MRP2-4, from drug resistance to physiology

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Chapter 6

Steroids and bile acids are substrates of human multidrug resistance protein (MRP) 4 (ATP-binding cassette C4)

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

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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 (E17βG), 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 E17βG. Inhibition by oestradiol 3,17-disulphate and taurodeoxycholate 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 0.6 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 proteins mediate the transport of various substrates across membranes at the expense of ATP hydrolysis [1,2]. The human ABC subfamily contains nine members that are referred to as multidrug-resistance proteins (MRPs) 1–9, in addition to sulphophyloane 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 (E17βG), 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 (Spon/Bsep) [16]. This finding provides a possible link between bile acid homeostasis 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 E17βG 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; E17βG, oestradiol 17-β-D-glucuronide; MRP, multidrug-resistance protein; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; OATP, organic anion-transporting polypeptide.

These authors contributed equally to this work.

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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

[\textsuperscript{3}H]DHEAS (40.5 Ci/mmol) and [\textsuperscript{3}H]E,17/\textsubscript{G} (40.5 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 an 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\textsubscript{2} under humidifying conditions. The generation of baculovirus containing MRP1 and MRP3 cDNA constructs was reported in Zeiler et al. [18]. The baculovirus containing MRP2 cDNA was a gift from Balazs Sarkadi (Institute of Enzymology, Budapest, Hungary) [19] and that containing MRP4 cDNA was from Remon van Aubei (Nijmegen Centre 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\textsubscript{2}, and 10 mM Tris/Cl, 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 J19 (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 M R P 1-R1 (1:1000) and NK1-12C4 (1:10), respectively, followed by a rabbit anti-rat horseradish peroxidase conjugate (1:1000). The monoclonal antibody NK1-12C4 does not cross-react with MRP1 or MRP5 [17]. Signals were visualized by enhanced chemiluminescence (ECL\textsuperscript{+}, 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 100 000 g for 40 min at 4 °C and the pellet was homogenized in ice-cold TS buffer (50 mM Tris/Cl, 250 mM sucrose, pH 7.4) using a tight-fitting Dounce homogenizer. After centrifugation at 500 g for 10 min, the supernatant was centrifuged at 100 000 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 S9 insect cells infected with an MRP4 cDNA-containing baculovirus (Figure 1, inset). We found a clear ATP- and time-dependent transport of E,17/\textsubscript{G} in membranes containing MRP4 (results not shown), a previously characterized substrate of MRP4 [9,13]. In the livers of F344 rats, which display 8-fold-increased serum bile acid levels as a result of a decrease in the expression of
of the main canalicular bile-salt-efflux pump (Bsep/Sppg), 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,17/17G, 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,17/17G transport. The results are summarized in Table 1. Sulphated bile acids inhibited the MRP4-mediated transport of E,17/17G at a low concentration ([S]$_{50}$ 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,17/17G transport (Table 2). Conjugated steroids inhibited the MRP4-mediated transport to different degrees. The inhibition of MRP4-mediated E,17/17G transport by both taurolocholic acid 3-sulphate and oestradiol 3,17-disulphate (at concentrations of 10 and 1 µM, respectively) was competitive, as at an increased substrate (E,17/17G) concentration the inhibition could be overcome; both compounds inhibited MRP4-mediated transport of 5 µM E,17/17G by approx. 50%, whereas at a concentration of 50 µM E,17/17G no inhibition was seen.

### Table 1: Effect of bile acids on MRP4-mediated E,17/17G transport

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Approx. IC$_{50}$ (µM)</th>
</tr>
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<tbody>
<tr>
<td>Cholic</td>
<td>250</td>
</tr>
<tr>
<td>Glycocholic acid</td>
<td>400</td>
</tr>
<tr>
<td>Taurocholic acid</td>
<td>350</td>
</tr>
<tr>
<td>Taurodeoxycholic acid</td>
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</tr>
<tr>
<td>Taurokohodihydrocholic acid</td>
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</tr>
<tr>
<td>Taurokohodihydrocholic acid</td>
<td>20</td>
</tr>
<tr>
<td>Taurokohodihydrocholic acid</td>
<td>10</td>
</tr>
<tr>
<td>Glycocholic acid</td>
<td>10</td>
</tr>
<tr>
<td>Lithocholic acid</td>
<td>10</td>
</tr>
</tbody>
</table>

Membrane vesicles prepared from HEK-293 cells overexpressing MRP4 were incubated at 37°C for 10 min with 30 µM [H]E,17/17G 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.

### Table 2: Effect of conjugated steroids on MRP4-mediated E,17/17G transport

Membrane vesicles prepared from Sf9 insect cells overexpressing MRP4 were incubated at 37°C for 5 min with 1 µM [H]E,17/17G 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.

<table>
<thead>
<tr>
<th>Inhibitor</th>
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<tbody>
<tr>
<td>DHEAS</td>
<td>2</td>
</tr>
<tr>
<td>DHEAS 3-glucuronic acid</td>
<td>50</td>
</tr>
<tr>
<td>Oestrone 3-sulphate</td>
<td>45</td>
</tr>
<tr>
<td>Oestradiol 3-sulphate</td>
<td>50</td>
</tr>
<tr>
<td>Oestradiol 3-glucuronic acid</td>
<td>120</td>
</tr>
<tr>
<td>Oestradiol 3,17-disulphate</td>
<td>3</td>
</tr>
</tbody>
</table>

### Table 3: Summary of kinetic parameters for MRP4-mediated transport

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_{m}$ (µM)</th>
<th>$V_{max}$ (pmol/mg protein/min)</th>
<th>$V_{max}/K_{m}$</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>E,17/17G</td>
<td>30</td>
<td>102</td>
<td>3.4</td>
<td>[9]</td>
</tr>
<tr>
<td>DHEAS</td>
<td>10</td>
<td>2</td>
<td>0.21</td>
<td>[9]</td>
</tr>
<tr>
<td>cAMP</td>
<td>55</td>
<td>1</td>
<td>0.02</td>
<td>[9]</td>
</tr>
<tr>
<td>Mehtonine</td>
<td>1300</td>
<td>450</td>
<td>0.35</td>
<td>[13]</td>
</tr>
<tr>
<td>Methionine</td>
<td>220</td>
<td>240</td>
<td>1.1</td>
<td>[14]</td>
</tr>
<tr>
<td>Amin acid</td>
<td>170</td>
<td>690</td>
<td>0.3</td>
<td>[14]</td>
</tr>
<tr>
<td>Lysinervin</td>
<td>640</td>
<td>1950</td>
<td>3.0</td>
<td>[14]</td>
</tr>
<tr>
<td>DHEAS$^*$</td>
<td>2</td>
<td>45</td>
<td>22</td>
<td>Present study</td>
</tr>
</tbody>
</table>

$^*$ Kinetic values determined at 27°C.

### 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 22°C with 25 nM [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 1 µM ATP from that in the presence of 4 mM ATP in the central 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.

### Figure 1: Kinetic parameter comparison of MRP4 and other transporters

![Kinetic parameter comparison of MRP4 and other transporters](image)

Transport of DHEAS by MRP4

Interestingly, MRP4-mediated transport of E,17/17G 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 transporters. 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 S19 insect cells overexpressing MRP4 were incubated at 27°C for 2 min with 25 nM [14C]DHEAS in the presence of conjugated steroids. Inhibition of the ATP-dependent uptake was measured in duplicate (n = 2 or 3). An approximate IC\textsubscript{50} value is presented. The approximate IC\textsubscript{50} values were extrapolated from determinations performed at three different concentrations spanning the IC\textsubscript{50} range. The complete data set for Table 4 can be obtained from the corresponding author (P.B.) upon request.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Approx. IC\textsubscript{50} (\mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E17\beta G</td>
<td>90</td>
</tr>
<tr>
<td>DHEA 3-glucuronate</td>
<td>80</td>
</tr>
<tr>
<td>Oestrone 3-sulphate</td>
<td>95</td>
</tr>
<tr>
<td>Oestradiol 3-sulphate</td>
<td>70</td>
</tr>
<tr>
<td>Oestradiol 3-glucuronate</td>
<td>80</td>
</tr>
<tr>
<td>Oestradiol 3,17-diol sulphate</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Figure 4  Transport of DHEAS by MRP1 and MRP2

Membrane vesicles from S19 insect cells infected with a wild type A: MRP1, B: MRP3, C: MRP4 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 (1 mM). Each bar represents the mean ± S.D. from triplicate done in duplicate.

Figure 3  Detection of MRPs in human adrenal gland

(A) Each lane was loaded with 50 µg of total tissue protein. MRP1 and MRP4 were detected with specific monoclonal antibodies as described in the Experimental section. Total cellular protein (10–20 µg) from cells stably expressing MRP1 or MRP4 was used as a positive control (+). (B) 20 µg of total cellular HEK293, HEK293/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. (C) Membrane vesicles from S19 insect cells infected with a wild type A: MRP1, B: MRP3, C: MRP4 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 (1 mM). Each bar represents the mean ± S.D. from triplicate done in duplicate.

Not shown). Non-linear regression analysis yielded apparent K\text{m} and V\text{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\text{max}/K\text{m}) for DHEAS are the highest reported for any MRP4 substrate to date (Table 3). As found with E\text{17G}, steroid analogues inhibited the MRP4-mediated DHEAS transport to varying degrees (Table 4). In contrast to a recent report by Lai and Tan [29], 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\text{17G} by MRP4 (results not shown). This suggests that any requirement for GSH is not a general feature of MRP4-mediated transport.
Transport of DHEAS by MRP1-3

In the adrenal gland MRP1 and MRP3 were readily detected, as were low levels of MRP4 (Figure 5). 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 $5 \pm 1 \mu M$ and $73 \pm 6 \text{pmol/mg min}$, 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 $10 \mu M$ 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 E17G 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 $50 \mu M$ (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 E17G 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, E17G and DHEAS, the latter with high affinity. (ii) MRP4 is found in several tissues important in steroid metabolism [25]. (iii) Transport of DHEAS and E17G 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 For−/− mice suggests yet another intriguing role for MRP4. These mice lack the nuclear hormone receptor FXR, which is a key regulator of bile acid homeostasis, 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-export 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 For−/− 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 MRPl-mediated oestriadiol glucuronide transport at low concentrations (IC$_{50}$, approx. 10 μM) in a competitive manner, suggesting that they represent substrates of MRPl. 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 MRPl in hepatocytes (1.4). 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 DHA-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 MRPl. Clearly, the potential role of MRPl in mitigating cholestatic liver disease could be important, but must be verified in further studies.

The inhibition of the MRPl-mediated E17G and DHEAS transport (Tables 1, 2 and 4) reveals that the structural requirements for recognition by MRPl 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 oestriadiol 3-sulphate. Interestingly, an oestriadiol derivative with sulphate groups in positions 3 and 17 is a very potent inhibitor of MRPl-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 MRPl 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 E17G, as DHEAS has the higher affinity for MRPl. Not all putative MRPl substrates behave in this fashion, however, as shown in Tables 2 and 4. Oestradiol 3,17-disulphate, for instance, inhibits DHEAS transport more than E17G at all concentrations tested. This may reflect a complexity in the substrate-binding pockets of MRPs, as deduced from studies on MRPl and MRPl2 [33,34]. Binding of oestradiol 3,17-disulphate to MRPl may be more similar to binding of DHEAS than E17G and therefore inhibit transport of the former more effectively. We also find that GSH is not required by MRPl to transport either DHEAS or E17G. This is in stark contrast to the requirement of GSH or a GSH analogue for DHEAS transport by MRPl. A similar stringent requirement has been found for transport by MRPl of 4-methyltritosamine-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 MRPl [36,37]. Whether this is also the case for DHEAS and NNAL glucuronide remains to be seen.

The physiology of DHEAS 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 transport DHEAS transport [41-44], they are believed to operate via an ion-exchange mech-

unism (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 MRPl and MRPI mediate high-affinity transport of DHEAS at physiologically relevant concentrations (2-10 μM [46]) raises the possibility that they represent the major transporters) mediating the secretion of DHEAS from the adrenal gland. Both transporters are detected in the human adrenal gland, and the high levels of MRPl 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 MRPl 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 MRPl [22], is required to determine whether their localization coincides with the DHEAS synthesis machinery.

The involvement of other MRPl family members in the physiology of DHEAS is controversial. We find that neither MRPl2 nor MRPl3 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 MRPl2 in polarized MDCK-II cells transfected with an OATPl construct to allow entry of DHEAS into the cells at the basolateral membrane. In contrast, Sasaki et al. [49] observed no DHEAS transport with a MDCK-II clone co-transfected with OATPl2 and MRPl2, even though the rate of apical transport of E17G and leukotriene C4 in this clone was comparable with that of the line of Cui et al. [48]. We find no transport of DHEAS by MRPl2 in vesicular transport assays, supporting the observations of Sasaki et al. [49]. The TR rats, which lack functional MRPl2, offer a good experimental system to settle this issue.

In conclusion, we find that MRPl4 and MRPl1 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 MRPl4 as well, raising the possibility that it has a role in steroid and bile acid physiology. The MRPl4 mouse recently generated in the Schuetz laboratory will be a useful model to study the contribution of MRPl4 to the physiology of steroid and bile acid conjugates.

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Steroid and bile acid transport by MRP4


