembles the TR^- phenotype (Paulusma *et al*., 1997).

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Database analysis revealed that rat Mrp2 is strongly related to the human multidrug resistance-associated protein MRP1, a member of the superfamily of ABC proteins (Büchler *et al*., 1996; Paulusma *et al*., 1996; Ito *et al*., 1997). Originally, MRP1 was identified due to its overexpression in a multidrug-resistant cell line and its ability to confer resistance to chemotherapeutic drugs (Loe *et al*., 1996). Using isolated membrane vesicles from *MRP1*-transfected cells, it has been shown that MRP1 is also capable of transporting anionic conjugates in an ATP-dependent manner. Although MRP1 and MRP2 share substrate specificity, these transporters show differences in their tissue distribution. MRP1 is expressed, predominantly intracellularly, in numerous tissues such as lung, heart, and kidney (Flens *et al*., 1996). In contrast, MRP2 was detected in small intestine and apical (canalicular) membranes of hepatocytes and cells of renal proximal tubules (Büchler *et al*., 1996; Paulusma *et al*., 1996; Schaub *et al*., 1997).

We cloned a rabbit cDNA encoding an ABC-transporter

ABBREVIATIONS: MRP2, multidrug resistance-associated protein 2; cMOAT, canalicular multispecific organic anion transporter; TR^-, transport-deficient rat; EHBR, Eisai hyperbilirubinemic rat; MRP1, multidrug resistance-associated protein 1; ABC, ATP-binding cassette; CFTR, cystic fibrosis transmembrane conductance regulator; LTC_4, leukotriene C_4; $E_2$17βG, 17β-estradiol-17-β-D-glucuronide; FL, fluorescein; MTX, methotrexate; FL-MTX, fluorescein methotrexate; CsA, cyclosporin A; MK571, 3-([{3-(2-[7-chloro-2-quinolinyl]ethenyl)phenyl}-{(3-dimethyl-amino-3-oxopropyl)-thio}-methyl]thio)propanoic acid.

that on basis of similar tissue distribution and highest amino acid homology was considered to be the rabbit homolog of rat Mrp2 (van Kuijck *et al*., 1996, 1997). On injection of its cRNAs in *Xenopus laevis* oocytes, we observed in a few cases a cAMP-dependent chloride conductance (van Kuijck *et al*., 1996). To investigate whether rabbit Mrp2 functions as an ATP-dependent organic anion transporter similar to rat Mrp2, we expressed rabbit Mrp2 in Sf9 cells using recombinant baculovirus and studied uptake of the anionic conjugates $E_2 17\beta G$ and LTC_4 into isolated membrane vesicles. In addition, the effect of various inhibitors on Mrp2-mediated [^{3}H]$E_2 17\beta G$ transport was investigated.

Experimental Procedures

Materials. [14,15,19,20-^{3}H]LTC_4 (165 Ci/mmol) and [6,7-^{3}H]$E_2 17\beta G$ (55 Ci/mmol) were purchased from NEN Life Science Products (Hoofddorp, The Netherlands). ATP, 5′-AMP, LTC_4, $E_2 17\beta G$, MTX, CsA, α-naphthyl-β-D-glucuronide, phenolphthalein glucuronide, and *p*-nitrophenyl-β-D-glucuronide were purchased from Sigma (Zwijndrecht, The Netherlands). FL-MTX and FL were purchased from Molecular Probes (Leiden, The Netherlands). Creatine phosphate and creatine kinase were purchased from Boehringer-Mannheim (Almere, The Netherlands). CELLFECTIN and competent DH10BAC *Escherichia coli* cells were purchased from Life Technologies (Breda, The Netherlands). PNGase F was purchased from New England Biolabs (Westburg, Leiden, The Netherlands). MK571 was a generous gift of Dr. A. W. Ford-Hutchinson (Merck Frosst, Center for Therapeutic Research, Quebec, Canada).

Preparation of antibodies. Rabbit polyclonal antibodies were directed against two different epitopes of rabbit Mrp2 (van Kuijck *et al*., 1996). Antiserum k78mrp2 was obtained by immunizing rabbits with a glutathione-*S*-transferase fusion protein containing the 159 carboxyl-terminal amino acids (1405–1564) of Mrp2. Antiserum k51mrp2 was obtained by immunizing rabbits with a synthetic peptide (FQKRQQKKSQKNSRLQGL) corresponding to amino acids 257–274 of Mrp2 coupled to keyhole limpet hemocyanin. Rabbits were immunized with 400 μg of either the fusion protein or the synthetic peptide mixed with Freund's complete adjuvants. At 3-week intervals after priming, rabbits were boosted with 200 μg of proteins supplemented with incomplete adjuvants. Test bleedings were checked for the presence of Mrp2-specific antibodies using enzyme-linked immunosorbent assay.

Expression construct. The vector pFASTBAC1 (Life Technologies) contains an expression cassette that consists of a polyhedrin promoter, a multiple cloning site, and an SV40 poly(A)$^+$ signal inserted between the left and right arms of the bacterial transposon Tn7. Cloning of a rabbit mrp2 cDNA into pFASTBAC1 was accomplished in two steps: (1) from the pBluescript KS$^+$ construct pBSmrp2, which contains the entire rabbit *mrp2* coding sequence (nucleotides 347-5038) (van Kuijck *et al*., 1996), a 2.7-kb *Xba*I/*Pst*I fragment (nucleotides 2690–5407) was cloned into the *Xba*I and *Pst*I sites of the multiple cloning site of pFASTBAC1 to create pFASTBAC-m1; and (2) to minimize the 5′-untranslated region, the 5′ coding sequence of rabbit Mrp2 was amplified by polymerase chain reaction using the forward primer Mrp2-F1 (5′-ATGCTGGATAAGTTCTGCAAC-3′; nucleotides 347–368), which contains the ATG start codon (underlined), and the reverse primer Mrp2-R1 (5′-GCAGGAGTAGGCCAGATTAG-3′; nucleotides 844–824). The resulting polymerase chain reaction product of 498 bp was cloned into the *Sma*I site of pBluescript KS$^+$, and its sequence was verified by dideoxy sequence analysis (Sanger *et al*., 1977). From this construct, a *Sty*I/*Hin*cII fragment was removed and replaced by a *Sty*I/*Eco*RV fragment (nucleotides 480-3007) from pBSmrp2. Next, a 2.5-kb *Bam*HI fragment of this construct, containing the 5′-region of *mrp2* (nucleotides 347-2868), was cloned into the *Bam*HI site of pFASTBAC1-m1, and its orientation was determined. The selected construct, designated pFBmrp2, contains a full-length rabbit *mrp2* cDNA with the ATG start codon immediately downstream of the polyhedrin promoter.

Production of recombinant baculovirus and viral infection. Baculovirus encoding rabbit Mrp2 was generated using the Bac-to-Bac baculovirus-expressing system (Life Technologies). Competent DH10BAC *E. coli* cells harboring a baculovirus shuttle vector (bacmid) with a Tn7 attachment site were transformed with the pFBmrp2 construct. On transposition between the Tn7 sites, recombinant bacmids were selected and isolated according to the manufacturer. Subsequently, insect Sf9 cells were transfected with recombinant bacmids using CELLFECTIN reagent. After 3 days, culture medium was collected and used to infect fresh Sf9 cells. Four days after infection, stocks of amplified virus were made. Sf9 cells (10^6/ml) were grown as 100-ml suspension cultures and infected at a multiplicity of infection of 1–5 with recombinant baculovirus encoding Mrp2. For control experiments, Sf9 cells were infected with recombinant baculovirus encoding β-glucuronidase (Life Technologies) or the β-subunit of H^+/K^+-ATPase (Klaassen *et al*., 1993). Three days after infection, membrane fractions were isolated (see below).

Isolation of membrane fractions. Crude membrane fractions and membrane vesicles from infected Sf9 cells were isolated as described by Leier *et al*. (1994) with modifications. Briefly, cells were collected and resuspended in hypotonic buffer (0.5 mM sodium phosphate, 0.1 mM EDTA, pH 7.0) supplemented with protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM pepstatin). Cells were stirred gently on ice for 90 min, and the resulting lysate was centrifuged at 100,000 × *g* for 40 min at 4°. The pellet of crude membranes was resuspended in TS-buffer (10 mM Tris-HEPES, 250 mM sucrose, pH 7.4) using a Potter homogenizer, and the homogenate was centrifuged at 12,000 × *g* for 10 min at 4°. The postnuclear supernatant was centrifuged at 100,000 × *g* for 40 min at 4°, and the pellet obtained was resuspended in TS-buffer with a tight-fitting Dounce (type B) homogenizer. The suspension was layered over 38% sucrose in 5 mM HEPES/KOH, pH 7.4, and centrifuged at 100,000 × *g* for 2 hr at 4°. The interphase was collected and homogenized on ice with a tight-fitting Dounce (type B) homogenizer, and the suspension was centrifuged at 100,000 × *g* for 40 min at 4°. The resulting pellet was resuspended in TS-buffer and passed through a 27-gauge needle 30 times. Membrane vesicles were frozen and stored at −80° until use. Sidedness of membrane vesicles was assessed by measuring 5′-nucleotidase activity (Doige and Sharom, 1991), and it was determined that ~65% of the vesicles were orientated inside-out.

Rabbit liver and kidney were excised, and renal proximal tubular cells were isolated by immunodissection as described previously (Rose *et al*., 1993). Crude membrane fractions were isolated as described previously (Marples *et al*., 1995). Briefly, liver, kidney, and renal proximal tubular cells were homogenized in buffer A (300 mM sucrose, 25 mM imidazole, 1 mM EDTA, pH 7.2) supplemented with protease inhibitors as described above. Homogenates were centrifuged at 500 × *g* for 15 min at 4°, followed by centrifugation of the supernatant at 200,000 × *g* for 60 min at 4°. Subsequently, the pellet was resuspended in buffer A. For all membrane preparations, protein concentration was determined using the BioRad protein assay (BioRad Laboratories, Veenendaal, The Netherlands).

Deglycosylation studies and immunoblot analysis. Crude membrane fractions from Sf9 cells infected with Mrp2-encoding baculovirus and from rabbit kidney were treated with PNGase F according to the manufacturer. Protein-equivalents (see figure legends) were solubilized in Laemmli's sample buffer supplemented with 100 mM dithiothreitol, heated for 10 min at 65°, subjected to SDS-polyacrylamide gel electrophoresis, and transferred to Hybond-C pure nitrocellulose membrane (Amersham, Buckinghamshire, UK) as described previously (Deen *et al*., 1996). Transfer of proteins was confirmed by the reversible staining of the membrane with Ponceau Red. Subsequently, the blot was blocked for 60 min with 5% nonfat dry milk powder in Tris-buffered saline supplemented with 0.3%

Tween-20 (TBS-T) and washed twice with TBS-T. To detect rabbit Mrp2 proteins, the membrane was incubated overnight at 4° with antiserum k78mrp2 or k51mrp2 diluted 1:5000 in TBS-T. After two times washing for 5 min with TBS-T, the blot was blocked for 30 min as described above. The blot was then washed twice with TBS-T and incubated at room temperature for 60 min with affinity-purified horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma Immunochemicals, St. Louis, MO) diluted 1:5000 in TBS-T. Finally, the blot was washed twice for 5 min with TBS-T and TBS, respectively. Proteins were visualized using enhanced chemiluminescence (Pierce, Rockford, IL).

Transport studies in membrane vesicles. Uptake of [^{3}H]LTC_4 into membrane vesicles was measured by using a rapid filtration technique (Leier *et al.*, 1994). Briefly, membrane vesicles (20 μg protein-equivalent) were rapidly thawed and incubated at 37° in the presence of 4 mM MgATP, 10 mM $MgCl_2$, 10 mM creatine phosphate, 100 μg/ml creatine kinase, and 3 nM [^{3}H]LTC_4 in a final volume of 120 μl of TS-buffer (10 mM Tris-HEPES, 250 mM sucrose, pH 7.4). At indicated times, 20-μl samples were taken from the reaction mixture, diluted in ice-cold TS-buffer and filtered through nitrocellulose filters (0.45-μm pore size, Schleicher & Schuell, Dassel, Germany) using a filtration device (Millipore, Bedford, MA). Filters were washed once with 5 ml of TS-buffer and dissolved in liquid scintillation fluid to determine the bound radioactivity. In control experiments, 4 mM MgATP was replaced by 4 mM 5′-AMP. Net ATP-dependent transport was calculated by subtracting values in the presence of 5′-AMP from those in the presence of ATP. Uptake of [^{3}H]$E_2$17βG at a final concentration of 50 nM was done similarly as described for [^{3}H]LTC_4, except that a 50 μg protein-equivalent of membrane vesicles was used.

Results

Sf9 insect cells were infected with recombinant baculovirus encoding rabbit Mrp2 or control baculovirus. Crude membranes were prepared and subjected to immunoblot analysis using antiserum k78mrp2 and k51mrp2. Both antisera detected a protein of ~180 kDa in membranes from cells infected with baculovirus encoding Mrp2 (Fig. 1A, Sf9-Mrp2) but not in membranes from cells infected with control baculovirus (Fig. 1A, Sf9-c). This size is smaller than cMoat/Mrp2 detected in liver and kidney, which has been reported to have a molecular weight of ~190 kDa (Paulusma *et al.*, 1996; Büchler *et al.*, 1996; Schaub *et al.*, 1997). To investigate whether this difference in molecular weight can be attributed to differences in post-translational modifications, crude membrane fractions from rabbit kidney and Sf9-Mrp2 cells were treated with or without PNGase F and analyzed by immunoblotting using antiserum k78mrp2 (Fig. 1B). Deglycosylation reduced the molecular weight of rabbit kidney Mrp2 from ~190 to 175 kDa, which is the size that can be deduced from the rabbit *mrp2* cDNA sequence (van Kuijck *et al.*, 1996, 1997). Treatment with PNGase F reduces the molecular mass of Mrp2 in Sf9 cells only slightly to 175 kDa. This indicates that in Sf9 cells, Mrp2 is less glycosylated than in rabbit kidney.

To determine the expression level of rabbit Mrp2 in Sf9 cells, we subjected crude membrane fractions from Sf9-Mrp2 cells and rabbit liver and rabbit renal proximal tubular cells to immunoblot analysis using antiserum k78mrp2 (Fig. 2). A 1 μg protein-equivalent of crude membranes from Sf9-Mrp2 cells was sufficient to detect Mrp2. Approximately 20 μg protein-equivalent of crude membranes from liver and renal proximal tubules was needed to detect a similar amount of Mrp2 protein as present in 4 μg protein-equivalent of Sf9-Mrp2 crude membranes.

To investigate whether recombinant rabbit Mrp2 is functional, we investigated uptake of [^{3}H]LTC_4 and [^{3}H]$E_2$17βG into Sf9-Mrp2 and Sf9-c membrane vesicles. Sf9-Mrp2 membrane vesicles exhibit net ATP-dependent uptake of both [^{3}H]LTC_4 (Fig. 3, *left*) and [^{3}H]$E_2$17βG (Fig. 3, *right*), which was at the 2-min time point ~11-fold higher than in Sf9-c membrane vesicles. In the presence of 5′-AMP, transport of either substrate was hardly detectable in Sf9-Mrp2 membrane vesicles and was similar to that in Sf9-c membrane vesicles in the presence of 5′-AMP or ATP (not shown). Initial rates of uptake for 3 nM [^{3}H]LTC_4 and 50 nM [^{3}H]$E_2$17βG were 75 and 450 fmol/mg/min, respectively.

To confirm that vesicle-associated increase of ligand reflects transport into a vesicular space rather than aspecific binding to the membrane, the medium osmolarity dependence of [^{3}H]$E_2$17βG uptake was investigated. By increasing the extravesicular sucrose concentration from 250 mM (isotonic condition) to 1000 mM, membrane vesicle space will shrink resulting in decreased uptake. As shown in Fig. 4A,

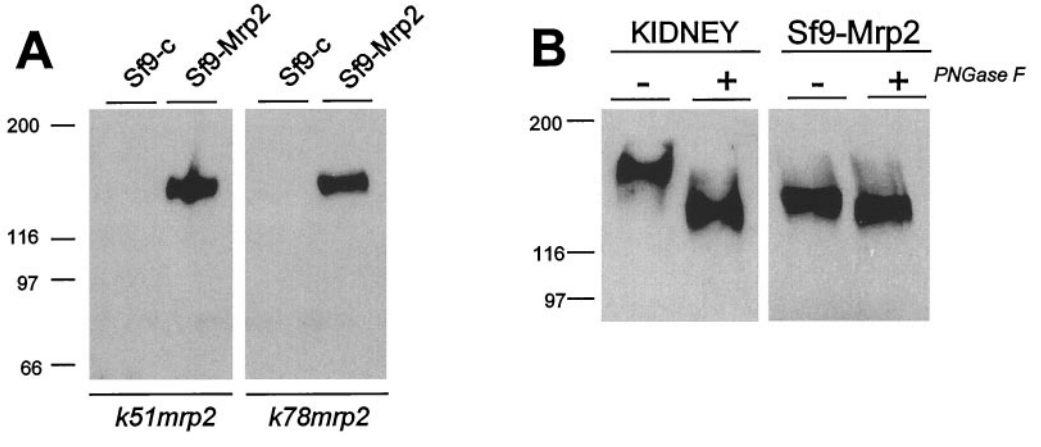

Fig. 1. Immunoblot analysis of Sf9 cells infected with recombinant baculovirus encoding Mrp2. A, Crude membrane fractions were isolated from Sf9 cells infected with a control baculovirus (*Sf9-c*) or recombinant baculovirus encoding Mrp2 (*Sf9-Mrp2*). Proteins (5 μg) were separated on a 6% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and incubated with Mrp2 polyclonal antiserum k78mrp2 or k51mrp2. Proteins were visualized using enhanced chemiluminescence. Sizes of protein standards are indicated in kilodaltons. B, Protein-equivalents of crude membrane fractions from rabbit kidney (20 μg) and from Sf9 cells infected with recombinant baculovirus encoding Mrp2 (5 μg) were treated without (−) or with (+) PNGase F and subjected to immunoblot analysis using antiserum k78mrp2 as described in A.

initial rates of [^{3}H]$E_2$17βG uptake in Sf9-Mrp2 membrane vesicles decreased linearly with increasing concentrations of sucrose. Transport in Sf9-Mrp2 membrane vesicles should also be dependent on the extravesicular concentration of ATP. Fig. 4B shows that initial rates of [^{3}H]$E_2$17βG uptake increased with ATP concentrations according to Michaelis-Menten kinetics, yielding an apparent K_m value of 623 ± 131 μM and V_{max} value of 563 ± 32 fmol/mg/min.

To characterize the inhibitor specificity of rabbit Mrp2, we studied the effect of various compounds on [^{3}H]$E_2$17βG uptake by Sf9-Mrp2 membrane vesicles (Table 1). Phenolphthalein glucuronide exerted a profound inhibition, whereas the other two glucuronides (α-naphthyl-β-D-glucuronide, p-nitrophenyl-β-D-glucuronide) and FL, a substrate of the classic organic anion transport system (Sullivan *et al.*, 1990), did not inhibit transport up to 1 mM. Uptake was also susceptible to inhibition by LTC_4, MTX, and FL-MTX. Furthermore, we tested the LTD_4-receptor antagonist MK571 (Jones *et al.*, 1989) and the immunosuppressive agent CsA, both of which are inhibitors of human MRP1 and rat Mrp2 (Leier *et al.*,

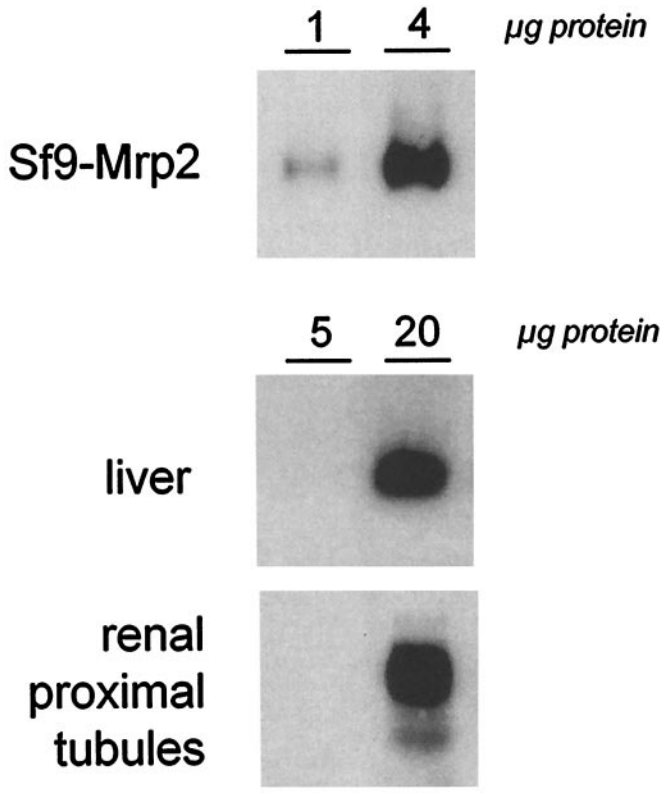

Fig. 2. Comparison of Mrp2 protein levels in Sf9 cells and liver and renal proximal tubules. Protein-equivalents of crude membrane fractions isolated from Sf9-Mrp2 cells (1, 4 μg), rabbit liver (5, 20 μg), and rabbit renal proximal tubules (5, 20 μg) were separated on a 7.5% SDS-polyacrylamide gel and subjected to immunoblot analysis using antiserum k78mrp2. Proteins were visualized using enhanced chemiluminescence.

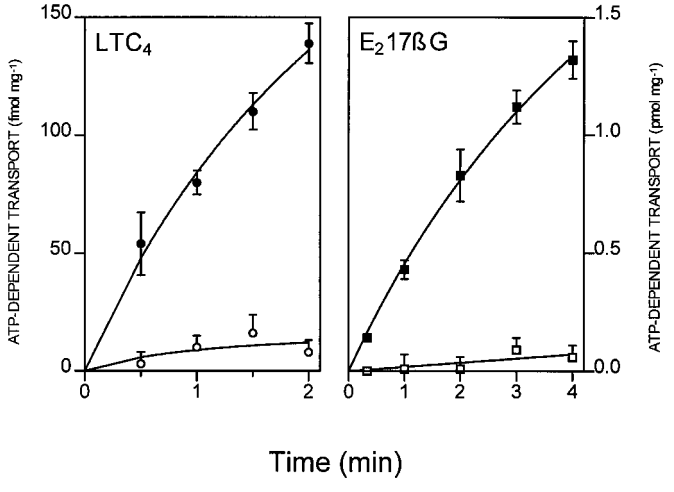

Fig. 3. Time course of net ATP-dependent uptake of [^{3}H]LTC_4 and [^{3}H]$E_2$17βG in Sf9-Mrp2 and Sf9-c membrane vesicles. Membrane vesicles from Sf9-c (○, □) and Sf9-Mrp2 (●, ■) were incubated in TS-buffer (10 mM Tris·HCl, 250 mM sucrose, pH 7.4) at 37° for the times indicated. Uptake was determined for [^{3}H]LTC_4 (*left*) and [^{3}H]$E_2$17βG (*right*) at final concentrations of 3 and 50 nM, respectively. Net ATP-dependent transport was calculated by subtracting values in the presence of 4 mM 5′-AMP from those in the presence of 4 mM ATP. Data points represent the mean ± standard error of three or four determinations in a typical experiment.

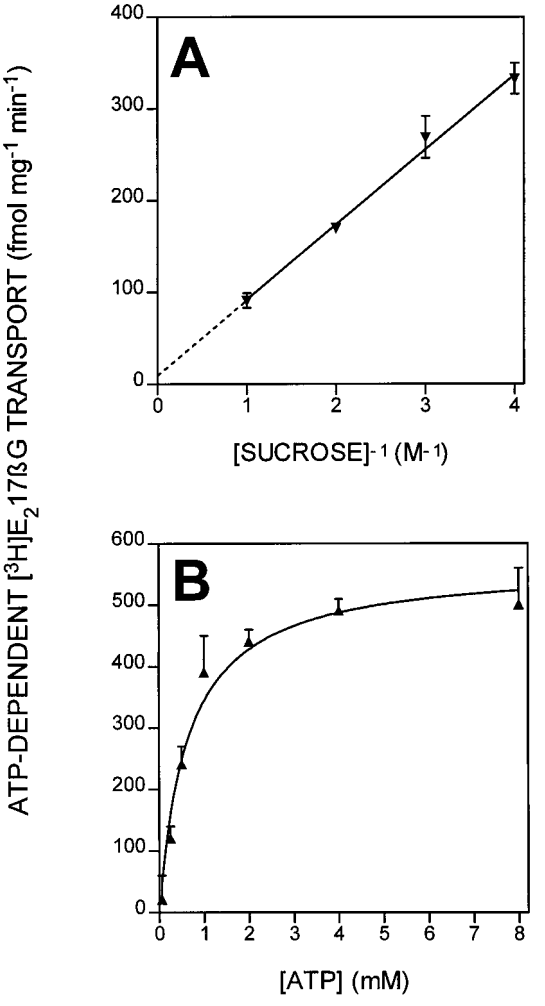

Fig. 4. Osmolarity dependence and effect of ATP concentration on [^{3}H]$E_2$17βG uptake in Sf9-Mrp2 membrane vesicles. Membrane vesicles from Sf9-Mrp2 cells were incubated with 50 nM [^{3}H]$E_2$17βG at 37° for 1 min. A, ATP-dependent transport was determined in the presence of sucrose concentrations ranging from 250 mM (isotonic condition) to 1000 mM. Initial rates of ATP-dependent [^{3}H]$E_2$17βG uptake were plotted against the inverse sucrose concentration in the reaction mixture. B, ATP-dependent transport was determined at various concentrations of ATP (60–8000 μM). The graph was plotted by fitting the obtained data according to the Michaelis-Menten equation using Prism (GraphPAD, San Diego, CA). Data points in all cases represent the mean ± standard error of three determinations in a typical experiment.

1994; Büchler *et al.*, 1996) and proved to be inhibitors of rabbit Mrp2.

Discussion

Mrp2 mediates ATP-dependent elimination of conjugated organic anions from liver and has recently been cloned from rat (Büchler *et al.*, 1996; Paulusma *et al.*, 1996; Ito *et al.*, 1997; Madon *et al.*, 1997) and human (Taniguchi *et al.*, 1996; Paulusma *et al.*, 1997). We cloned a rabbit cDNA encoding an ABC-transporter that on basis of similar tissue distribution and highest amino acid homology was considered as the rabbit homolog of rat Mrp2 (van Kuijck *et al.*, 1996, 1997). On injection of its cRNAs in *Xenopus laevis* oocytes, we observed in a few cases a cAMP-dependent chloride conductance (van Kuijck *et al.*, 1996). The recent finding that substrates of Mrp2 activate a chloride conductance in hepatocytes of normal rats but not in TR^- hepatocytes (Weinman and Carruth, 1997) indicates that this phenomenon warrants further investigation.

To investigate Mrp2-mediated transport, we expressed rabbit Mrp2 in Sf9 cells using recombinant baculovirus. In these cells, Mrp2 is highly expressed, although less glycosylated compared with kidney Mrp2. This is in line with results from other studies in which CFTR and MRP1 were expressed in insect cells and detected as an underglycosylated product (Kartner *et al.*, 1991; Gao *et al.*, 1996). Results of functional studies on MRP1- and CFTR-expressing insect cells are comparable to those obtained from transfected eukaryotic cells, indicating that underglycosylation has no significant effect on its function (Kartner *et al.*, 1991; Gao *et al.*, 1996). This was further corroborated by inhibition of glycosylation with tunicamycin in drug-resistant MRP1-expressing human cells (Bakos *et al.*, 1996) and, on basis of our studies, can also be concluded for Mrp2.

Based on studies with intact rats and liver canalicular membranes, the conjugates $E_2$17βG and LTC_4 are considered to be substrates for rat Mrp2 (Büchler *et al.*, 1996; Takikawa *et al.*, 1996). In addition, ATP-dependent transport of LTC_4 has been demonstrated in membrane vesicles isolated from NIH/3T3 cells transfected with a rat *mrp2* cDNA, and LTC_4 efflux was found in Mrp2-expressing *Xenopus* oocytes and COS-7 cells (Madon *et al.*, 1997; Ito *et al.*, 1998). In the current study, we unambiguously demonstrated that rabbit Mrp2 mediates ATP-dependent uptake of both [^{3}H]$E_2$17βG and [^{3}H]LTC_4. The initial uptake rates for [^{3}H]LTC_4 and [^{3}H]$E_2$17βG, as well as the V_{max} value for ATP using [^{3}H]$E_2$17βG as a cosubstrate, are lower than the values described for rat canalicular membrane vesicles (Büchler *et al.*, 1996; Vore *et al.*, 1996) and membrane vesicles from *mrp2*-transfected NIH/3T3 cells (Ito *et al.*, 1998). This difference, however, may be explained by the substantially lower substrate concentrations that we used. Uptake of [^{3}H]$E_2$17βG in Sf9-Mrp2 membrane vesicles was inhibited by LTC_4 and phenolphthalein glucuronide. α-Naphthyl-β-D-glucuronide and *p*-nitrophenyl-β-D-glucuronide had no significant effect on uptake, although both compounds are thought to be Mrp2 substrates. ATP-dependent uptake of *p*-nitrophenyl-β-D-glucuronide into rat canalicular membrane vesicles has been described, whereas in TR^- rat livers, α-naphthyl-β-D-glucuronide excretion was impaired (de Vries *et al.*, 1989; Kobayashi *et al.*, 1991). These findings suggest that α-naphthyl-β-D-glucuronide and *p*-nitrophenyl-β-D-glucuronide are transported with low affinity by Mrp2 and consequently are poor competitive inhibitors themselves. MK571 and CsA are inhibitors of human MRP1 and rat Mrp2 (Leier *et al.*, 1994; Büchler *et al.*, 1996) and, as shown in this study, also inhibit rabbit Mrp2-mediated [^{3}H]$E_2$17βG transport. However, it remains to be elucidated whether these compounds are Mrp2 substrates.

Mrp2 is expressed not only in liver canalicular membranes but also in small intestine and brush-border membranes of renal proximal tubular cells (Büchler *et al.*, 1996; Paulusma *et al.*, 1996; Schaub *et al.*, 1997). However, the functional identification of an ATP-dependent organic anion transporter in membrane vesicles from renal proximal tubular cells, such as in liver canalicular membranes, has never been documented (Pritchard and Miller, 1993). This is mainly due to technical limitations because membrane vesicles of renal proximal tubular cells are exclusively orientated right-side out (Haase *et al.*, 1978). Recently, Masereeuw *et al.* (1996) identified an energy-dependent transport mechanism for organic anions in isolated renal proximal tubules from killifish using FL-MTX as a substrate. The excretory pathway of FL-MTX was characteristic for its sensitivity to LTC_4, MTX, CsA, and probenecid. In addition, the energy-dependency of this pathway was confirmed by treating cells with KCN, which did not influence FL-MTX uptake but completely abolished luminal excretion. This suggests that FL-MTX may be an Mrp2-substrate for which we provide evidence in this study because FL-MTX strongly inhibits [^{3}H]$E_2$17βG uptake in Sf9-Mrp2 membrane vesicles. In contrast, FL did not inhibit [^{3}H]$E_2$17βG uptake, whereas MTX was only partially inhibitory. It remains to be established whether Mrp2 directly mediates ATP-dependent uptake of FL-MTX.

Besides Mrp2, additional organic anion transporters might be present in brush-border membranes of renal proximal tubular cells. For example, excretion of FL-MTX was shown to be only partially inhibited by probenecid, whereas the probenecid-insensitive mechanism was inhibited completely by verapamil (Masereeuw *et al.*, 1996). In addition, it has been shown that TR^- rats have impaired hepatic excretion of the conjugates α-naphthyl-β-D-glucuronide and LTC_4, whereas urinary excretion is hardly affected (Huber *et al.*, 1987; de Vries *et al.*, 1989), suggesting that the deficiency of

TABLE 1

Effect of various compounds on Mrp2-mediated [^{3}H]$E_2$17βG transport

Sf9-Mrp2 membrane vesicles were incubated with 50 nM [^{3}H]$E_2$17βG at 37° for 1 min with or without (control) the compounds as indicated. Net ATP-dependent transport was calculated by subtracting values in the presence of 4 mM 5′-AMP from those in the presence of 4 mM ATP. Transport was expressed as percent of the control uptake. Data represent mean values of three to six determinations (± standard error).

Compound	Concentration	[^{3}H]$E_2$17βG uptake
	μM	% control
None (control)		100
LTC_4	10	22 ± 7[b]
α-Naphthyl-β-D-glucuronide	1000	94 ± 1
p-Nitrophenyl-β-D-glucuronide	1000	97 ± 5
Phenolphthalein glucuronide	1000	5 ± 4[b]
MK571	5	33 ± 3[a]
CsA	10	48 ± 1[a]
FL	1000	94 ± 4
MTX	1000	55 ± 4[a]
FL-MTX	100	22 ± 7[b]

[a] $p < 0.05$.
[b] $p < 0.01$ (analysis of variance with Bonferroni's correction).

Mrp2 in the kidney can be compensated for by other organic anion transporters. Possible candidates might be the organic anion transporters Oatp1 and Oat-k1, which are both localized in brush-border membranes of renal proximal tubular cells (Bergwerk *et al*., 1996; Masuda *et al*., 1997). Although Oatp1 and Oat-k1 are structurally not related to Mrp2, these proteins mediate transport of Mrp2-substrates, such as $E_2$17βG, LTC_4, and MTX (Kanai *et al*., 1996; Saito *et al*., 1996; Li *et al*., 1997). Furthermore, the recently identified family members of human MRP1 (i.e., MRP3, MRP4, and MRP5) are all expressed to some extent in the kidney and might also be involved in renal organic anion transport (Kool *et al*., 1997).

In conclusion, we demonstrated ATP-dependent transport by recombinant rabbit Mrp2 and provided new data on inhibitor specificity. In future studies, this expression system will be used for identification and characterization of Mrp2-substrates, with emphasis on compounds that are excreted by the kidney.

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