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Localization of the murine reduced folate carrier as assessed by immunohistochemical analysis

Yanhua Wang, Rongbao Zhao, Robert G. Russell, I. David Goldman *

Departments of Medicine and Molecular Pharmacology, Albert Einstein Comprehensive Cancer Center, Albert Einstein College of Medicine, Chanin Two, 1300 Morris Park Avenue, Bronx, NY 10461, USA

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Abstract

The reduced folate carrier (RFC1) is a major route for the transport of folates in mammalian cells. The localization of RFC1 in murine tissues was evaluated by immunohistochemical analysis using a polyclonal antibody to the C-terminus of the carrier. There was expression of RFC1 in the brush-border membrane of the jejunum, ileum, duodenum and colon. RFC1 was localized to the basolateral membrane of the renal tubular epithelium. Carrier was detected on the plasma membrane of hepatocytes but not in bile duct epithelial cells. In the choroid plexus RFC1 was highly expressed at the apical surface. It was also expressed in axons and dendrites and on the apical membrane of cells lining the spinal canal. In spleen, RFC1 was detected only in the cells of the red pulp. These data provide insights into the role that RFC1 plays in folate delivery in a variety of tissues. In particular, the localization of carrier may elucidate the role of RFC1 in the vectorial transport of folates across epithelia. The data also indicate that in kidney tubules and choroid plexus the sites of RFC1 expression are different from what has been reported previously for the folate receptor; and while RFC1 is expressed in small intestine, folate receptor is not.

Keywords: Reduced folate carrier; Folate carrier localization; Folate carrier immunohistochemistry

1. Introduction

Folates are key one-carbon donors critical to the synthesis of purines, thymidylate and methionine [1]. Folate-dependent enzymes have long been exploited as chemotherapeutic targets for antimetabolites – classically methotrexate (MTX), a potent inhibitor of dihydrofolate reductase [2]. More recently antifolates have been developed that are direct inhibitors of folate-dependent enzymes required for purine and thymidylate biosynthesis [3–5]. The membrane transport of folates and antifolates has been an area of considerable interest because of the role that these processes play in intestinal folate absorption, the delivery of physiological folates to peripheral tissues and as determinants of tumor cell cytotoxicity, selectivity and resistance to antifolates [6,7].

Folates are transported into mammalian cells via several distinct systems. Folate receptors mediate transport by an endocytotic, energy-requiring process and several folate receptor isoforms have been cloned from human and murine tissues [6,8–11]. The re-

Abbreviations: RFC1, the reduced folate carrier; PBS, phosphate-buffered saline; MTX, methotrexate; MRP, multidrug resistance protein

* Corresponding author. Fax: +1-718-430-8550;
E-mail: igoldman@aecom.yu.edu

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duced folate carrier (RFC1), a classical facilitative mechanism, delivers folates to hematopoietic and a variety of other tissues and plays a key role in mediating transport of antifolates [2,7,12]. This system is capable of producing uphill transport of folates into cells via an anion exchange mechanism with organic phosphates that are concentrated within cells [13]. There are, in addition, transport routes for folates that operate optimally at low pH [14,15] along with families of organic anion transporters some of which have affinities for folates in the range of those observed with RFC1 [16–19]. Folates and antifolates are also substrates for energy-dependent exporters [20,21]. Cells that express multidrug resistance proteins (MRPs) are resistant to MTX as well as other drugs and in some cases export of MTX has been demonstrated [22–25].

The distribution of folate receptors in the tissues of different species has been demonstrated by immunohistochemistry [26–31]. Although the functional properties of RFC1 have been studied in detail in many different cell systems, little is known about the tissue distribution of RFC1 protein and its disposition within cells beyond its apical localization in retinal pigment epithelium [29]. This report is the first description of RFC1 protein localization in mouse tissues.

2. Materials and methods

2.1. Mouse

C57BL/6 adult mice (18–22 g) were fed a normal diet and killed by cervical dislocation. The intestines were immediately removed and flushed repeatedly with ice-cold 0.01 M PBS buffer. Jejunum, duodenum, ileum and colon were cut into small fragments, laid onto foil, and quickly placed into a deiced solution. Liver, spleen and kidney were also cut into blocks and treated the same way. The skull was opened and the whole brain, with the very upper part of spinal cord, was removed gently and placed in deiced solution. All the samples were then stored at −80°C before processing for continuous cryosectioning at 5-μm thickness. The brain was similarly cryosectioned from cerebrum to medulla oblongata and into the proximal spinal canal.

2.2. Antibody

This laboratory has developed a polyclonal antibody, AE390, targeted to the distal C-terminus of mouse RFC1 (Met^{499} through Ala^{512}). The peptide was synthesized by the Proteomics Facility at the Albert Einstein College of Medicine Comprehensive Cancer Center and coupled to keyhole limpet hemocyanin (Pierce). Antisera was produced in rabbits by Covance (Denver, PA) and antibody was purified on an affinity column to which the corresponding peptide antigen was covalently linked to SulfoLink Coupling gel (Pierce) [32].

2.3. Western blot

A murine L1210 leukemia cell line (R16) with a high level of RFC1 expression [33] was used to assess the RFC1 antibody. Total lysate from 2×10^7 cells containing proteinase inhibitor (Sigma) was sonicated on ice for 20 s. The protein concentration was determined with the BCA Protein Assay kit (Pierce); 2.5 μg of protein was then utilized for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The loading buffer consisted of 62.5 mM Tris–HCl (pH 6.8) containing 10% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.00125% bromophenol blue without dithiothreitol. Protein was dissolved in the loading buffer at room temperature [32]. The samples were electrophoresed on 10% SDS–PAGE gels and transferred to PVDF filters. The blots were probed with affinity-purified rabbit anti-mouse RFC1 antibody (AE390) or preimmune serum, obtained from rabbits before immunization, followed by peroxidase-conjugated goat anti-rabbit IgG (Promega, Madison, WI). The blots were enhanced by chemiluminescence detection according to the manufacturer's instructions (Amersham).

2.4. Immunohistochemical staining

Cryosections were dried at room temperature, fixed in cold acetone for 20 min, then washed in phosphate-buffered saline (PBS, pH 7.4) for 15 min. Endogenous peroxidase activity was quenched by immersion of slides into 3% H_2O_2 for 15 min. After rinsing with PBS three times, tissue was incubated with 5% normal goat serum at room temperature

for 30 min before loading primary antibody (AE390) overnight at 4°C. Control slides were treated in the same way with preimmune serum. Each slide was then washed three times with PBS buffer, incubated with biotin-labeled goat anti-rabbit IgG, then washed thrice and incubated with an avidin–biotin complex. The reaction was enhanced with the TSA Signal Increasing System according to the kit instructions (NEN Life Science). The reaction was then developed with 3,3′-diaminobenzidine tetrahydrochloride. Slides were counterstained with Mayer's hematoxylin and mounted in cytoseal™ 280.

3. Results

3.1. Western blot

To confirm the specificity of the polyclonal antibody, total lysate from R16 cells was immunoblotted with the AE390 antibody. A single strong band was detected at 58 kDa which represents murine RFC1 [32]. No band was detected with the preimmune serum, indicating that the AE390 antibody was specific for RFC1 (Fig. 1).

3.2. RFC1 distribution and tissue localization

3.2.1. Intestine

The AE390 antibody produced a strong immunopositive signal localized to the brush-border membrane of jejunum (Fig. 2A). The brush-border membranes of ileum, duodenum and colon were also positive for the AE390 antibody (not shown). No staining was detected in the sections incubated with the preimmune serum. No signal was detected in the lamina propria, muscularis mucosa, submucosa, muscularis externa, or smooth muscle cells of the large and small intestine.

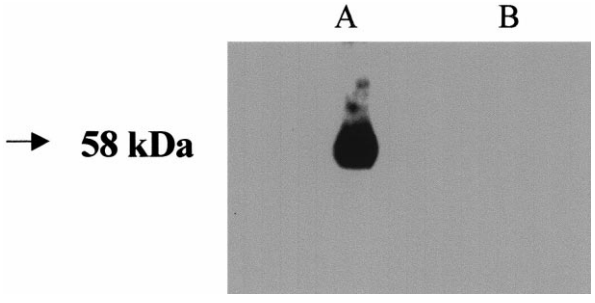

Fig. 1. Specificity of the AE390 antibody against the mouse RFC1 C-terminus by Western blot analysis. RFC1 from total R16 cell lysate was immunoblotted by AE390 (A) or the preimmune rabbit serum (B) (see Section 2.3).

3.2.2. Kidney

RFC1 was detected in the basolateral membrane of cortical (Fig. 2B) and medullary (not shown) renal tubular epithelial cells. The cellular distribution was most apparent from the oil-immersion high-power view (Fig. 2C). No signal was detected in the glomeruli.

3.2.3. Liver

RFC1 was evenly distribution on hepatocyte membranes within the liver lobule (Fig. 2D). No RFC1 signal was found in the bile duct epithelial or stromal cells. There was no reaction in the presence of the preimmune serum. RFC1 was not detected within the hepatocytes.

3.2.4. Brain

The choroid plexus is a polarized tissue with microvilli facing the cerebrospinal fluid and the basal membrane in proximity to the capillary bed. RFC1 was detected only on the brush-border membrane. Carrier was also detected in axons and dendrites (Fig. 2E inset). RFC1 was noted at the brush-border membrane of cells lining the spinal canal (not shown). No positive signal was detected with the preimmune serum.

3.2.5. Spleen

RFC1 was detected on the membrane of cells in the red pulp but not on the cells in the white pulp of the spleen (Fig. 2F). No signal was detected in spleen incubated with the preimmune serum.

4. Discussion

Dietary folates are delivered into the intestinal lumen as polyglutamates where they are hydrolyzed to monoglutamates then transported into enterocytes within the small intestinal villus. This is followed by transport across the basolateral membrane into the submucosa, diffusion into the portal capillary network, then transport to, and storage in, the liver

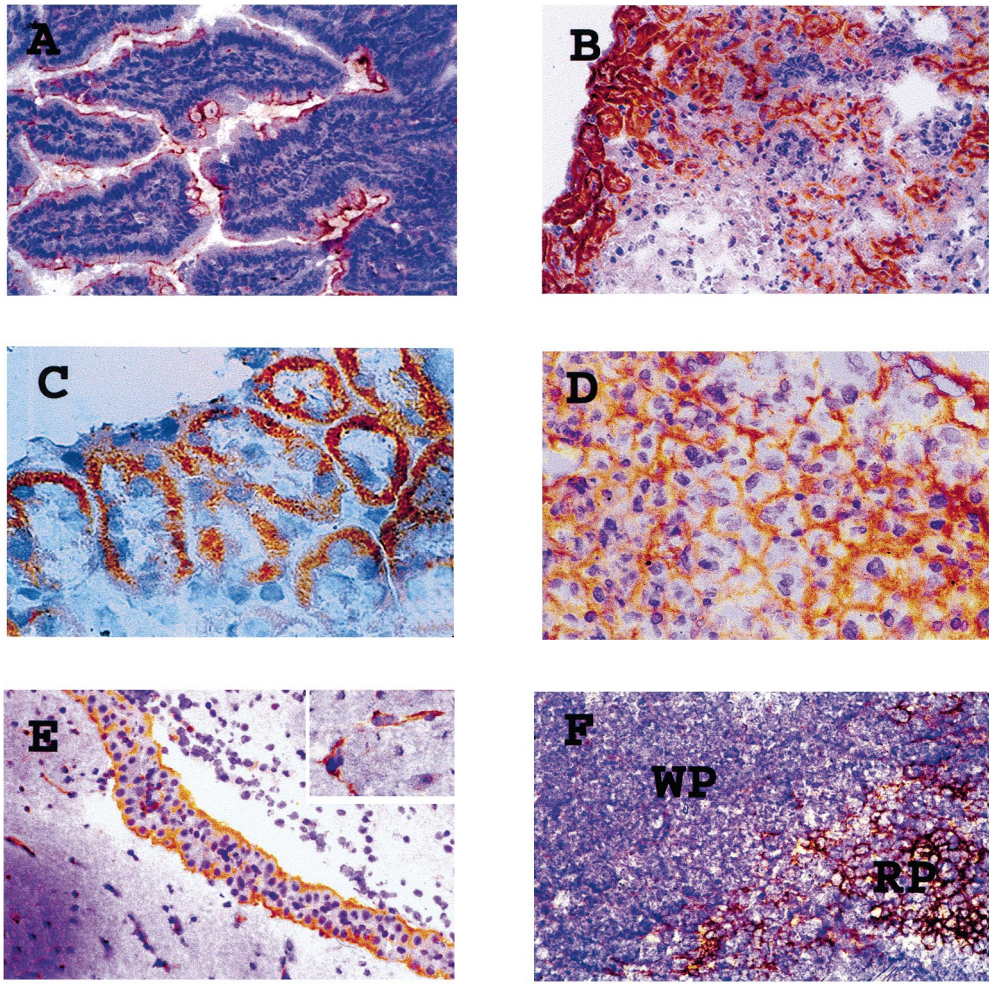

Fig. 2. Immunohistochemical analysis of mouse tissues exposed to RFC1 C-terminus antibody AE390. (A) Jejunum (250×); RFC1 localized to the brush-border membrane. (B) Renal cortex (250×); RFC1 localized to the tubules, not glomeruli. (C) Renal cortex (1000×) with RFC1 located at the basolateral membrane of renal tubule cells. (D) liver (400×); RFC1 signal is localized to the hepatocyte plasma membrane. (E) Choroid plexus (250×); RFC1 signal is on the brush-border membrane of choroid plexus epithelial cells; inset shows an RFC1-positive axon and dendrite (400×). (F) Spleen (100×); RFC1-positive cells are located in red pulp (RP) not in the white pulp (WP).

[34]. Folates are then released from liver and delivered via the systemic circulation to peripheral tissues. Folates delivered to the kidney are largely filtered at the glomerulus then reabsorbed by the renal tubular epithelial cells. Within the central nervous system folates are transported across the choroid plexus [35] and a gradient is maintained between blood and cerebrospinal fluid [36]. The goal of the present study was to determine the localization of RFC1 in several tissues that have a high requirement for folates, serve as storage sites, or are important to folate homeostasis. Of particular interest was the localization of RFC1 within cells as a basis for better understanding the role of this transporter in the processes that govern the vectorial flows of folates across epithelial tissues.

These studies demonstrate that RFC1 is expressed in many different tissues but the site of expression within cells varies from tissue to tissue. RFC1 is localized to the brush-border membrane of small and large intestinal epithelial cells with the most intense signal in the jejunum. Since RFC1 generates transmembrane gradients for folates across cell membranes [13,33,37] it is possible that transport across the apical brush-border membrane generates a small intracellular folate electrochemical potential with

subsequent downhill flow of folates across the basolateral membrane into the submucosal capillary network. In addition, MRP exporters that transport folates [38,39], (MRP2 is expressed in the basolateral membrane of murine intestinal epithelial cells [40]), could produce the major driving force for folate absorption by pumping folates out of cells into the submucosal space. Folate receptors are not expressed in the small intestine and hence play no role in the absorptive process [26,28,41].

The pattern of RFC1 distribution in the kidney was different from that of the gut. Carrier was expressed in the basolateral membrane. Murine folate receptors, on the other hand, are expressed at the brush-border membrane of renal tubular epithelial cells [26,31]. This suggests that the receptors play a major role in the extraction of folates from the glomerular filtrate and their delivery into renal tubular epithelial cells by endocytosis and that this is followed by RFC1 mediated transport across the basolateral membrane. This is a somewhat surprising sequence since folate receptor turnover is quite slow, indeed, an order of magnitude slower than that of RFC1 [42], and an unlikely mechanism to efficiently extract substrates from filtrate as it flows through the tubules. Besides these systems there is a family of organic anion facilitative carriers expressed in the kidney (OATK1, OATK2), that are localized to the brush-border membrane [17,18]. These transporters may also contribute to folate reabsorption in the kidney.

RFC1 expression was uniformly pronounced in the hepatocyte plasma membrane within the liver lobule as observed also for folate receptor [41]. It was not expressed in bile duct epithelial cells. It is of interest that transport of folates in isolated hepatocytes manifests properties that are different from RFC1-mediated transport in hematopoietic and other cells [43–45]. This may be due to tissue-specific factors that modulate RFC1 function as appears to occur in the intestine where RFC1 is predominantly a process with a low pH optimum and high affinity for folic acid whereas in hematopoietic cells transport is optimum at pH 7.4 and the affinity for folic acid is two orders of magnitude lower [46]. The canalicular multispecific organic anion transporter (cMOAT-MRP2) and MRP3 may play important roles in biliary excretion of folates [25,47].

There was high-level RFC1 expression on the apical membrane of the bulbous microvilli of the choroid plexus epithelium adjacent to the ventricular cavity. On the other hand, folate receptor is localized to the basolateral membrane of the choroid plexus [27]. RFC1 was also detected in the apical membrane of the epithelial cells lining the spinal canal and high-level expression was also found in dendrites and axons. The latter is of particular interest since high folate requirement is usually associated with rapidly proliferating cells. It may be that these one-carbon donors play other important roles in meeting the specialized needs of neural cells [48,49].

RFC1 was localized to the red pulp of the spleen, which consists primarily of venous sinusoids, splenic cords, and a variety of free cells of hematopoietic origin. This is consistent with the marked atrophy of the red pulp of the spleen and other hematopoietic organs in mice in which RFC1 has been inactivated by homologous recombination [50]. No RFC1 was detected in cells of the white pulp of the spleen, suggesting that resting T and B lymphocytes lack carrier expression. However, unlike resting cells, mitogen-activated lymphocytes are sensitive to MTX [51,52] and lymphoblasts express RFC1 and transport the drug [53,54]. It is of interest that folate receptors are found in lymph organs and some leukemia cells suggesting that transport by this route also plays a role in the delivery of folates to hematopoietic tissues [55–57].

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