The H^+-dependent reduced folate carrier 1 of humans and the sodium-dependent methotrexate carrier-1 of the rat are orthologs

Carsten Kneuer, Walther Honscha*

Institute of Pharmacology, Pharmacy and Toxicology, An den Tierkliniken 15, University of Leipzig, 04103 Leipzig, Germany

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Abstract Previously, two different carrier systems for uptake of reduced folates and the antifolate methotrexate (Mtx) were described: the pH-dependent folate sensitive reduced folate carrier 1 (RFC1) from human, hamster and mouse and a sodium-dependent and folate insensitive Mtx carrier-1 (MTX-1) from rat. It was found that all critical residues of the homologous amino acid sequence were identical. RFC1- as well as MTX-1-mediated uptake of a marker substrate into suitable human and rat cell lines increased with proton concentration, was sodium-dependent at neutral pH, and inhibited by folate at acidic pH. It is concluded that RFC1 and MTX-1 are orthologs.

Keywords: Reduced folate carrier 1; Methotrexate carrier-1; Folate; Methotrexate; Fluorescein-methothrexate; Transport

1. Introduction

It has long been established, that the uptake of hydrophilic antifolates like methotrexate (Mtx) and reduced folates including tetrahydrofolate and 5-formyltetrahydrofolate into mammalian cells is mediated by an active (reduced folate) carrier system [1]. Changes in the reduced folate transport capacity were linked to antifolate resistance in tumor therapy [2]. In 1994, the isolation of a cDNA coding for the reduced folate carrier protein (RFC1) from mouse and hamster by expression cloning in a transport deficient cancer cell line was reported [3,4]. The authors utilized the increased resistance against the lipophilic antifolate trimetrexate that was conferred by RFC1 mediating the cellular uptake of antagonistic 5-formyltetrahydrofolate or the ability to grow in low (2 nM) folinic acid medium as selection marker. Using this sequence as probe, the human homolog (hRFC1) was isolated from a cDNA library [5,6]. In the course of its functional characterization, it was found that RFC1 function may be modulated by the tissue environment. While a pH optimum around 7.5 was reported in the L1210 leukemia cells [7], reduced folates and Mtx were more efficiently transported at acidic pH in liver and intestinal cells, and this transport was inhibited by folic acid (FA) at pH 5.5 but not pH 7.4 [8,9]. Sodium dependency of RFC1 was until now not shown.

In rat, a similar carrier system for Mtx with sensitivity to reduced folates was demonstrated and cloned [10,11]. The activity of this transporter was described as strictly sodium-dependent when examined in freshly isolated hepatocytes, where it accounts for 90% of the Mtx uptake, as well as after heterologous expression. It was therefore termed as the rat sodium-dependent Mtx carrier-1 (MTX-1). So far, there are no reports on the pH/proton dependency of this protein. Further, there is apparently no inhibition of transport by FA, at least at neutral pH [10,12]. This is in contrast to the observations made with human and mouse RFC1, which are inhibited by FA, at least at acidic pH.

Therefore, we systematically compared the sequences of human and mouse RFC1 with rat MTX-1 giving special consideration to amino acids that have been linked to substrate binding or transport. Furthermore, a human and a rat cell line expressing high levels of RFC1 or MTX-1 activity, respectively, were analyzed with regard to influence of pH, sodium dependency and inhibition by FA at acidic pH.

2. Materials and methods

All chemicals including media and supplements were obtained from Sigma–Aldrich (Deisenhofen, Germany) unless stated otherwise.

2.1. Cell culture and uptake experiments

The human adenocarcinoma cell line Caco-2 was purchased from ATCC (Rockville, MD) and the rat hepatocytoma cell line HPCT-1E3 was donated by E. Petzinger (Institute of Pharmacology and Toxicology, Justus-Liebig-University Giessen, Germany). Caco-2 cultures were grown in DMEM containing 10% FCS (Greiner, Frickenhausen, Germany), 2 mM glutamine and 1× non-essential amino acids, while HPCT-1E3 medium was further supplemented with 10 μg/ml insulin, 10 μg/ml inosin and 1.5 μM dexamethasone. Upon confluency, the cells were sub-cultured using trypsin/EDTA for detachment.

For uptake studies, freshly trypsinized cells were seeded onto 96 well plates at a density of 150 000 (Caco-2) or 45 000 (HPCT-1E3) cells/cm^2 and grown until cells displayed a differentiated phenotype after 5 (Caco-2) or 3 (HPCT-1E3) days. The medium was replaced for a 15 min equilibration period with Tyrode salt solution (137 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 5.5 mM glucose) or sodium-free Choline-Tyrode salt solution (137 mM choline chloride, 3 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 5.5 mM glucose) buffered with 20 mM MES (pH 5.0–6.5) or 20 mM HEPES (pH 7.0–8.0). The transport substrate fluorescein-methotrexate (FMTX, Molecular Probes, Eugene, NL) was added at a concentration of 10 μM and uptake was allowed for 30 min at 37 °C. Linearity was shown in a preliminary experiment over 5, 10, 20, 30 and 60 min. Incubation was stopped by transfer onto ice and two washing steps with ice-cold PBS. Finally,

* Corresponding author. Fax: +49-341-9738149.
E-mail address: honscha@vetmed.uni-leipzig.de (W. Honscha).

Abbreviations: RFC1/rfc1, reduced folate carrier 1; MTX-1, methotrexate carrier-1; Mtx, methotrexate

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cells were lysed with 1% SDS in PBS and assayed for fluorescence in a suitable microplate reader. The buffering capacity of the washing and lysis solutions did compensate pH effects of the incubation and avoided artifacts due to the pH dependency of FMTX fluorescence. FMTX uptake over 30 min was expressed as picomoles per well using a standard.

For inhibition studies, FA was added at various concentrations to the equilibration as well as the incubation buffer.

2.2. Sequence analysis

Amino acid sequences for human RFC1, rat MTX-1, mouse and hamster RFC1 were obtained from NCBI. A multiple sequence alignment was performed using DNAStar MegAlign v5.06 (DNAStar Inc., Madison, WI) according to the method of Hein [13] with a gap penalty of 11, a gap length penalty of 3, a Ktuple of 2 and residue weighting according to the PAM250 table. Sequence similarity was expressed pairwise as percentage of identical residues.

2.3. Statistical analysis

Uptake activities are given as means ± SD of at least six replicate measurements. The significance of the results was determined using the *F*-test. Statistical significance was assumed at P values of <0.05.

3. Results and discussion

3.1. Sequence similarity

Comparison of human, mouse and hamster RFC1 with rat MTX-1 revealed 66–67% amino acid identity between human RFC1 on one, and RFC1 of mouse, hamster and rat MTX-1 on the other side (Table 1). The percentage of sequence identity between the proteins from rat, mouse and hamster was considerably higher, with values between 80% and 90%. This relation strongly suggests that the rat MTX-1 is more closely related, from the phylogenetic view, to the mouse and hamster RFC than these are to the human carrier. This pattern also questions the distinction which has been made between RFC1 and MTX-1 on the functional level.

Table 1
Amino acid sequence similarity of human RFC1, rat MTX-1 and mouse and hamster RFC1

[% identity]	Human RFC1	Rat MTX-1	Mouse RFC1	Hamster RFC1
Human RFC1		66.7	67.3	66.1
Rat MTX-1			90.0	83.6
Mouse RFC1				80.6
Hamster RFC1				

A multiple sequence alignment according to the Hein method was performed using DNAStar. Sequence similarity is expressed pairwise as percentage of identical residues.

Therefore, we further examined the amino acids at positions which were known to be involved in protein functionality from earlier analyses of human and mouse RFC1 mutants. However, of the 33 amino acids that have been identified as important for Mtx transport by RFC1 [14–19] only Ser 317 was exchanged for an alanine in rat MTX-1 (Fig. 1). Replacement of Ser 317 by the bulky amino acid phenylalanine was reported to reduce the transport of Mtx more than 100-fold [17]. As Ser 317 is predicted to be localized within transmembrane domain 8, where it may form part of an aqueous channel, it appears likely that replacement by phenylalanine would cause pore blocking while an exchange with alanine should be of minor significance.

In accordance, the apparent affinities of human and mouse RFC1 and rat MTX-1 for Mtx that have been determined in uptake studies are very similar. For human RFC1, K_m or K_t values (t for transport) between 3.15 and 5.1 μM were reported in hRFC1 transfectants and leukemia cells expressing endogenous RFC1 [5,19,20]. For mouse RFC1, the K_m/K_t values were in essentially the same range 3.1–7.5 μM [15,16,18], while they were only little higher for rat MTX-1: 23 μM in isolated hepatocytes, 41 μM in transfected MDCK cells [10,12].

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1   MVPSSPAVEKQVPVEPGPDPELRSWRHLVCYLCFYGFMAQIRPGESFITPYLLGPDKNFTREQVTNEITPVLSYSYLAVL human RFC1
1   MVPTGQVAEKQACEEPRQDRELKSWRWLVFYLCFFGFMAQLRPGESFITPYLL--ERNFTKEQVTNEIIPMLPYSHLAVL rat MTX-1
1   MVPTGQVAEKQAYEEPRQDHELKSWRCLVFYLCFFGFMAQLRPGESFITPFLL--ERKFTKEQVTNEIIPMLPYSHLAVL mouse rfc1

81  VPVFLLTDYLRYTPVLLLQGLSFVSVWLLLLLGHSVAHMQLMELFYSVTMAARIAYSSYIFSLVRPARYQRVAGYSRAAV human RFC1
79  VPIFLLTDYLRYKPVLVLQCLSFVCVWLLLLLGTSVVHMQLMEVFYSITMAARIAYSSYIFSLVQPSRYQRMASYSRAAV rat MTX-1
79  VPVFLLTDYLRYKPVLVLQCLSFVCVWLLLLLGTSVVHMQLMEVFYSVTMAARIAYSSYIFSLVHPSRYQRMASYSRAAV mouse rfc1

161 LLGVFTSSVLGQLL--VTVGRVSFSTLNYISLAFLTFSVVLALFLKRPKRSLFFNRDDRGRCETSASELERMNPGPG--- human RFC1
159 LLGVFISSVLGQVL--VTLGGISTYMLNCISLGFILFSLSLSLFLKRPKRSLFFNRSALVQ-GALPCELDQMHPGPGRPE rat MTX-1
159 LLGVFISSVLGQAL--VTVGHISTYTLNCVSLGFILFSLVLSLFLKRPKRSLFFNRSTLAR-GALPCELDQMHPGPDRPE mouse rfc1

236 -GKLGHALRVACGDSVLARMLRELGDSLRRPQLRLWSLWWVFNSAGYYLVVYYVHILW-NEVDPTTNSARVYNGAADAAS human RFC1
236 PRKLERML-GTCRDSFLVRMLSELVKNVRQPQLRLWCLWWVFNSAGYYLITYYVHVLWK-----ITDSRLNYNGAVDAAS rat MTX-1
236 TRKLDRML-GTCRDSFLVRMLSELVENARQPQLRLWCLWWVFNSSGYYLITYYVHVLWR-----STDSSLSYNGAVDAAS mouse rfc1

314 TLLGAITSFAAGFVKIRWARWSKLLIAGVTATQAGLVF---LLAHTRHPSSIWLCYAAFVLFRGSYQFLVPIATFQIASS human RFC1
310 TLLSAITAFTAGFVNIRWALWSKLVIASVIAIQAGLVFCMF------QIPDIWVCYVTFVLFRGAYQFLVPIATFQIASS rat MTX-1
310 TLLSAITSFSAGFLSIRWTLWSKLVIAGVIAIQASLVFCMF------QIRDIWVCYVTFVLFRGAYQFLVPIATFQIASS mouse rfc1

391 LSKELCALVFGVNTFFATIVKTIITFIVSDVRGLGLPVRKQFQLYSVYFLILSIIYFLGAMLDGLRHCQRGHHPRQPPAQ human RFC1
384 LSKELCALVFGINTFLATALKTSITLVVSDKRGLGLQVHQQFRIYFMYFLTLSIICLAWAGLDGLRYYRRGRHQPLAQAQ rat MTX-1
384 LSKELCALVFGINTFLATALKTCITLVVSDKRGLGLQVRDQFRIYFIYFLMLSITCFAWAGLDGLRYCQRGRHQPLAQAQ mouse rfc1
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Fig. 1. Alignment of the amino acid sequence of human RFC1, rat MTX-1 and mouse RFC1. Multiple sequence alignment was performed using MegAlign v5.06 (DNAStar Inc.) according to the method of Hein with a gap penalty of 11, a gap length penalty of 3, a Ktuple of 2 and residue weighting according to the PAM250 table. Positions known to influence rfc function when mutated are shaded in gray and framed and marked with an asterisk when not identical in all three proteins.

3.2. pH and sodium dependency of FMTX uptake in Caco-2 and HPCT-1E3 cells

So far, pH dependency of RFC1 and sodium dependency of MTX-1 was thought to be a major functional criterion for the distinction of the two proteins. We therefore compared the pH dependency of FMTX uptake into human Caco-2 and rat HPCT-1E3 cells. FMTX is an easily detectable marker substrate of RFC1 and MTX-1 and the Caco-2 cells show an enterocyte-like phenotype with high RFC1 expression [21], while HPCT-1E3 cells are a good model for the study of rat MTX-1 [10]. Although carriers of the OAT and human OATP families are also known to transport FMTX and Mtx, its uptake into Caco-2 and HPCT cells is dominated by RFC1 or rat MTX-1, respectively, as a preliminary analysis showed. RT-PCR detected no transcripts of OAT1-4 and OATP1B1, and very low levels of OATP1B3 in Caco-2 cells, while RFC1 was expressed strongly. In HPCT cells, OAT2 was found in addition to MTX-1. However, Mtx uptake in this cell type was typically 60–95% sodium-dependent, which is not a property of OAT-mediated uptake. Furthermore, OAT inhibition with up to 1 mM salicylate never resulted in more than 25% reduction on FMTX uptake.

As depicted in Fig. 2, the human and the rat cell line showed increased activity at acidic pH. In both cases, the level of substrate uptake at pH 5.5 was approximately 3-fold higher than at pH 7.5. Between pH 5.5 and 6.5, FMTX uptake into Caco-2 showed a pronounced drop, while uptake into HPCT decreased more gradually in the range between pH 5.5 and 7.5. The observations confirm the well established pH dependency of human RFC1 and, for the first time, demonstrate such a dependency of rat MTX-1.

We further re-evaluated the sodium dependency of both carriers at neutral and acidic pH (Fig. 3). Interestingly, this uptake activity was clearly sodium-dependent at neutral pH, but not at acidic pH (pH 5.5) in Caco-2 cells. The same observations were made using the rat cell line HPCT-1E3. While the sodium dependency of rat MTX-1 at pH 7.5 is only a confirmation of previous knowledge, this is the first report of the influence of sodium on FMTX uptake by RFC1 in a human cell line. The mechanism behind this effect of sodium on FMTX uptake by RFC1 and MTX-1, as well as the reason for the obviously different situation at acidic pH remains a subject of speculation. Possibly, a sodium-proton exchange may be

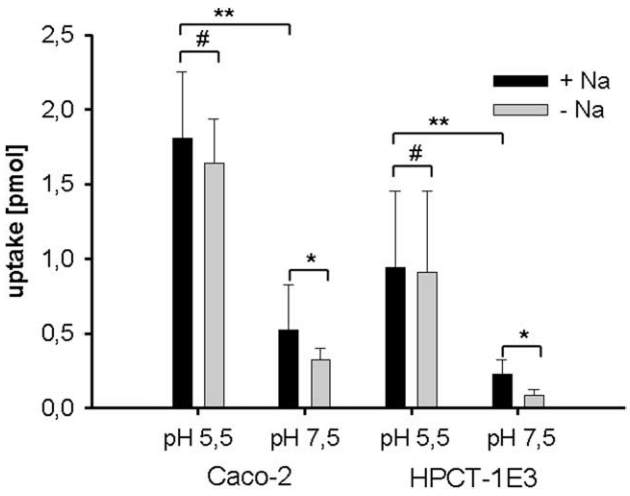

Fig. 3. Influence of pH and sodium on RFC1 and MTX-1-mediated FMTX uptake into Caco-2 and HPCT-1E3 cells. (# not significant, * $P < 0.05$, ** $P < 0.01$) Cells were seeded at a density of 50 000 (Caco-2) or 15 000 (HPCT-1E3) per well and grown until they displayed a differentiated phenotype. Medium was replaced with Tyrode salt solution (137 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 5.5 mM glucose) buffered with 20 mM MES (pH 5.5) or 20 mM HEPES (pH 7.5) or sodium free choline Tyrode salt solution. FMTX (10 μM) was added after 15 min of equilibration and uptake over 30 min was quantified by fluorescence spectrophotometry ($n \geqslant 6$).

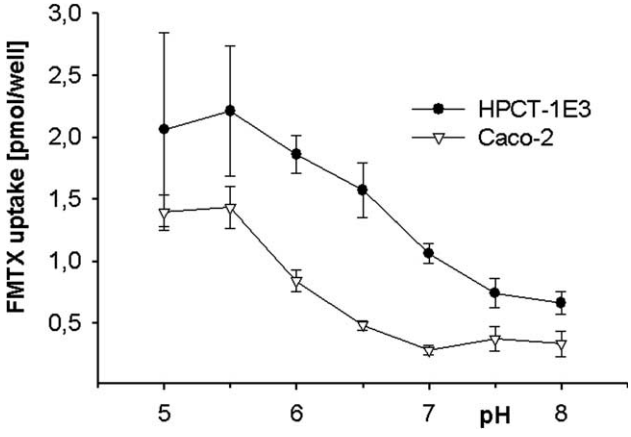

Fig. 2. pH dependency of FMTX uptake into Caco-2 and HPCT-1E3 cells. Cells were seeded at a density of 50 000 (Caco-2) or 15 000 (HPCT-1E3) per well and grown until they displayed a differentiated phenotype. Medium was replaced with Tyrode salt solution (137 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 5.5 mM glucose) buffered with 20 mM MES (pH 5.0–6.5) or 20 mM HEPES (pH 7.0–8.0) and 10 μM FMTX was added after 15 min of equilibration. Uptake was quantified after 30 min by fluorescence spectrophotometry ($n \geqslant 6$).

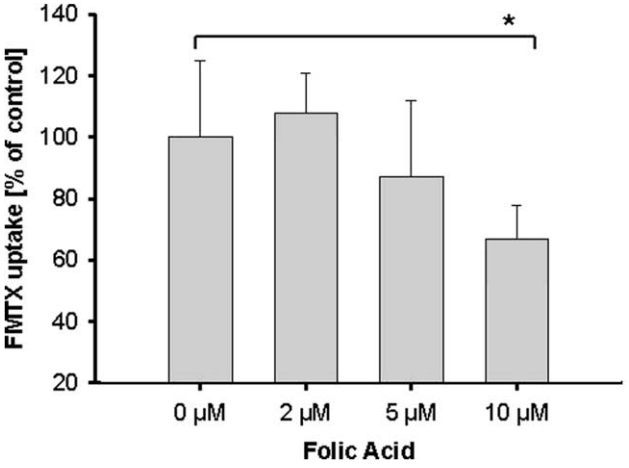

Fig. 4. Inhibition of MTX-1-mediated FMTX uptake into HPCT-1E3 cells by FA at pH 5.5. (* $P < 0.05$) HPCT cells were seeded at a density of 15 000 per well and grown for 3 days until they displayed a differentiated phenotype. Medium was replaced with Tyrode salt solution (137 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5.5 mM glucose and 20 mM MES, pH 5.5) containing increasing concentrations of FA. FMTX (10 μM) was added after 15 min of equilibration and uptake over 30 min was quantified by fluorescence spectrophotometry ($n \geqslant 6$).

required in a neutral environment to build a local proton or OH^- gradient necessary to drive the transport process [22].

3.3. FA inhibits FMTX uptake by rat MTX-1 at acidic pH

Previously, FA was not classified as inhibitor of rat MTX-1, but known to decrease Mtx and FMTX uptake by the RFC. In fact, incubation with 100 μM FA did not result in measurable inhibition of Mtx uptake in rat liver hepatocytes at pH 7.4 [10], while FA is a known substrate and inhibitor of RFC1 in cells of non-hematopoietic origin, at least under acidic conditions [8,9]. To resolve this apparent discrepancy, we tested the sensitivity of FMTX uptake to FA at acidic pH in the MTX-1 expressing HPCT-1E3 rat hepatocytoma cells. As shown in Fig. 4, there was a concentration-dependent and significant inhibition of MTX-1 activity at pH 5.5. Similar concentrations (10 μM) did not have any effect on FMTX uptake at neutral pH in HPCT-1E3 cells nor in MDCK canine kidney cells that were transfected with recombinant rat MTX-1 [12].

In conclusion, rat MTX-1 is more closely related to mouse and hamster RFC1 than these are to human RFC1 on the amino acid level. The only amino acid exchange at a sensitive position was Ser317Ala. Although it remains unclear whether this exchange is of similar significance as the Ser317Phe transition that reduced the activity of mouse RFC1 more than 100-fold, our functional comparison did so far not reveal a major effect. Furthermore, the apparent affinities of human and mouse RFC1 and rat MTX-1 for Mtx are in the range 3–41 μM. Finally, human RFC1 activity has now been recognized to be sodium-dependent at neutral pH in a cellular context, as it was known for MTX-1. Vice versa, rat MTX-1 activity is promoted at acidic pH, where it is also sensitive to FA, as reported for RFC1. On the basis of these five overlapping properties, we suggest to regard MTX-1 as the rat ortholog of human RFC1.

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