Steroid and bile acid conjugates are substrates of human multidrug-resistance protein (MRP) 4 (ATP-binding cassette C4)

Noam ZELCER*[1], Glen REID*[1], Peter WIELINGA*, Annemieke KUIL*, Ingrid VAN DER HEIJDEN*, John D. SCHUETZ† and Piet BORST*[2]

*Division of Molecular Biology and Center for Biomedical Genetics, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands, and †St. Jude Children's Research Hospital, 501 St. Jude Place, Memphis, TN 38105, U.S.A.

Human multidrug-resistance protein (MRP) 4 transports cyclic nucleotides and when overproduced in mammalian cells mediates resistance to some nucleoside analogues. Recently, it has been shown that *Mrp4* is induced in the livers of *Fxr*$^{(-/-)}$ mice, which have increased levels of serum bile acids. Since MRP4, like MRP1–3, also mediates transport of a model steroid conjugate substrate, oestradiol 17-β-D-glucuronide ($E_2$17βG), we tested whether MRP4 may be involved in the transport of steroid and bile acid conjugates. Bile salts, especially sulphated derivatives, and cholestatic oestrogens inhibited the MRP4-mediated transport of $E_2$17βG. Inhibition by oestradiol 3,17-disulphate and taurolithocholate 3-sulphate was competitive, suggesting that these compounds are MRP4 substrates. Furthermore, we found that MRP4 transports dehydroepiandrosterone 3-sulphate (DHEAS), the most abundant circulating steroid in humans, which is made in the adrenal gland. The ATP-dependent transport of DHEAS by MRP4 showed saturable kinetics with K_m and V_{max} values of 2 μM and 45 pmol/mg per min, respectively (at 27 °C). We further studied the possible involvement of other members of the MRP family of transporters in the transport of DHEAS. We found that MRP1 transports DHEAS in a glutathione-dependent manner and exhibits K_m and V_{max} values of 5 μM and 73 pmol/mg per min, respectively (at 27 °C). No transport of DHEAS was observed in membrane vesicles containing MRP2 or MRP3. Our findings suggest a physiological role for MRP1 and MRP4 in DHEAS transport and an involvement of MRP4 in transport of conjugated steroids and bile acids.

Key words: adrenal gland, bile acid, cholestasis, dehydroepiandrosterone 3-sulphate (DHEAS), multidrug-resistance protein (MRP), steroid.

INTRODUCTION

Members of the ATP-binding cassette (ABC) family of membrane pumps mediate the transport of various substrates across membranes at the expense of ATP hydrolysis [1,2]. The human ABCC subfamily contains nine members that are referred to as multidrug-resistance proteins (MRPs) 1–9, in addition to sulphonylurea receptors SUR1 and SUR2, and cystic fibrosis transmembrane conductance regulator ('CFTR') [3,4]. Initial interest in the MRP members of this subfamily was driven by their possible involvement in clinical drug resistance. MRP1 (ABCC1), the first member of this subfamily to be cloned, confers resistance to multiple and structurally unrelated anticancer drugs when overexpressed in cells [5]. It quickly became evident, however, that MRPs transport a wide variety of other organic anions and compounds that are conjugated to sulphate, glucuronide or GSH (reviewed in [4,6]). Mutations in MRP genes are the cause of two genetic diseases. Mutations in *MRP2* cause Dubin–Johnson syndrome (reviewed in [6]), whereas mutations in *MRP6* lead to pseudoxanthoma elasticum, a disorder of connective tissue (reviewed in [2]). Both examples emphasize the importance of understanding the role of MRPs in normal and aberrant physiology. A first step to this end is a proper characterization of the substrate specificity of each member of this family.

MRPs share an overlapping substrate specificity despite different patterns of tissue distribution, localization in polarized cells and protein size (reviewed in [6,7]). The antifolate drug methotrexate and the oestradiol metabolite oestradiol 17-β-D-glucuronide ($E_2$17βG), organic anions that are often used to study transport by MRPs, are transported by MRP1–4 with comparable affinities. In addition, MRP4 and its closest homologue MRP5 have a substrate specificity distinct from the other MRPs characterized to date in that they transport cAMP and cGMP [8,9]. This has led to the speculation that they might be involved in the regulation of the intracellular concentration of these important second messengers. Moreover, mammalian cells that overproduce either MRP4 or MRP5 are resistant to nucleoside analogue drugs such as 9-(2-phosphonylmethoxyethyl) adenine ('PMEA') [8–14]. Unexpectedly, it was recently shown that *Mrp4* is induced in livers of *Fxr*$^{(-/-)}$ mice [15]. Absence of the nuclear receptor FXR leads to elevated levels of bile acids in the livers and serum of these mice due to the reduction in the expression of the major canalicular bile salt pump (*Spgp*/*Bsep*) [16]. This finding provides a possible link between bile acid homoeostasis and MRP4 and suggests that cholestatic bile acids or structurally related compounds (e.g. steroids) might be endogenous substrates of MRP4.

Using vesicular transport assays with membrane vesicles containing MRP4, we found high-affinity transport of dehydroepiandrosterone 3-sulphate (DHEAS), the major circulating steroid in humans, which is made in the adrenal gland. Both cholestatic bile acids and steroids competitively inhibited the MRP4-mediated transport of $E_2$17βG and DHEAS. We also found that like MRP4, MRP1 transports DHEAS with high affinity, but in a glutathione-dependent manner. In contrast, no

Abbreviations used: ABC, ATP-binding cassette; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone 3-sulphate; $E_2$17βG, oestradiol 17-β-D-glucuronide; MRP, multidrug-resistance protein; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; OATP, organic anion-transporting polypeptide.

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed (e-mail p.borst@nki.nl).

transport of DHEAS by MRP2 or MRP3 was found. MRP1 and MRP4 may represent the as yet uncharacterized transporters that release DHEAS from the adrenal gland. Our results extend the substrate specificity of MRP4 and suggest a physiological role for MRP4 in the transport of conjugated steroids and bile acids.

EXPERIMENTAL

Materials

[^{3}H]$E_2$17βG (40.5 Ci/mmol) and [^{3}H]DHEAS (60 Ci/mmol) were purchased from NEN Life Science Products (Boston, MA, U.S.A.). Creatine phosphate and creatine kinase were obtained from Boehringer Mannheim (Almere, The Netherlands) and RC-L55 and ME25 filters were from Schleicher and Schuell (Dassel, Germany). All other chemicals and reagents were purchased from Sigma (St. Louis, MO, U.S.A.).

Cell lines and culture conditions

The transfection of HEK-293 cells with a MRP4 cDNA construct and generation of clones overproducing MRP4 have been reported previously [17]. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 100 units/ml penicillin/streptomycin. Cells were grown at 37 °C with 5% CO_2 under humidifying conditions. The generation of baculovirus containing MRP1 and MRP3 cDNA constructs was reported in Zelcer et al. [18]. The baculovirus containing MRP2 cDNA was a gift from Balasz Sarkadi (Institute of Enzymology, Budapest, Hungary) [19] and that containing MRP4 cDNA was from Remon van Aubel (Nijmegen Center for Molecular Life Sciences, Nijmegen, The Netherlands) [13].

Protein analysis and immunohistochemistry

Total cell lysates from cell lines and membrane vesicle preparations were prepared in a hypotonic buffer (10 mM KCl, 1.5 mM $MgCl_2$ and 10 mM Tris/HCl, pH 7.4), supplemented with a cocktail of protease inhibitors used at the concentration recommended by the manufacturer (Roche, Mannheim, Germany). Normal human tissues collected post-mortem were obtained from the tissue bank of the Netherlands Cancer Institute (Amsterdam, The Netherlands) and after homogenization were processed as above. The indicated amount of protein was separated on a SDS/polyacrylamide (7.5%) gel and subsequently blotted overnight in a tank blotting system. MRP3 was detected with the monoclonal antibody M_3II9 (1:250) followed by a rabbit anti-mouse horseradish peroxidase conjugate (1:1000), as described previously [18]. MRP1 and MRP4 were detected with monoclonal antibodies MRP1-R1 (1:1000) and NKI-12C4 (1:10), respectively, followed by a rabbit anti-rat horseradish peroxidase conjugate (1:1000). The monoclonal antibody NKI-12C4 does not cross-react with MRP1–3 or MRP5 [17]. Signals were visualized by enhanced chemiluminescence (ECL®; Amersham Biosciences, Little Chalfont, Bucks., U.K.).

Preparation of membrane vesicles

The HEK-293 control cells and HEK-293/4.63 cells that overproduce MRP4 were grown as described above, and membrane vesicles were prepared as described below. Membrane vesicles from *Spodoptera frugiperda* (Sf9) cells were obtained after infection with an MRP1, MRP2, MRP3 or MRP4 cDNA-containing baculovirus at a multiplicity of infection of 1. After incubation at 27 °C for 3 days, cells were harvested by centrifugation at 500 ***g*** for 5 min. The pellet was resuspended in ice-cold hypotonic buffer (0.5 mM sodium phosphate/0.1 mM EDTA, pH 7.4) supplemented with protease inhibitors (2 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin and 10 μM pepstatin) and incubated at 4 °C for 90 min. The suspension was centrifuged at 100000 ***g*** for 40 min at 4 °C and the pellet was homogenized in ice-cold TS buffer (50 mM Tris/HCl, 250 mM sucrose, pH 7.4) using a tight-fitting Dounce homogenizer. After centrifugation at 500 ***g*** at 4 °C for 10 min, the supernatant was centrifuged at 100000 ***g*** for 40 min at 4 °C. The pellet was resuspended in TS buffer and passed 25 times through a 27-gauge needle. The vesicles were dispensed in aliquots, frozen in liquid nitrogen and stored at −80 °C until use.

Vesicular transport assays

The time- and concentration-dependent uptake of various substrates into membrane vesicles was studied following the rapid filtration method as described previously [18]. Concentration-dependent uptake was analysed using a non-linear regression algorithm.

RESULTS

MRP4 is competitively inhibited by conjugated steroids and bile acids

To study the substrate specificity of MRP4 we used membrane vesicles from HEK-293 cells that overexpress *MRP4* and Sf9 insect cells infected with an MRP4 cDNA-containing baculovirus (Figure 1, inset). We found a clear ATP- and time-dependent transport of $E_2$17βG in membranes containing MRP4 (results not shown), a previously characterized substrate of MRP4 [9,13]. In the livers of *Fxr*$^{(-/-)}$ mice, which display 8-fold-increased serum bile acid levels as a result of a decrease in the expression

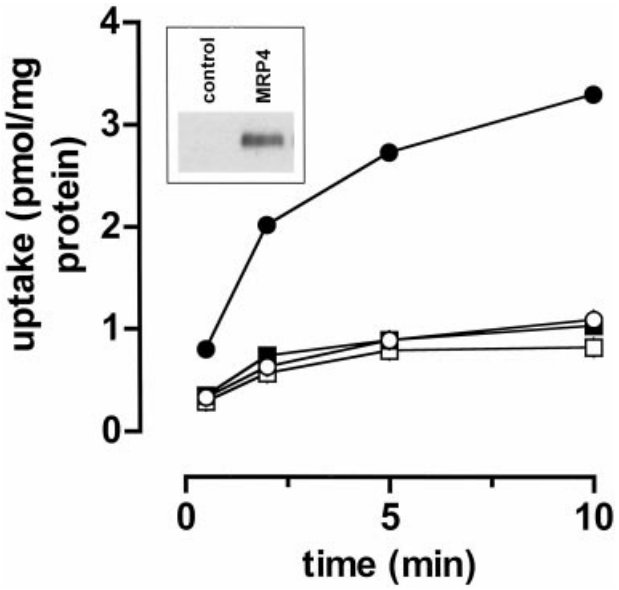

Figure 1 Time- and ATP-dependent transport of DHEAS by MRP4

Membrane vesicles containing MRP4 (●, ○) or control membranes (■, □) from Sf9 insect cells were incubated at 27 °C with 25 nM DHEAS in the presence of 4 mM ATP (●, ■) or AMP (○, □). Results are the means ± S.D. from experiments performed in triplicate. Inset: Western blot showing MRP4 in Sf9 membrane vesicles. Control Sf9 (4 μg/per lane) or Sf9-MRP4 (0.5 μg/per lane) membrane vesicles were loaded. MRP4 was detected using monoclonal antibody NKI-12C4 [17].

Table 1 Effect of bile acids on MRP4-mediated $E_2 17\beta G$ transport

Membrane vesicles prepared from HEK-293 cells overexpressing *MRP4* were incubated at 37 °C for 10 min with 30 μM [^{3}H]$E_2 17\beta G$ in the presence of bile acids. Inhibition of the ATP-dependent uptake was measured in duplicate ($n = 2$). An approximate IC_{50} is presented. The approximate IC_{50} values were extrapolated from determinations performed at three different concentrations spanning the IC_{50} range. The complete data set for Table 1 can be obtained from the corresponding author (P.B.) upon request.

Inhibitor	Approx. IC_{50} (μM)
Cholate	250
Glycocholate	400
Taurocholate	350
Taurodeoxycholate	60
Taurochenodeoxycholate	55
Taurolithocholate	20
Taurolithocholic acid sulphate	10
Glycolithocholic acid sulphate	10
Lithocholic acid sulphate	10

Table 2 Effect of conjugated steroids on MRP4-mediated $E_2 17\beta G$ transport

Membrane vesicles prepared from Sf9 insect cells overexpressing *MRP4* were incubated at 37 °C for 5 min with 1 μM [^{3}H]$E_2 17\beta G$ in the presence of conjugated steroids. Inhibition of the ATP-dependent uptake was measured in duplicate ($n = 2$ or 3). The approximate IC_{50} values were extrapolated from determinations performed at three different concentrations spanning the IC_{50} range. The complete data set for Table 2 can be obtained from the corresponding author (P.B.) upon request.

Inhibitor	Approx. IC_{50} (μM)
DHEAS	3
DHEA 3-glucuronide	80
Oestrone 3-sulphate	45
Oestradiol 3-sulphate	50
Oestradiol 3-glucuronide	120
Oestradiol 3,17-disulphate	2

of the main canalicular bile-salt-efflux pump (*Bsep*/*Spgp*), *Mrp4* is induced [15,16]. This might suggest that MRP4 is involved in the removal of cholestatic bile acids from the liver across the sinusoidal membrane, where it is localized, into the circulation (J. D. Schuetz, unpublished work). Bile acids, like the steroid $E_2 17\beta G$, contain a cholesterol backbone structure and may thus represent physiological substrates of MRP4. We tested this hypothesis by studying the capacity of non-radioactive compounds to inhibit MRP4-mediated $E_2 17\beta G$ transport. The results are summarized in Table 1. Sulphated bile acids inhibited the MRP4-mediated transport of $E_2 17\beta G$ at a low concentration (IC_{50}, approx. 10 μM). Much higher concentrations were required to achieve 50 % inhibition with the other bile acids used.

We also tested conjugated steroids, another class of compounds that share the cholesterol backbone structure, for their ability to inhibit the MRP4-mediated $E_2 17\beta G$ transport (Table 2). Conjugated steroids inhibited the MRP4-mediated transport to different degrees. The inhibition of MRP4-mediated $E_2 17\beta G$ transport by both taurolithocolic acid 3-sulphate and oestradiol 3,17-disulphate (at concentrations of 10 and 1 μM, respectively) was competitive, as at an increased substrate ($E_2 17\beta G$) concentration the inhibition could be overcome; both compounds inhibited MRP4-mediated transport of 5 μM $E_2 17\beta G$ by approx. 50 %, whereas at a concentration of 50 μM $E_2 17\beta G$ no inhibition was seen.

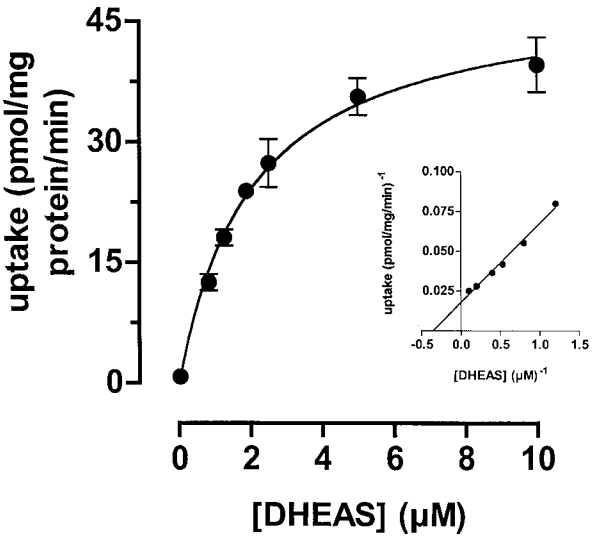

Figure 2 Concentration dependence of DHEAS transport by MRP4

Membrane vesicles from Sf9 insect cells infected with a MRP4 or with a wild-type baculovirus were incubated for 1 min at 27 °C with 25 nM [^{3}H]DHEAS and unlabelled DHEAS to the final concentrations shown in the figure. The ATP-dependent transport by MRP4 was calculated by subtracting the transport of DHEAS in the presence of 4 mM AMP from that in the presence of 4 mM ATP in the control vesicles from that in the Sf9-MRP4 vesicles. Each point is the mean ± S.D. from an experiment performed in triplicate. Inset: Lineweaver–Burk transformation of the data.

Table 3 Summary of kinetic parameters for MRP4-mediated transport

Substrate	K_m (μM)	V_{max} (pmol/mg per min)	V_{max}/K_m	Reference
$E_2 17\beta G$	30	102	3.4	[9]
cGMP	10	2	0.21	[9]
cAMP	45	4	0.09	[9]
Methotrexate	1300	430	0.33	[13]
Methotrexate	220	240	1.1	[14]
Folic acid	170	680	4.0	[14]
Leucovorin	640	1950	3.0	[14]
DHEAS*	2	45	22	Present study

* Kinetic values determined at 27 °C.

Transport of DHEAS by MRP4

Interestingly, MRP4-mediated transport of $E_2 17\beta G$ was inhibited by low concentrations of DHEAS. Synthesis of DHEAS occurs within cells of the zona reticularis in the adrenal gland. Its release into the circulation is mediated by as yet unidentified transporter(s). In membrane vesicles from Sf9 insect cells we found that MRP4 transported DHEAS at a concentration of 25 nM in a time- and ATP-dependent manner with a rate of 0.8 pmol/mg per min (Figure 1). Similar transport of DHEAS was also observed in membrane vesicles prepared from HEK-293/MRP4 cells (results not shown). At 37 °C the reaction rate for MRP4-mediated transport of DHEAS in membrane vesicles from Sf9 insect cells was too fast to enable the reliable determination of initial rates of transport and to overcome this problem we carried out all reactions at 27 °C. The transport of DHEAS by MRP4 was decreased by increasing the sucrose concentration in the transport buffer, indicating that DHEAS is transported into an osmotically sensitive compartment (results

Table 4 Effect of conjugated steroids on MRP4-mediated DHEAS transport

Membrane vesicles prepared from Sf9 insect cells overexpressing *MRP4* were incubated at 27 °C for 2 min with 25 nM [^{3}H]DHEAS in the presence of conjugated steroids. Inhibition of the ATP-dependent uptake was measured in duplicate ($n = 2$ or 3). An approximate IC_{50} is presented. The approximate IC_{50} values were extrapolated from determinations performed at three different concentrations spanning the IC_{50} range. The complete data set for Table 4 can be obtained from the corresponding author (P.B.) upon request.

Inhibitor	Approx. IC_{50} (μM)
$E_2$17βG	30
DHEA 3-glucuronide	80
Oestrone 3-sulphate	95
Oestradiol 3-sulphate	70
Oestradiol 3-glucuronide	80
Oestradiol 3,17-disulphate	0.2

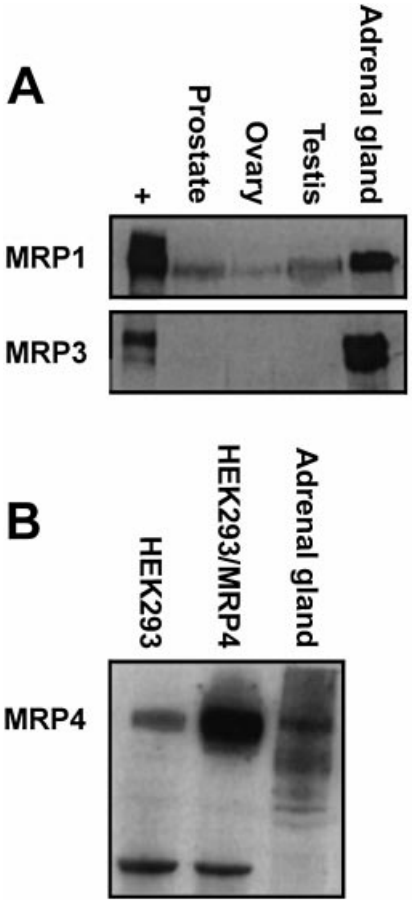

Figure 3 Detection of MRPs in human adrenal gland

(**A**) Each lane was loaded with 50 μg of total tissue protein. MRP1 and MRP3 were detected with specific monoclonal antibodies as described in the Experimental section. Total cellular protein (10–20 μg) from cells stably expressing MRP1 or MRP3 was used as a positive control (+). (**B**) 20 μg of total cellular HEK-293, HEK-293/MRP4 or 30 μg of a normal adrenal gland collected post-mortem were loaded per lane. MRP4 was detected as described in the Experimental section.

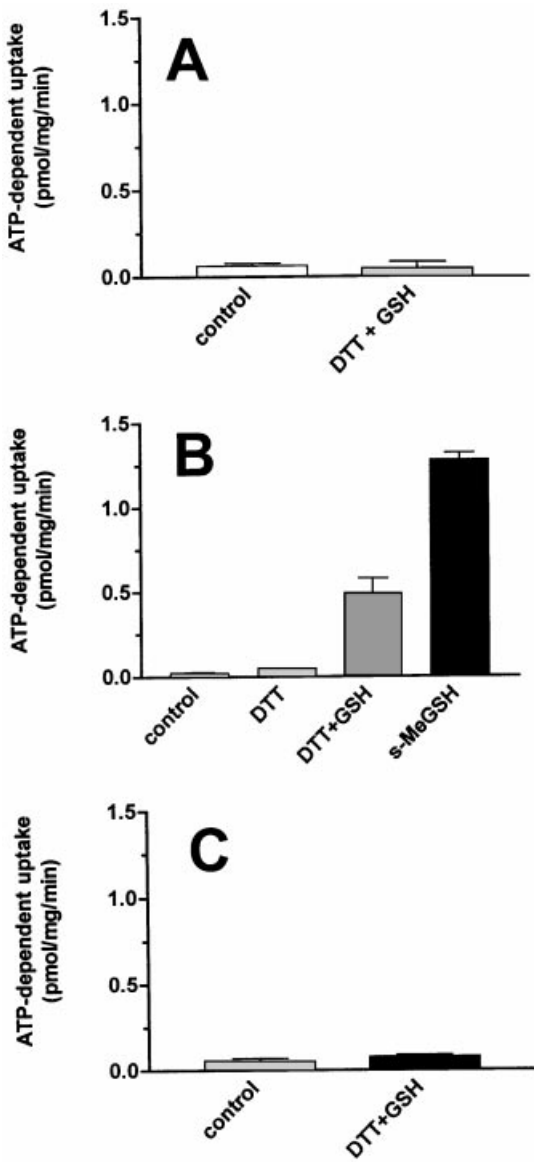

Figure 4 Transport of DHEAS by MRP1 and MRP2

Membrane vesicles from Sf9 insect cells infected with a wild-type (**A**), MRP1 (**B**) or MRP2 (**C**) baculovirus were incubated with 25 nM DHEAS at 27 °C for 1 min in the presence of the reducing agents dithiothreitol (DTT; 10 mM) or GSH (1 mM), or with the GSH derivative *S*-methyl-GSH (s-MeGSH; 1 mM). The control shows the uptake in the presence of 4 mM ATP alone. The ATP-dependent transport was calculated by subtracting the transport in the presence of 4 mM AMP from that in the presence of ATP. Each bar represents the mean $\pm$ S.D. from three experiments done in duplicate.

not shown). Non-linear regression analysis yielded apparent K_m and V_{max} values of 1.9 ± 0.2 μM and 45 ± 6 pmol/mg per min, respectively (Figure 2). Both the affinity and the catalytic efficiency (V_{max}/K_m) for DHEAS are the highest reported for any MRP4 substrate to date (Table 3). As found with $E_2$17βG, steroid analogues inhibited the MRP4-mediated DHEAS transport to varying degrees (Table 4). In contrast to a recent report by Lai and Tan [20], addition of 1 mM GSH had no effect on MRP4-mediated transport of DHEAS, whereas we find that an equivalent concentration of *S*-methyl-GSH inhibited transport by approx. 40 % (results not shown). Similarly, GSH at concentrations of up to 10 mM had no effect on transport of $E_2$17βG by MRP4 (results not shown). This suggests that any requirement for GSH is not a general feature of MRP4-mediated transport.

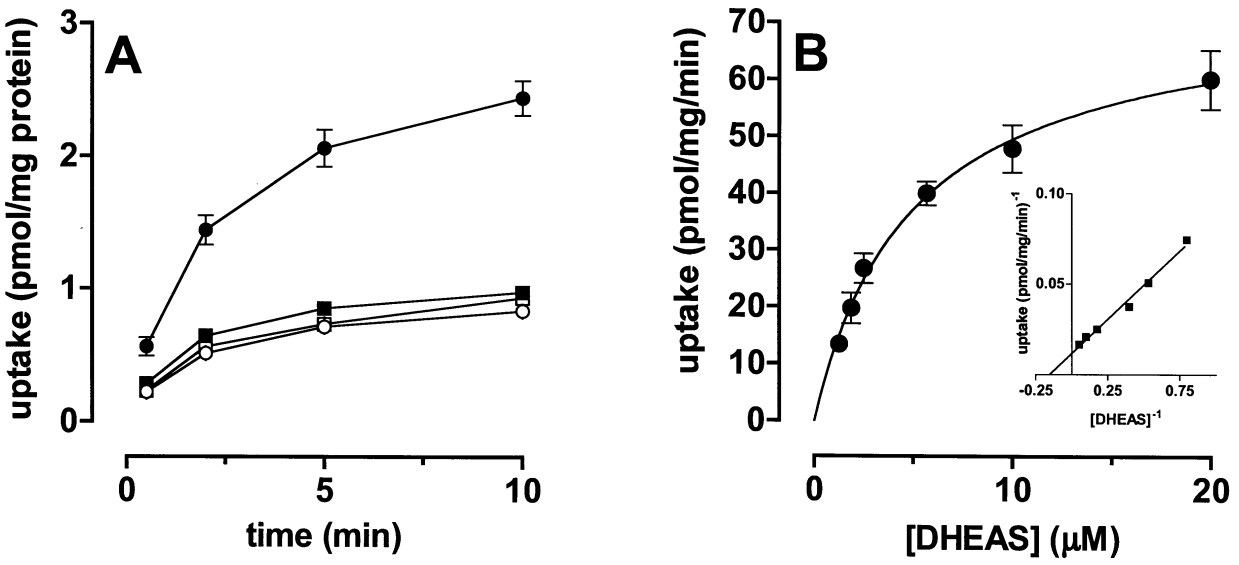

Figure 5 Characterization of DHEAS transport by MRP1

(**A**) Membrane vesicles from Sf9 insect cells infected with a MRP1 (●, ○) or a wild-type (■, □) baculovirus were incubated with 25 nM DHEAS at 27 °C in the presence of dithiothreitol (10 mM) and GSH (1 mM) and either 4 mM ATP (●, ■) or AMP (○, □). Results are the means ± S.D. from experiments performed in triplicate. (**B**) Membrane vesicles from Sf9 insect cells infected with a MRP1 baculovirus or with a wild-type baculovirus were incubated for 1 min at 27 °C with 25 nM [^{3}H]DHEAS and unlabelled DHEAS to the final concentrations shown in the figure in the presence of dithiothreitol (10 mM) and GSH (1 mM). The ATP-dependent transport by MRP1 was calculated as described in Figure 2. Each point is the mean ± S.D. from an experiment done in triplicate. Inset: Lineweaver–Burk transformation of the data.

Transport of DHEAS by MRP1–3

In the adrenal gland MRP1 and MRP3 were readily detected, as were low levels of MRP4 (Figure 3). However, with immunohistochemistry we have been unable to localize MRP4 in the adrenal gland with the antibodies available so far. In contrast, MRP1 has been localized to the adrenal cortex [21] and MRP3 has been localized to cells of the zona reticularis [22], the site of DHEAS synthesis and release.

To test whether MRP1–3, like MRP4, can transport DHEAS, we used vesicular transport assays with membrane vesicles from Sf9 insect cells that had been infected with a baculovirus containing the cDNA of these transporters. With Sf9 membrane vesicles containing MRP1 we observed transport of DHEAS only in the presence of GSH or the non-reducible GSH analogue *S*-methyl-GSH (Figure 4B), similar to transport of other substrates by this transporter [23,24]. We further characterized the time- and ATP-dependent transport of DHEAS by MRP1 (Figure 5A) and found saturation kinetics with apparent K_m and V_{max} values of 5 ± 1 μM and 73 ± 6 pmol/mg per min, respectively (Figure 5B).

The transport rate of DHEAS in Sf9 membranes containing MRP2 (Figure 4C) was not higher than that found with wild-type Sf9 membranes (Figure 4A). No MRP2-mediated transport was observed at 10 μM DHEAS (results not shown). Furthermore, adding GSH to the reaction did not result in transport of DHEAS by MRP2. These results are not the consequence of low MRP2 activity in these vesicles, as $E_2 17\beta G$ was transported at rates comparable with those observed with MRP1 and MRP3 vesicles (results not shown). We conclude that DHEAS is not recognized as a substrate by MRP2. Similarly, although the expression of *MRP3* in the adrenal cortex is high [22], we did not detect any transport of DHEAS by MRP3. Additionally, transport of etoposide glucuronide, a high-affinity substrate of MRP3 [18], was not inhibited by DHEAS concentrations up to 50 μM (results not shown).

DISCUSSION

We used vesicular transport assays to further delineate the substrate specificity of human MRP4. Previous studies reported MRP4 to be a high-affinity transporter of cGMP and cAMP (K_m values of 10 and 45 μM, respectively), similar to that found for its closest homologue, MRP5 [8,9]. However, unlike MRP5, MRP4 also transports $E_2 17\beta G$ and methotrexate [9,13,14], substrates that it shares with MRP1–3. Our results indicate that the substrate specificity of MRP4 is even broader and that DHEAS is the highest-affinity substrate (K_m, 2 μM) reported for MRP4 to date, suggesting that a major function of MRP4 may be the transport of DHEAS and/or structurally related steroids. Several lines of evidence support this idea. (i) MRP4 transports the two structurally related steroids, $E_2 17\beta G$ and DHEAS, the latter with high affinity. (ii) MRP4 is found in several tissues important in steroid metabolism [25]. (iii) Transport of DHEAS and $E_2 17\beta G$ by MRP4 is inhibited to various degrees by several steroid derivatives in a competitive manner, suggesting that these derivatives are also MRP4 substrates. The recent report by Schuetz et al. [15] that *Mrp4* is induced in the livers of *Fxr*$^{(-/-)}$ mice suggests yet another intriguing role for MRP4. These mice lack the nuclear hormone receptor FXR, which is a key regulator of bile acid homoeostasis, and display, among many other abnormalities, elevated bile acid levels in their serum as a result of diminished expression of the major canalicular bile-salt-efflux pump, *Bsep* [16]. In addition to increased levels of MRP4 in the basolateral membranes of hepatocytes, two mouse ESTs homologous to human dehydroepiandrosterone (DHEA)-sulphotransferase, which is capable of sulphating bile acids, are also up-regulated in *Fxr*$^{(-/-)}$ mice (J. D. Schuetz, unpublished work). This combination of increased MRP4 and sulphotransferases may serve as an escape route for toxic bile acids and organic anions under conditions where their hepatic concentration is elevated, similar to the proposed role of MRP3 in the TR$^-$ rats and in humans suffering from Dubin–Johnson

syndrome [26–29]. Sulphated bile acids, which are increased in cholestatic liver conditions [30], inhibited MRP4-mediated oestradiol glucuronide transport at low concentrations (IC_{50}, approx. 10 μM) in a competitive manner, suggesting that they represent substrates of MRP4. Monovalent bile acids, on the other hand, were poor inhibitors. As sulphation of bile acids and their urinary excretion is increased under cholestatic conditions, the basolateral localization of MRP4 in hepatocytes (J. D. Schuetz, unpublished work) and the apical localization in proximal tubule cells of the kidney [13] offer a possible pathway for their elimination. Moreover, bile acids induce the expression of DHEA-sulphotransferase via FXR, as well as being substrates for conjugation by this enzyme [31,32]. The sulphated bile acids that are formed via this pathway might be subsequently transported out of the liver by MRP4. Clearly, the potential role of MRP4 in mitigating cholestatic liver disease could be important, but must be verified in further studies.

The inhibition of the MRP4-mediated $E_2 17\beta G$ and DHEAS transport (Tables 1, 2 and 4) reveals that the structural requirements for recognition by MRP4 are not straightforward. A glucuronide or sulphate group at position 3 of the A ring of the steroidal backbone (as is found in DHEAS) is not sufficient to result in substantial inhibition at a low concentration, as is evident from results obtained with DHEA 3-glucuronide and oestradiol 3-sulphate. Interestingly, an oestradiol derivative with sulphate groups in positions 3 and 17 is a very potent inhibitor of MRP4-mediated DHEAS transport (IC_{50}, approx. 1 μM). It is not clear at present if this is the result of the charge distribution or of an optimal structure for recognition. Moreover, if MRP4 has a simple substrate binding site in which all substrates or inhibitors fit in a similar fashion, one would expect that a given competitor concentration would inhibit transport of DHEAS less than that of $E_2 17\beta G$, as DHEAS has the higher affinity for MRP4. Not all putative MRP4 substrates behave in this fashion, however, as shown in Tables 2 and 4. Oestradiol 3,17-disulphate, for instance, inhibits DHEAS transport more than $E_2 17\beta G$ at all concentrations tested. This may reflect a complexity in the substrate-binding pockets of MRPs, as deduced from studies on MRP1 and MRP2 [33,34]. Binding of oestradiol 3,17-disulphate to MRP4 may be more similar to binding of DHEAS than $E_2 17\beta G$ and therefore inhibit transport of the former more effectively. We also find that GSH is not required by MRP4 to transport either DHEAS or $E_2 17\beta G$. This is in stark contrast to the requirement of GSH or a GSH analogue for DHEAS transport by MRP1. A similar stringent requirement has been found for transport by MRP1 of 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanol (NNAL) glucuronide [23]. In contrast, transport of other acidic compounds, such as oestrone 3-sulphate [24] or etoposide glucuronide [35], is stimulated by GSH, but not totally dependent on GSH or a GSH analogue. For several non-acidic anticancer drugs (e.g. vincristine), GSH is co-transported with the drug by MRP1 [36,37]. Whether this is also the case for DHEAS and NNAL glucuronide remains to be seen.

The physiology of DHEA and its sulphated derivative DHEAS, the major circulating hormones in humans [38–40], are not well understood although they were identified in the 1930s. Both are synthesized in cells of the zona reticularis in the adrenal gland and elicit a variety of responses in target tissues. To exert its effects, DHEAS must be released into the circulation after its synthesis in the adrenal gland. As DHEAS is poorly membrane-permeable, this process must be carrier mediated. Although members of the organic anion-transporting polypeptide (OATP) family of transporters mediate high-affinity DHEAS transport [41–44], they are believed to operate via an ion-exchange mechanism (e.g. in exchange for GSH), implying that they are most likely to be responsible for uptake into target tissues rather than secretion [45]. Our finding that both MRP1 and MRP4 mediate high-affinity transport of DHEAS at physiologically relevant concentrations (2–10 μM [46]) raises the possibility that they represent the major transporter(s) mediating the secretion of DHEAS from the adrenal gland. Both transporters are detected in the human adrenal gland, and the high levels of MRP1 in this tissue, specifically in the adrenal cortex [21], strongly suggests that it is an important adrenal DHEAS efflux pump. Despite the low level of MRP4 in the adrenal gland, it might be expressed at high levels in the cells that synthesize and release DHEAS. Precise immunohistochemical localization of both transporters, as has been done for MRP3 [22], is required to determine whether their localization coincides with the DHEAS synthesis machinery.

The involvement of other MRP family members in the physiology of DHEAS is controversial. We find that neither MRP2 nor MRP3 transports DHEAS. An early study demonstrated the presence of DHEAS in human bile [47]. In line with this finding, Cui et al. [48] reported significant transport of DHEAS by MRP2 in polarized MDCK-II cells transfected with an OATP8 construct to allow entry of DHEAS into the cells at the basolateral membrane. In contrast, Sasaki et al. [49] observed no DHEAS transport with an MDCK-II clone co-transfected with OATP2 and MRP2, even though the rate of apical transport of $E_2 17\beta G$ and leukotriene C_4 in this clone was comparable with that of the line of Cui et al. [48]. We find no transport of DHEAS by MRP2 in vesicular transport assays, supporting the observations of Sasaki et al. [49]. The TR^- rats, which lack functional MRP2, offer a good experimental system to settle this issue.

In conclusion, we find that MRP4 and MRP1 transport DHEAS with high affinity at physiologically relevant concentrations. Both transporters might mediate the release of DHEAS from the adrenal gland and could have an effect on the distribution of DHEAS in the body. It is plausible that, like DHEAS, other steroid and bile acid conjugates are substrates of MRP4 as well, raising the possibility that it has a role in steroid and bile acid physiology. The *Mrp4*$^{(-/-)}$ mice recently generated in the Schuetz laboratory will be a useful model to study the contribution of MRP4 to the physiology of steroid and bile acid conjugates.

We thank our colleagues Henk de Vries, Nobuhito Ono, Hein te Riele (Department of Molecular Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands), Jan Wijnholds (Eye Institute, University of Amsterdam, Amsterdam, The Netherlands) and Ronald Oude Elferink (University of Amsterdam) for critical reading of the manuscript. We are grateful to Puck Knipscheer (Department of Molecular Carcinogenesis, The Netherlands Cancer Institute) for assisting us in culturing Sf9 insect cells, and Balasz Sarkadi (Institute of Enzymology, Budapest, Hungary) and Remon van Aubel (Nijmegen Center for Molecular Life Sciences, Nijmegen, The Netherlands) for providing us with baculovirus containing MRP2 and MRP4 cDNA. This work was supported by the Dutch Cancer Society grants NKI 2001-2473, 2001-2474 and 1998-1794 to P. B.

REFERENCES

1 Higgins, C. F. (1992) ABC transporters: from microorganisms to man. Annu. Rev. Cell Biol. **8**, 67–113
2 Borst, P. and Elferink, R. O. (2002) Mammalian ABC transporters in health and disease. Annu. Rev. Biochem. **71**, 537–592
3 Dean, M., Rzhetsky, A. and Allikmets, R. (2001) The human ATP-binding cassette (ABC) transporter superfamily. Genome Res. **11**, 1156–1166
4 Renes, J., de Vries, E. G., Jansen, P. L. and Muller, M. (2000) The (patho)physiological functions of the MRP family. Drug Resist. Update **3**, 289–302
5 Cole, S. P., Bhardwaj, G., Gerlach, J. H., Mackie, J. E., Grant, C. E., Almquist, K. C., Stewart, A. J., Kurz, E. U., Duncan, A. M. and Deeley, R. G. (1992) Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science **258**, 1650–1654

6 Konig, J., Nies, A. T., Cui, Y., Leier, I. and Keppler, D. (1999) Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity, and MRP2-mediated drug resistance. Biochim. Biophys. Acta **1461**, 377–394

7 Borst, P., Evers, R., Kool, M. and Wijnholds, J. (2000) A family of drug transporters: the multidrug resistance-associated proteins. J. Natl. Cancer Inst. **92**, 1295–1302

8 Jedlitschky, G., Burchell, B. and Keppler, D. (2000) The multidrug resistance protein 5 functions as an ATP-dependent export pump for cyclic nucleotides. J. Biol. Chem. **275**, 30069–30074

9 Chen, Z. S., Lee, K. and Kruh, G. D. (2001) Transport of cyclic nucleotides and estradiol 17-beta-D-glucuronide by multidrug resistance protein 4. Resistance to 6-mercaptopurine and 6-thioguanine. J. Biol. Chem. **276**, 33747–33754

10 Wijnholds, J., Mol, C. A., van Deemter, L., de Haas, M., Scheffer, G. L., Baas, F., Beijnen, J. H., Scheper, R. J., Hatse, S., De Clercq, E. et al. (2000) Multidrug-resistance protein 5 is a multispecific organic anion transporter able to transport nucleotide analogs. Proc. Natl. Acad. Sci. U.S.A. **97**, 7476–7481

11 Schuetz, J. D., Connelly, M. C., Sun, D., Paibir, S. G., Flynn, P. M., Srinivas, R. V., Kumar, A. and Fridland, A. (1999) MRP4: A previously unidentified factor in resistance to nucleoside-based antiviral drugs. Nat. Med. **5**, 1048–1051

12 Lee, K., Klein-Szanto, A. J. and Kruh, G. D. (2000) Analysis of the MRP4 drug resistance profile in transfected NIH3T3 cells. J. Natl. Cancer Inst. **92**, 1934–1940

13 van Aubel, R. A., Smeets, P. H., Peters, J. G., Bindels, R. J. and Russel, F. G. (2002) The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human kidney proximal tubules: putative efflux pump for urinary cAMP and cGMP. J. Am. Soc. Nephrol. **13**, 595–603

14 Chen, Z. S., Lee, K., Walther, S., Raftogianis, R. B., Kuwano, M., Zeng, H. and Kruh, G. D. (2002) Analysis of methotrexate and folate transport by multidrug resistance protein 4 (ABCC4): MRP4 is a component of the methotrexate efflux system. Cancer Res. **62**, 3144–3150

15 Schuetz, E. G., Strom, S., Yasuda, K., Lecureur, V., Assem, M., Brimer, C., Lamba, J., Kim, R. B., Ramachandran, V., Komoroski, B. J. et al. (2001) Disrupted bile acid homeostasis reveals an unexpected interaction among nuclear hormone receptors, transporters, and cytochrome P450. J. Biol. Chem. **276**, 39411–39418

16 Sinal, C. J., Tohkin, M., Miyata, M., Ward, J. M., Lambert, G. and Gonzalez, F. J. (2000) Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. Cell **102**, 731–744

17 Wielinga, P. R., Reid, G., Challa, E. E., Van Der Heijden, I., Van Deemter, L., De Haas, M., Mol, C., Kuil, A. J., Groeneveld, E., Schuetz, J. D. et al. (2002) Thiopurine metabolism and identification of the thiopurine metabolites transported by MRP4 and MRP5 overexpressed in human embryonic kidney cells. Mol. Pharmacol. **62**, 1321–1331

18 Zelcer, N., Saeki, T., Reid, G., Beijnen, J. H. and Borst, P. (2001) Characterization of drug transport by the human multidrug resistance protein 3 (ABCC3). J. Biol. Chem. **276**, 46400–46407

19 Bakos, E., Evers, R., Sinko, E., Varadi, A., Borst, P. and Sarkadi, B. (2000) Interactions of the human multidrug resistance proteins MRP1 and MRP2 with organic anions. Mol. Pharmacol. **57**, 760–768

20 Lai, L. and Tan, T. M. (2002) Role of glutathione in the multidrug resistance protein 4 (MRP4/ABCC4)-mediated efflux of cAMP and resistance to purine analogues. Biochem. J. **361**, 497–503

21 Flens, M. J., Zaman, G. J., van der Valk, P., Izquierdo, M. A., Schroeijers, A. B., Scheffer, G. L., van der Groep, P., de Haas, M., Meijer, C. J. and Scheper, R. J. (1996) Tissue distribution of the multidrug resistance protein. Am. J. Pathol. **148**, 1237–1247

22 Scheffer, G. L., Kool, M., de Haas, M., de Vree, J. M., Pijnenborg, A. C., Bosman, D. K., Elferink, R. P., van der Valk, P., Borst, P. and Scheper, R. J. (2002) Tissue distribution and induction of human multidrug resistant protein 3. Lab. Invest. **82**, 193–201

23 Leslie, E. M., Ito, K., Upadhyaya, P., Hecht, S. S., Deeley, R. G. and Cole, S. P. (2001) Transport of the beta-O-glucuronide conjugate of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) by the multidrug resistance protein 1 (MRP1). Requirement for glutathione or a non-sulfur-containing analog. J. Biol. Chem. **276**, 27846–27854

24 Qian, Y. M., Song, W. C., Cui, H., Cole, S. P. and Deeley, R. G. (2001) Glutathione stimulates sulfated estrogen transport by multidrug resistance protein 1. J. Biol. Chem. **276**, 6404–6411

25 Lee, K., Belinsky, M. G., Bell, D. W., Testa, J. R. and Kruh, G. D. (1998) Isolation of MOAT-B, a widely expressed multidrug resistance-associated protein/canalicular multispecific organic anion transporter-related transporter. Cancer Res. **58**, 2741–2747

26 Ogawa, K., Suzuki, H., Hirohashi, T., Ishikawa, T., Meier, P. J., Hirose, K., Akizawa, T., Yoshioka, M. and Sugiyama, Y. (2000) Characterization of inducible nature of MRP3 in rat liver. Am. J. Physiol. **278**, G438–G446

27 Donner, M. G. and Keppler, D. (2001) Up-regulation of basolateral multidrug resistance protein 3 (Mrp3) in cholestatic rat liver. Hepatology **34**, 351–359

28 Meier, P. J. and Stieger, B. (2002) Bile salt transporters. Annu. Rev. Physiol. **64**, 635–661

29 Zelcer, N., Saeki, T., Bot, I., Kuil, A. and Borst, P. (2002) Transport of bile acids in multidrug resistance protein 3 over-expressing cells co-transfected with the ileal sodium-dependent bile acid transporter. Biochem. J. **369**, 23–30

30 Ostrow, J. D. (1993) Metabolism of bile salts in cholestasis in humans. In Hepatic Transport and Bile Secretion (Tavoloni, N. and Berk, P. D., eds.), pp. 673–712, Raven Press, New York

31 Song, C. S., Echchgadda, I., Baek, B. S., Ahn, S. C., Oh, T., Roy, A. K. and Chatterjee, B. (2001) Dehydroepiandrosterone sulfotransferase gene induction by bile acid activated farnesoid X receptor. J. Biol. Chem. **276**, 42549–42556

32 Radominska, A., Comer, K. A., Zimniak, P., Falany, J., Iscan, M. and Falany, C. N. (1990) Human liver steroid sulphotransferase sulphates bile acids. Biochem. J. **272**, 597–604

33 Zhang, D. W., Cole, S. P. and Deeley, R. G. (2001) Identification of an amino acid residue in multidrug resistance protein 1 critical for conferring resistance to anthracyclines. J. Biol. Chem. **276**, 13231–13239

34 Ito, K., Suzuki, H. and Sugiyama, Y. (2001) Single amino acid substitution of rat MRP2 results in acquired transport activity for taurocholate. Am. J. Physiol. **281**, G1034–G1043

35 Sakamoto, H., Hara, H., Hirano, K. and Adachi, T. (1999) Enhancement of glucuronosyl etoposide transport by glutathione in multidrug resistance-associated protein-overexpressing cells. Cancer Lett. **135**, 113–119

36 Evers, R., de Haas, M., Sparidans, R., Beijnen, J., Wielinga, P. R., Lankelma, J. and Borst, P. (2000) Vinblastine and sulfinpyrazone export by the multidrug resistance protein MRP2 is associated with glutathione export. Br. J. Cancer **83**, 375–383

37 Loe, D. W., Deeley, R. G. and Cole, S. P. (1998) Characterization of vincristine transport by the *M*, 190,000 multidrug resistance protein (MRP): evidence for cotransport with reduced glutathione. Cancer Res. **58**, 5130–5136

38 Kroboth, P. D., Salek, F. S., Pittenger, A. L., Fabian, T. J. and Frye, R. F. (1999) DHEA and DHEA-S: a review. J. Clin. Pharmacol. **39**, 327–348

39 Yen, S. S. (2001) Dehydroepiandrosterone sulfate and longevity: new clues for an old friend. Proc. Natl. Acad. Sci. U.S.A. **98**, 8167–8169

40 Gurnell, E. M. and Chatterjee, V. K. (2001) Dehydroepiandrosterone replacement therapy. Eur. J. Endocrinol. **145**, 103–106

41 Kullak-Ublick, G. A., Fisch, T., Oswald, M., Hagenbuch, B., Meier, P. J., Beuers, U. and Paumgartner, G. (1998) Dehydroepiandrosterone sulfate (DHEAS): identification of a carrier protein in human liver and brain. FEBS Lett. **424**, 173–176

42 Hsiang, B., Zhu, Y., Wang, Z., Wu, Y., Sasseville, V., Yang, W. P. and Kirchgessner, T. G. (1999) A novel human hepatic organic anion transporting polypeptide (OATP2). Identification of a liver-specific human organic anion transporting polypeptide and identification of rat and human hydroxymethylglutaryl-CoA reductase inhibitor transporters. J. Biol. Chem. **274**, 37161–37168

43 van Montfoort, J. E., Schmid, T. E., Adler, I. D., Meier, P. J. and Hagenbuch, B. (2002) Functional characterization of the mouse organic-anion-transporting polypeptide 2. Biochim. Biophys. Acta **1564**, 183–188

44 Eckhardt, U., Schroeder, A., Stieger, B., Hochli, M., Landmann, L., Tynes, R., Meier, P. J. and Hagenbuch, B. (1999) Polyspecific substrate uptake by the hepatic organic anion transporter Oatp1 in stably transfected CHO cells. Am. J. Physiol. **276**, G1037–G1042

45 Li, L., Meier, P. J. and Ballatori, N. (2000) Oatp2 mediates bidirectional organic solute transport: a role for intracellular glutathione. Mol. Pharmacol. **58**, 335–340

46 Orentreich, N., Brind, J. L., Rizer, R. L. and Vogelman, J. H. (1984) Age changes and sex differences in serum dehydroepiandrosterone sulfate concentrations throughout adulthood. J. Clin. Endocrinol. Metab. **59**, 551–555

47 Laatikainen, T., Peltokallio, P. and Vihko, R. (1968) Steroid sulfates in human bile. Steroids **12**, 407–421

48 Cui, Y., Konig, J. and Keppler, D. (2001) Vectorial transport by double-transfected cells expressing the human uptake transporter slc21a8 and the apical export pump abcc2. Mol. Pharmacol. **60**, 934–943

49 Sasaki, M., Suzuki, H., Ito, K., Abe, T. and Sugiyama, Y. (2002) Transcellular transport of organic anions across a double-transfected Madin-Darby canine kidney II cell monolayer expressing both human organic anion-transporting polypeptide (OATP2/SLC21A6) and multidrug resistance-associated protein 2 (MRP2/ABCC2). J. Biol. Chem. **277**, 6497–6503

Received 5 December 2002/8 January 2003; accepted 10 January 2003
Published as BJ Immediate Publication 10 January 2003, DOI 10.1042/BJ20021886