Sodium-dependent methotrexate carrier-1 is expressed in rat kidney: cloning and functional characterization

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Submitted 18 July 2003; accepted in final form 8 November 2003

Kneuer, Carsten, Kerstin U. Honscha, and Walther Honscha. Sodium-dependent methotrexate carrier-1 is expressed in rat kidney: cloning and functional characterization. Am J Physiol Renal Physiol 286: F564–F571, 2004. First published November 11, 2003; 10.1152/ajprenal.00257.2003.—Previous Northern blot studies suggested strong expression of a homolog to the sodium-dependent hepatocellular methotrexate transporter in the kidneys. Here, we report on the cloning of the cDNA for the renal methotrexate carrier isoform-1 (RK-MTX-1) and its functional characterization. Sequencing revealed 97% homology to the rat liver methotrexate carrier with an identical open reading frame. Differences were located in the 5′ untranslated region and resulted in the absence of putative regulatory elements (Barbie box, Ah/ARNT receptor) identified in the cDNA for the hepatocellular carrier. For functional characterization, MTX-1 cDNA was stably expressed in Madin-Darby canine kidney (MDCK) cells. A sodium-dependent transport of methotrexate with a Kₘ of 41 μM and a Vₗₘₜₕ of 337 pmol·mg protein⁻¹·min⁻¹ was observed. This uptake was blocked by the reduced folates dihydro- and tetrahydrofolate as well as by methotrexate itself. Folate was inhibiting only weakly, whereas 5-methyltetrahydrofolate was a strong inhibitor. Further inhibitors of the methotrexate transport included the bile acids cholate and taurocholate and xenobiotics like bumetanide and BSP. PAH, ouabain, bumetanide, cholate, taurocholate, and acetyl salicylic acid were tested as potential substrates. However, none of these substances was transported by MTX-1. Furthermore, expression of RK-MTX-1 in MDCK cells enhanced methotrexate toxicity in these cells fivefold. Analysis of a fusion protein of RK-MTX-1 and the influenza virus hemagglutinin epitope by immunoblotting revealed a major band at 72 kDa within the cell membrane but not in the soluble fraction of transfected MDCK. Indirect immunofluorescence staining revealed an exclusive localization of the carrier in the plasma membrane, and by confocal laser-scanning microscopy we were able to demonstrate that the protein is expressed in the serosal region of MDCK tubules grown in a morphogenic collagen gel model. AMONG THE VARIOUS DRUGS in use for the treatment of malignancies, the group of antimetabolites is of significant practical relevance. The antifolate methotrexate (Mtx) is also successfully used in various nononcological indications like rheumatoid arthritis and psoriasis (6, 8, 19, 23, 33). However, chemotherapy with Mtx is confronted with adverse effects such as bone marrow suppression, hepatotoxicity, as well as acute and long-term nephrotoxicity. Especially, the interest in chemotherapy-induced nephrotoxicity has grown, because an increasing number of individuals with curable cancer are potentially at risk for long-term renal sequelae (21). Furthermore, it has been reported that the concentration of Mtx in the kidney is 80-fold higher than in the liver and 28-fold higher than in the bone marrow under high-dose Mtx therapy (18). Taken together, this may suggest the involvement of active Mtx transport in the kidney.

Previously, two different classes of Mtx transporting carriers were identified in normal tissue from rat liver and kidney: one group of currently four families of sodium-independent proteins including organic anion transporter (OAT) kidney 1/2 (OAT-K1/OAT-K2), reduced folate carrier (rfc), the ABC pump mrp2, and the sodium-dependent rat liver Mtx transporter-1 (RL-MTX-1).

Sekine et al. (34) and Sweet et al. (39) independently cloned the first member of the sodium-independent OAT family (OAT1) from rat kidney. Its mRNA is translated into a protein of 551 amino acids with a calculated molecular weight of 60.7 kDa. OAT1 was demonstrated to transport PAH, cAMP, cGMP, PGE2, urate, α-ketoglutarate, and Mtx across the serosal membrane. Other family members that can interact with Mtx to different extents have been described since and include OAT2 (37), OAT3 (25), and OAT4 (3). All these transporters are expressed in the kidneys and other tissues including liver and placenta in a gender- and subtype-dependent manner (2, 3). The second family of sodium-independent Mtx facilitators is represented by OAT-K1 and -2, cloned by Saito et al. (31) and Masuda et al. (26). Both proteins are located in the apical (luminal) membrane of proximal tubule epithelial cells and mediate the exchange of folate and Mtx (26, 27, 31).

Moreover, it is well known that the rfc1 are also able to facilitate the influx of Mtx. In humans, rfc1 mRNA expression has been reported in liver, placenta, and to a lesser extent in the kidneys, leucocytes, lung, and other tissues (44). A highly homologous transporter has also been reported in mouse (43) and hamster (45). The rfc-mediated Mtx uptake has been described as sodium independent but sensitive to pH, folates, and reduced folates.

Finally, the multidrug-resistance-related protein 2 (MRP2) is known to be involved in the active transport of Mtx (1). MRP2 expression in the kidneys has been reported for various species, where it localizes on the luminal membrane of tubule epithelial cells (32) to mediate ATP-driven export of unconjugated Mtx into the proximal tubule.

Recently, we demonstrated the existence of a sodium-dependent Mtx transport system in rat liver (14). In freshly isolated hepatocytes, this pathway dominates Mtx uptake to 90% and...
can be inhibited by reduced folates, bile acids, xenobiotics like ochratoxin A and humate, but not by folate. Cloning of the cDNA for the carrier involved in this sodium-dependent process revealed two transcripts corresponding to rat liver Mtx carrier 1 and 2 (RL-MTX-1/2). RL-MTX-1 and -2 transported Mtx in a sodium-dependent manner by expression in Xenopus laevis oocytes (13). Northern blot analysis further suggested that a homologous mRNA was strongly expressed in the kidneys. We, therefore, now cloned and sequenced this isoform and characterized its substrate spectrum in a suitable mammalian expression system. In addition, we aimed to confirm the localization of the protein within the cell membrane and to collect information about potential involvement of the transporter in kidney toxicity of the drug.

**MATERIALS AND METHODS**

**Preparation of mRNA.** Total RNA was prepared from frozen rat kidney according to the method of Chomczynski and Sacchi (5) where mRNA was isolated from total RNA with a Qiagen Oligotex mRNA kit (Qiagen, Hilden, Germany).

cDNA synthesis and rapid amplification of cDNA ends-PCR. First- and second-strand cDNA synthesis and the ligation of the adaptors were performed using a Marathon cDNA amplification kit (Clontech, Heidelberg, Germany) according to the manufacturer’s instructions and as previously described (23). In brief, the first-strand cDNA synthesis was started with 1 μg poly A+ RNA and 10 pmol of the cDNA synthesis primer for 60 min at 42°C. Second-strand cDNA synthesis was performed at 16°C for 90 min using dNTPs, a mixture of Escherichia coli DNA polymerase I, E. coli DNA ligase, and E. coli RNAse H. Efficiency and yield were estimated by agarose gel electrophoresis and autoradiography. One-half of the product was ligated to 20 pmol Marathon cDNA adaptor using T4 DNA ligase (1 unit) at 16°C overnight.

The following gene-specific primers were used for amplification of the 5′- and 3′-rapid amplification of cDNA ends (RACE) products for the Mtx carrier: 5′-RACE, Mtx-2350: GACAAGCATAAAA-CGAAGACCCTC; 3′-RACE, Mtx-371: GCCG(AGT)CTCGGGGA(A-G)AGCTTCATCA.

For RACE-PCR, a master mix of PCR-buffer, dNTPs, and Taq Start antibody/polynucleotide (Expand high-fidelity polymerase, Boehringer, Mannheim, Germany) was prepared and added to 5 μl of diluted cDNA together with 10–20 pmol of the gene-specific primer and 10 pmol of the adaptor primer. For the final 3′-RACE-PCRs, the following protocol was used: 30 s 94°C, 5 cycles with 94°C (5 s), 70°C (30 s), 69°C (240 s), 10 cycles with an annealing temperature of 68°C, and 20 cycles with an annealing temperature of 66°C. The 5′-RACE-PCR was performed as follows: 30 s 94°C, 5 cycles with 94°C (5 s), 65°C (30 s), 69°C (240 s), 10 cycles with an annealing temperature of 63°C, and 20 cycles with an annealing temperature of 61°C. In both cases, a final elongation step over 7 min was added. Both final RACE products were cloned into the pCR 3.1 vector.

**Sequencing.** Different clones of the various RACE products were isolated and sequenced using the dye terminator cycle sequencing method on an ABI Prism (Applied Biosystems, Perkin Elmer, Weiterstadt, Germany). The final full-length cDNA clones (pRK-MTX-1) were generated by elimination of the overlapping DNA fragments through subcloning, and two resulting cDNA clones were sequenced. For generation of stably transfected cells, the RK-MTX-1 cDNA was subcloned into the pIREShneo vector (pIRES-MTX-1).

**Tagging of RK-MTX-1 with the influenza virus hemagglutinin epitope.** The TAG stop codon at position 1630 of the cDNA for RK-MTX-1 was replaced by PCR-directed mutagenesis using pRK-MTX-1 as template and a reverse primer that contained a 5′-overhang coding for the hemagglutinin (HA) epitope followed by a new stop codon: 5′-t gag aag cga age caa ggc tca tca gat gtt cca gat tag-3′ in combination with the T7 primer binding upstream in pRK-MTX-1. The PCR product was ligated into pCR4-TOPO-TA (Invitrogen, Karlsruhe, Germany), and the fusion construct was excised by EcoRI digestion and cloned into pCR3.1 to yield pRK-MTX1630HA.

**Cell culture and transfection.** Madin-Darby canine kidney (MDCK) cells were grown in DMEM medium (GIBCO-BRL, Egg- enstein, Germany) supplemented with 10% FCS under 10% carbon dioxide atmosphere at 37°C. Three-dimensional branching tubule structures were obtained by morphogenic culture of MDCK cells (seeding density 10⁶/ml) in a 0.2% type I collagen gel for 14 days as described by McAlteer et al. (28).

For transfection, 2 × 10⁶ MDCK cells were seeded into six-well plates 24 h before transfection, pIRES-MTX-1 plasmid DNA (2.4 μg), 19.2 μl Enhancer, and 15 μl Effectene (both Qiagen) or pRK-MTX1630HA (2 μg) and 20 μg polyethyleneimine (Sigma, Deisen- hofen, Germany) were mixed and left for 10 min at room temperature. Transfection was carried out overnight, followed by 2-day incubation in growth medium before this was replaced by selection medium containing 200–400 μg/ml G418 (Sigma). Resistant single-cell clones were individually cloned and propagated.

**Uptake experiments.** The uptake measurements were performed as previously described (15). In brief, cells from two culture flasks (75-cm² dishes) were collected by trypsinization and resuspended in fresh culture medium. After three washing cycles with Tyrode buffer (137 mM NaCl, 12 mM NaHCO₃, 2.7 mM KCl, 1.05 mM MgCl₂ * 6 H₂O, 1.8 mM CaCl₂ * 2 H₂O, 5.55 mM glucose, 0.42 mM NaH₂PO₄, pH 7.4 at 37°C), the cells were equilibrated in the same buffer at 37°C for 10 min in a shaking water bath under carbogen. In general, viability was 95–98% by trypan blue exclusion. After addition of 3[H]Mtx (2 × 10⁵ dpm/ml, 9.25 MBq/mmol in 2 μl nonlabeled Mtx; Amersham, Braunschweig, Germany) to 1.2 ml of cell suspension, 100-μl aliquots were removed at different time points (15, 45, 75, 180, 300, 600, and 1,200 s). The cells were separated by immediate centrifugation across a silicone oil gradient and lysed in 4 M KOH overnight. Cell-associated radioactivity was measured by liquid scintillation counting. To determine sodium dependency, the tyrode buffer was substituted for sodium-free choline buffer or sodium-free N-methyl-d-glucamine buffer. For estimation of the Km value, the concentration-dependent uptake of Mtx (2 to 20 μM) was measured in the presence and in the absence of sodium ions. After subtraction of the sodium-independent uptake, the linear range (0, 0.25, 0.75, 1.25, and 3 min) of the sodium-dependent uptake was analyzed by regression line.

**Immunoblotting.** Cell lysates were obtained by addition of 1 ml ice-cold lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) supplemented with 1/50 of protease inhibitor cocktail (Sigma) to 10⁷ freshly trypsinized cells, followed by homogenization on ice using an Ultra-Turrax (IKA, Staufen, Germany) at no more than 20,000 rpm. Large organelles were sedimented by centrifugation at 100 g for 5 min, and the membrane fraction was separated from the soluble proteins by ultracentrifugation at 50,000 g for 30 min at 4°C. The protein content of all fractions was determined using the Lowry assay. Aliquots of both fractions were mixed with 2× Laemmli denaturing SDS-PAGE sample buffer heated to 95°C for 5 min, and 40 μg total protein per lane were subjected to a standard Tris-Glycine polyacrylamide gel electrophoresis in 10 and 12% gels. Proteins were transferred onto a Millipore NC membrane by semidy blotting at 0.9 mA/cm² over 90 min. The membrane was blocked overnight at 4°C with 1% blot-qualified BSA in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl). A rabbit polyclonal anti-HA antiserum (Sigma) was diluted 1:1,000 in TBST (TBS, 0.05% Tween 20) and applied to the membrane for 60 min at RT. After at least three washing steps with TBS, a 3,000-fold diluted anti-rabbit-AP conjugate (Promega, Mannheim, Germany) was added for 60 min followed by another three washing steps. Alkaline phosphatase activity was detected using a freshly prepared mixture of 66 μl NBT- and 33 μl BCIP-substrate.
(both Promega) in 10 ml AP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl2).

Immunochemistry. For indirect immunofluorescent staining, MDCK cells were seeded onto glass coverslips, transfected as described above, and grown to confluence. Alternatively, polarized MDCK tubules were obtained by culture in collagen gel as described above. Fixation was achieved with freshly prepared 2% paraformaldehyde, followed by blocking with 50 mM ammonium chloride and permeabilization with 0.1% Triton X-100. Both rabbit polyclonal anti-HA antiserum and secondary FITC-labeled goat anti-rabbit (Fab2) fragment were obtained from Sigma and used at a dilution of 1:200 in PBS containing 1% BSA. Counterstaining of nuclei was performed where indicated by addition of 1 nM propidium iodide to the second-PBS containing 1% BSA. Counterstaining of nuclei was performed by conventional fluorescence microscopy or examined in PBS (collagen gels) using a confocal laser-scanning microscope (Zeiss LSM 410).

Cytotoxicity. The stably transfected cell line MDCK 3B and mock-transfected MDCK 7AP were seeded in 96-well plates at a density of 30,000 cells/cm² in growth medium. After adhesion overnight, the wells were washed and serial dilutions of Mtx in DMEM were added. Overall cellular activity was determined 72 h later as mitochondrial oxidation activities are given as means ± SD of at least triplicate measurements. The significance of the results was determined using Student’s t-test. Statistical significance was assumed at P values of <0.05.

RESULTS

Molecular cloning and expression of the full-length RK-MTX-1 cDNA. A Marathon cDNA library from rat renal mRNA was constructed and the 5' Marathon cDNA library from rat renal Information (USA) using the BLAST algorithm. Secondary structure analysis was performed on the server of the National Center for Biotechnology Information (NCBI) using the CLUSTAL W program. Homology searches were performed on the Weizman server (Rehovot, Israel).

STATISTICAL ANALYSIS. Transport activities are given as means ± SD of at least triplicate measurements. The significance of the results was determined using Student’s t-test. Statistical significance was assumed at P values of <0.05.

RESULTS

Molecular cloning and expression of the full-length RK-MTX-1 cDNA. A Marathon cDNA library from rat renal mRNA was constructed and the 5'-RACE and 3'-RACE PCR product were amplified with gene-specific primers deduced from the hepatocellular RL-MTX-1 clone rat kidney (RK)-MTX-1. RK-MTX-1 consists of 2,290 bp with a predicted open reading frame of 1,539 bp from nucleotide 95 to position 1633. The entire sequence shows a 97% homology to the rfc cDNA cloned from rat vascular smooth muscle cells, suggesting an exclusive localization of the protein near or within the outer cell membrane. To collect evidence about the polarization of MTX-1 expression, stably transfected MDCK cells were grown into three-dimensional tubules using a morphogenic model with correct sorting of apical proteins to the serosal/outer side (28).

Major differences between the rat liver and RK-MTX-1 mRNA sequence were found in the 5'-UTR. It is interesting to note that the arylhydrocarbon receptor/arylhydrocarbon receptor nuclear translocator binding site and the so-called Barbie box (barbiturate-responsive DNA element) present in the cDNA of the hepatocellular carrier are absent in RK-MTX-1. There was also a similar degree of homology (96%) to the rfc cDNA cloned from rat vascular smooth muscle (NCBI U38180).

Fig. 1. Alignment of the nucleotide sequences (nucleotide 1 to 450) of rat liver and rat kidney methotrexate carrier isoforms (1-RK-MTX-1 and RK-MTX-1, respectively). The coding regions of both cDNA clones are identical (start of translation is identified by an arrow). Differences between the cDNA clones are located in the 5'-untranslated regions. The Barbie box, a putative pheno-barbital-responsive DNA sequence, was identified in the cDNA of the methotrexate carrier in the liver but is disrupted in the renal cDNA. The binding site for the arylhydrocarbon/arylhydrocarbon nuclear translocator (AhARNT; dioxygen receptor binding site) is also lost in the renal cDNA.

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Functional characterization. So far, the activity of MTX-1 has only been characterized in X. laevis oocytes using rat liver cDNA for heterologous expression. MDCK cells provide a suitable mammalian model for transport studies as they can be grown into confluent polarized monolayers that maintain a differentiated phenotype and express only low levels of interfering transport activity (14). A cell line stably expressing RK-MTX-1 (MDCK 3B) as well as a mock-transfected clone (MDCK 7AP) were generated. [3 H]Mtx (5 μM) was actively taken up in MDCK 3B as shown Fig. 3 but not in MDCK 7AP or untransfected MDCK cells. This uptake was sodium dependent. Figure 3B shows the corresponding Lineweaver-Burk plot. The $K_m$ was determined at 41 ± 13 μM and the $V_{max}$ was 337 ± 79 pmol·mg$^{-1}$·min$^{-1}$. This uptake activity is likely to increase the susceptibility to Mtx toxicity. In fact, a significant difference was observed in dose-response between functionally expressing MDCK 3B and mock-transfected MDCK 7AP cells (Fig. 4). The corresponding LD$_{50}$ values differed fivefold (16 vs. 80 nM).

In a previous report, we showed that the uptake of Mtx into hepatocytes is inhibited by various substances (14). However, due to the presence of further transport proteins, it remained...
unclear whether this activity was mediated via MTX-1. Figure 5 depicts the inhibitory potential of various of these substances in the RK-MTX-1-expressing cell line MDCK 3B. The most prominent effects were observed with Mtx (>100 µM) and 5-methyltetrahydrofolate (50 µM). The IC₅₀ value of 5-methyltetrahydrofolate was estimated to be 44 µM by regression analysis of the inhibition at various concentrations of the inhibitor. Dihydrofolate (FH₂) and tetrahydrofolate (FH₄) inhibited the Mtx transport by 40.92 and 33.38% at concentrations of 200 and 100 µM, respectively. Folate inhibited the transport of Mtx only at very high concentrations, and PAH was only a weak inhibitor of RK-MTX-1 at concentrations up to 200 µM. Taurocholate, cholate, bromosulfophthalein (BSP), and ouabain showed an intermediate inhibitory effect.

We further tested whether the carrier is able to mediate the transport of other substrates like taurocholate, cholate, acetyl-salicylate, ouabain, and PAH. However, none of the tested compounds was taken up by RK-MTX-1-expressing cells compared with mock-transfected cells (Fig. 6).

DISCUSSION

In the past decade, more than 40 different carriers for organic anions and cations were identified and cloned. Most of these transporters were functionally characterized in detail. However, only a few sodium-dependent carriers could be isolated. The majority of carriers for anionic and zwitterionic drugs including organic anion transporting polypeptides ( oatp) 1–4 (25, 34, 37, 39), and OAT-K1/K2 (2, 3, 26, 27, 31) are solely facilitating drug transport. The first carrier known to be actively driven by a sodium gradient was cloned by Hediger et al. (12) and is able to mediate the transport of glucose. Hagenbuch et al. (11) then identified the sodium-dependent taurocholate transporter in the liver, and Wong et al. (46) cloned the sodium-dependent bile acid transporter from the ileum. All these carriers have a narrow substrate spectrum in contrast to the sodium-independent facilitators. The latter are characterized by a broad and sometimes overlapping substrate specificity.

In addition to the previously described sodium-independent transporters for Mtx, we now identified a sodium-dependent Mtx carrier in the kidneys. On the protein level, this rat kidney Mtx carrier is likely to be identical to the previously cloned sodium-dependent rat liver Mtx carrier (13) as cDNA sequence analysis predicted the same open reading frames. In contrast, major differences in the MTX-1 mRNAs produced in rat liver and kidneys were identified in the 5′-UTR. This strongly suggests that both mRNAs are transcribed from different, tissue-specific promoters. Furthermore, the observed apparent molecular weight of 72 kDa, which is 14 kDa above the calculated molecular weight, suggests glycosylation of the protein and potential phosphorylation at two sites also allows for protein modification. Hence, regulation at the transcrip-
tional and posttranscriptional level, as well as the lifetime expression course of MTX-1, is likely to vary between these two organs.

On the protein level, the rat kidney Mtx carrier-1 also shares high homology to rfc1 of rat (99%) and, to a lesser extent, mouse (88%) and human (62%). Of the 32 amino acids that have been identified as important for Mtx transport in murine and human rfc1 (7, 30, 35, 47, 48), only Ser 317 is mutated to alanine. Although in the rat rfc1 expression has only been reported in the vascular smooth muscle, murine rfc1 has been documented in kidney tubular cells by immunohistochemistry (43) and rfc1 mRNA expression was demonstrated in human placenta, liver, lung, and small intestine (29). However, it is not entirely clear whether the sodium-dependent Mtx carrier-1 contributes to renal elimination by tubular secretion.

To put the Mtx transport by MTX-1 into a clinical perspective, the drug plasma levels as well as the properties of other Mtx carriers have to be considered. As described by Wall et al. (42), high-dose therapy with Mtx results in plasma concentrations of 1,700 μM 1 h pi, followed by a rapid decrease that can be accelerated by dialysis or diuretics. After 5 to 6 days, concentrations of less than 0.3 μM were measured after hemodialysis. The facilitators hOAT1 and 3 are expressed at the basolateral membrane and have Km values of 554 and 21 μM for Mtx (40). Therefore, it seems likely that these facilitators play a major role during the first hours after infusion when high plasma levels and concentration gradients are still present. With decreasing drug concentrations, Mtx uptake into the kidney epithelium should be shifted toward the pathways mediated by the human ortholog of RK-MTX-1 and hOAT3. Importantly, RK-MTX-1 is able to mediate the sodium-dependent uptake of the drug against a concentration gradient in contrast to hOAT3. For effective renal secretion, the serosal uptake of Mtx into epithelial cells must be followed by export at the apical side. The transport proteins present there are MRP2, Km = 2.5 to 3 mM (1); MRP4, Km = 220 to 1,300 μM (4, 41); and hOAT4, Km = 18 μM (40). Interestingly, the carriers with high Km values (MRP2, 4) are energy driven and can work against a concentration gradient, whereas hOAT4 with a lower Km is solely facilitating transmembrane flux. It may therefore be speculated that the system hOAT1, 3/MPR2, 4 (influx/extrusion) is relevant at high plasma levels, whereas MTX-1/hOAT4 (import/export) is more prevalent at lower concentrations.

In conclusion, RK-MTX-1 is distinct from the hitherto cloned Mtx-transporting proteins of the OAT, OAT-K, and MRP families as we observed fundamental differences in sequence, ion, and energy dependency as well as the spectrum of substrates and inhibitors. In contrast to the homologous murine and human rfc1 proteins, MTX-1 is strictly sodium dependent. Whether this sodium dependency is due to changes in the amino acid sequence, or the result of tissue-specific modulation as suggested for murine rfc1 by Wang et al. (43), together with the observation that MTX-1 expression increases the susceptibility to Mtx, MTX-1 can be classified as an import carrier that mediates cellular uptake of its substrate and, as a result of its basolateral localization in kidney epithelial cells, contributes to renal elimination by tubular secretion.

For detailed characterization of the substrate specificity of MTX-1, we expressed the rat kidney cDNA in MDCK cells. In these cells, the Mtx uptake is sodium dependent to more than 90%. In accordance with previous results obtained on freshly isolated rat hepatocytes (14), Mtx uptake was inhibited by BSP, bile acids, and reduced folates like dihydro- and tetrahydrofolate, whereas folate as well as acetylsaliclyate and PAH were only weak inhibitors. Previously, cholate and taurocholate were classified as competitive inhibitors of Mtx uptake into hepatocytes (14). Therefore, we tested these as potential substrates. However, none of the two substances was taken up by RK-MTX-1-transfected MDCK cells. With the exception of Mtx itself, only 5-methyltetrahydrofolate showed a very strong inhibitory effect at a concentration of 50 μM. As previous studies described sodium-independent (10, 24) and sodium-dependent carrier systems for Mtx and 5-methyltetrahydrofolate in the liver (22), it might be speculated that 5-methyltetrahydrofolate is the physiological substrate of the sodium-dependent Mtx carrier. Expression of rat liver cDNA in X. laevis oocytes (13) resulted in an active bumetanide uptake, whereas the corresponding renal clone was inactive in MDCK cells. This discrepancy is likely to be due to an artifact of the oocyte expression system. A similar observation has been reported for OATP8, which accepted different bile salts when expressed in oocytes but not in MDCK cells (16, 17). In addition, bumetanide was identified to be only a noncompetitive inhibitor of Mtx uptake in primary hepatocytes (14).

Subcellular fractionation and immunofluorescence studies clearly identified MTX-1 as a membrane protein localized within or at least near the outer plasma membrane. This would be consistent with glycosylation as the suggested apparent molecular weight that exceeds the predicted value. Moreover, the sorting of this transporter was restricted to the basolateral side in polarized multicellular tubules of MDCK epithelial cells. The validity of this model was confirmed earlier by Simmons et al. (36), who showed correct sorting of the apical transporter P-glycoprotein to the luminal membrane, resulting in secretion of the substrate into the tubule lumen. Taken together with the observation that MTX-1 expression increases the susceptibility to Mtx, MTX-1 can be classified as an import carrier that mediates cellular uptake of its substrate and, as a result of its basolateral localization in kidney epithelial cells, contributes to renal elimination by tubular secretion.
remains unclear. The clinical relevance of the renal sodium-dependent Mtx carrier described here will also need to be elucidated further.

ACKNOWLEDGMENTS

The authors thank Prof. W. F. Flintoff, Department of Microbiology and Immunology, University of Western Ontario, London, Ontario, for the generous gift of the cDNA clone pMrx9, which allowed us to initiate this project. [H]PAH was supplied by the Center of Physiology and Pathophysiology, Georg August University Göttingen and [H]obain by the Institute for Biochemistry and Endocrinology, Justus Liebig University of Gießen. We thank N. Thomsen and C. Lakoma for excellent technical assistance. Part of the work was conducted at the Institute of Pharmacology and Toxicology, Justus Liebig University Gießen. Confocal laser-scanning microscopy was performed at the laboratory of Dr. U. Rothe, Institute for Physiological Chemistry, Martin Luther University Halle Wittenberg.

GRANTS

This study was supported by the Deutsche Forschungsgemeinschaft (Graduiertenkolleg “Molekulare Veterinaarmedizin” to K. U. Honscha) and a grant to W. Honscha (HO2103/1–1). The nucleotide sequence reported in this paper has been submitted to the National Institute of Biological Information/GenBank under accession number AF173642.

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AJP-Renal Physiol. • VOL 286 • MARCH 2003 • www.ajprenal.org


