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Rapid report

The reduced folate carrier in L1210 murine leukemia cells is a 58-kDa protein

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Abstract

The reduced folate carrier (RFC1) is a major transporter for both natural reduced folates and antifolate chemotherapeutics. Using polyclonal antibodies targeted to epitopes at the loop between the sixth and seventh predicted transmembrane domains or the distal C-terminus, we were able to demonstrate by Western blot analysis that the molecular size of RFC1 expressed in murine leukemia L1210 cells is 58 kDa as predicted by the open reading frame of its cDNA. 46- and 38-kDa proteins detected only in plasma membrane preparations were proteolytic degradation products that appeared during membrane preparation or treatment with the conventional SDS–PAGE loading buffer. These data resolve discrepancies reported previously for the molecular size of RFC1.

Keywords: Reduced folate carrier; Membrane proteolysis; Carrier proteolysis; Folate transport; Transport

The reduced folate carrier (RFC1) is a member of the major facilitator superfamily [1] and a major transport route for folates that are critical for one-carbon dependent biosynthetic reactions [2]. RFC1 mediates transport of a variety of antifolates as well and impaired membrane transport due to decreased expression, or mutations, of RFC1 is an important basis for intrinsic and acquired drug resistance [3–11]. Recently, RFC1 was also proposed to play a major role in folate absorption in small intestine [12,13]. Before RFC1 was cloned, affinity labeling was utilized to characterize the transporter. Using the *N*-hydroxysuccinimide ester of tritiated methotrexate (NHS-[^{3}H]MTX), a protein with a molecular weight of ~36 kDa was specifically labeled [14]. However, when N^{α}-(4-amino-4-deoxy-10-methylpteroyl)-N^{ε}-(4-azido-5-[^{125}I]iodosalicylyl)-L-lysine (APA-[^{125}I]ASA-Lys) was employed, a 46-kDa protein was identified, although a 38-kDa band was also sometimes visible on SDS–PAGE [15,16]. Affinity labeling with NHS-[^{3}H]aminopterin of an L1210 variant overexpressing this transporter also labeled a 46-kDa protein [17]. The murine RFC1 was cloned in 1994 and shown to encode a protein with a predicted molecular size of 58 kDa [18]. However, the peptide expressed when RFC1 cDNA was transfected into RFC1-null L1210 cells was also found to be a 46-kDa protein based upon Western blot analysis [8]. On the other hand, RFC1 expressed in murine small intestinal cells was identified as a 58-kDa protein [13].

This laboratory has developed polyclonal antibod-

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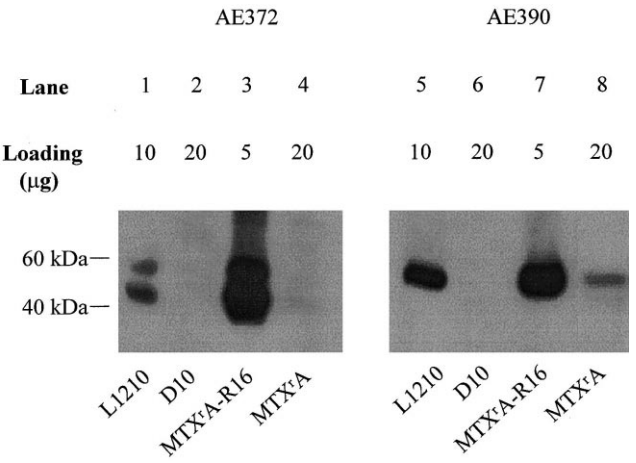

Fig. 1. A representative Western blot analysis of plasma membrane fractions using the AE372 antibody to the large loop between the sixth and seventh transmembrane domains, and the AE390 antibody to the C-terminus. Plasma membranes from L1210, D10, MTX^{r}A-R16 and MTX^{r}A cells were extracted as reported, except a protease inhibitor cocktail (P8340, Sigma) at a dilution of 1:1000 was used instead of 1 mM phenylmethylsulfonylfluoride [14]. Protein concentrations were determined with the BCA Protein Assay kit (Pierce). The membrane proteins were dissolved in a SDS–PAGE loading buffer (60 mM Tris, 10% glycerol (v/v), 2% SDS and trace bromophenol, pH 6.8) without heating at 95°C for 5 min and resolved on 12% SDS–polyacrylamide gel. The proteins were transferred to PVDF Transfer Membranes (Amersham) and processed by the ECL Plus Western blotting detection system from Amersham. The amounts of total plasma membrane protein loaded on the SDS–polyacrylamide gel are indicated in the figure. The data shown are representative of six separate experiments.

ies targeted to two different epitopes in RFC1. The AE372 antibody recognizes the loop between the predicted sixth and seventh transmembrane domains (Asp^{232} through Asp^{248}), while the AE390 antibody targets the distal C-terminus (Met^{499} through Ala^{512}) of RFC1. The peptides were synthesized by the Proteomics facility at the Albert Einstein College of Medicine Comprehensive Cancer Center and coupled to keyhole limpet hemocyanin (Pierce). Antisera were produced in rabbits by Covance (Denver, PA, USA) and the antibodies purified with affinity columns to which the corresponding peptide antigens were covalently linked to SulfoLink Coupling gel (Pierce). Cell plasma membranes were prepared by Dounce homogenization under hypotonic conditions and subsequent sucrose gradient centrifugation [14]. Protein concentrations were determined with the BCA Protein Assay kit (Pierce).

As shown in Fig. 1, lane 1, the AE372 antibody to the loop detected proteins at 58 and 46 kDa, while AE390 to the C-terminus detected only the 58-kDa protein (lane 5) in L1210 cells. Both proteins were missing in D10 cells (lanes 2 and 6), an MTX-resistant L1210 cell line which does not express RFC1 due to a homogeneous G→A mutation in the initiation codon of translation [9]. Protein was highly expressed in an RFC1-overproducing transfectant, MTX^{r}A-R16 (lanes 3 and 7), in which RFC1 activity and message were 9- and 5-fold greater than in L1210 cells, respectively [5,19]. Again, the 58- and 46-kDa proteins were detected with the loop antibody, but only the 58-kDa protein was identified with the C-terminus antibody. The amount of protein was decreased in MTX^{r}A cells from which MTX^{r}A-R16 was derived, as compared to that in L1210 cells. The signal produced with the AE390 antibody was stronger than that with the AE372 antibody. If MTX^{r}A-R16 samples were overloaded on SDS–PAGE, another product with a molecular weight of 12 kDa was detected only with AE390 antibody, presumably representing the C-terminus part of RFC1 (not shown). In Western blot analyses from six separate plasma membrane preparations from L1210 cells, the pattern of double bands with the AE372 antibody and a single band with the AE390 antibody was always observed; in two cases, the intensity of the 46-kDa band was much stronger than that of the 58-kDa band. These data, therefore, suggested that RFC1 encodes a protein with a molecular size of 58 kDa as predicted from its cDNA and the 46-kDa protein may be a proteolytic product lacking the C-terminus of RFC1 not detected by the AE390 antibody.

To establish whether partial degradation of RFC1 was associated with the method utilized to prepare the plasma membrane fraction, another technique was employed to determine if the degradation process could be minimized. Total lysates from MTX^{r}A-R16, L1210 and MTX^{r}A cells were prepared by brief sonication of intact cells and then analyzed by Western blots in the same way as the plasma membrane samples. As shown in Fig. 2, RFC1 protein expression from MTX^{r}A-R16 cells (lanes 1 and 4) was much higher than from L1210 cells (lanes 2 and 5). There was minimal detection of RFC1 in the MTX^{r}A cells (lanes 3 and 6). In all these cell lines,

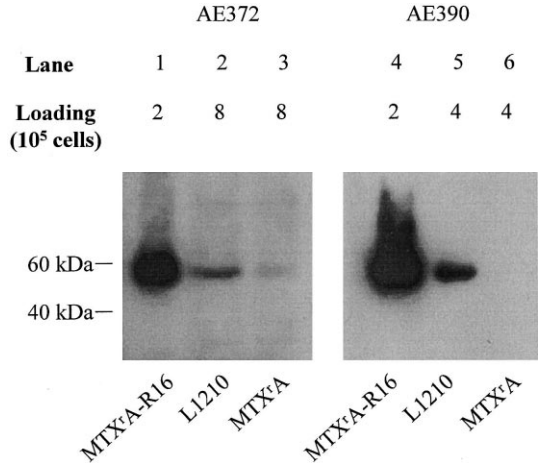

Fig. 2. Western Blot analysis of total lysates using the AE372 loop and AE390 C-terminus antibodies. MTX^{r}A-R16, L1210 and MTX^{r}A cells (3×10^7) were harvested, washed twice with HEPES-buffered saline (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose, pH 7.4) and suspended in 100 μl of the same solution containing 10 μl of protease inhibitor (P8340, Sigma). The cell suspension was sonicated for 20 s in a tube submerged in ice-water and mixed with 100 μl of 2×SDS–PAGE loading buffer (see the legend for Fig. 1) without heating. Western blot analysis was carried out according to the ECL Plus protocol from Amersham. The number of cells from which the lysate loaded on the SDS–polyacrylamide was obtained is indicated in the figure. Data are representative of two separate experiments.

however, only protein with a molecular size of 58 kDa was detected with the AE372 antibody. This was in contrast to the pattern found with the plasma membrane preparations (Fig. 1), consistent with the likelihood that 58-kDa protein was degraded during the process of plasma membrane isolation.

The conventional method of preparing samples for SDS–PAGE is to dissolve proteins in a loading buffer containing dithiothreitol (DTT) to break S–S bonds and heat the solution for 5 min at 95°C to denature the protein. However, both DTT and heat during preparation of samples profoundly affected the outcome of Western blot analysis of the plasma membrane samples (Fig. 3). When DTT was included in the loading buffer and the solution was heated at 95°C, the AE372 antibody identified only one protein at 38 kDa (lane 1), whereas AE390 labeled a weak band at 58 kDa (lane 5). When samples were not heated and DTT was omitted, there were bands at 46 and 58 kDa (lane 4) with the AE372 antibody and one strong band at 58 kDa with the AE390 antibody (lane 8) as in Fig. 1. The disappearance or decreased intensity of the 46- and 58-kDa bands with DTT and heat (lane 1 and lane 5) was probably due to oligomerization of proteins suggested by the smearing at higher molecular weights (lane 5). Oligomerization appeared to be accelerated by heating at 95°C, since the 46- and/or 58-kDa proteins were present when DTT was included but heating was omitted (lanes 2 and 6). Heat-activated oligomerization was not dependent on the presence of DTT as visualized in lane 7 of Fig. 3. Under these conditions, no specific proteins were identified. Hence, conditions in which heating and DTT were excluded were optimal for maximal preservation of RFC1 protein.

These observations may explain why only a ~38-kDa protein was detected when murine RFC1 was

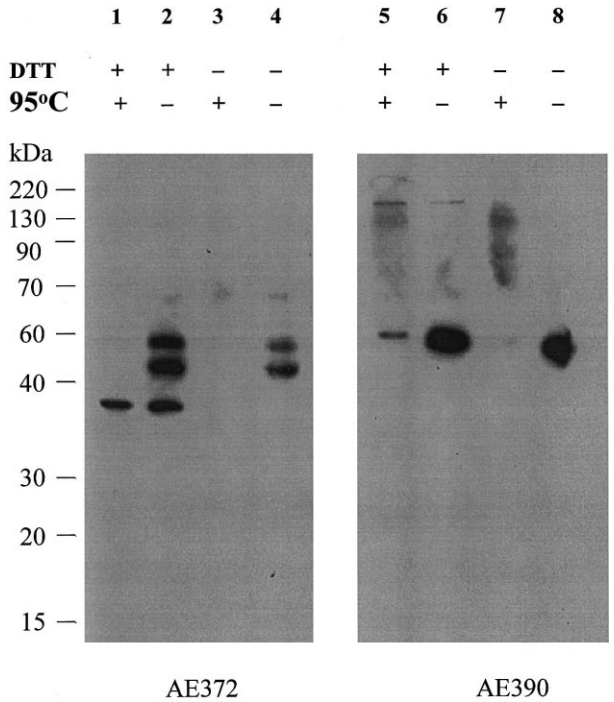

Fig. 3. Effect of DTT in the SDS–PAGE loading buffer and heating of the protein solution on Western blot analysis of plasma membrane fractions. L1210 cell plasma membranes (as described in the legend for Fig. 1) were treated with the SDS–PAGE loading buffer with or without addition of 1.5% DTT. The mixtures were either first heated at 95°C for 5 min then loaded on the 12% SDS–polyacrylamide gel, or loaded directly on the gel with heating. The subsequent steps for Western blot analysis follow the ECL Plus protocols from Amersham. The same amount of plasma membrane (10 μg) was loaded on each lane. The data are representative of the results from three separate experiments.

affinity-labeled with NHS-[^{3}H]MTX followed by SDS–PAGE with DTT and heating [14] or why when murine RFC1 was labeled with APA-[^{125}I]ASA-Lys, 38- and 46-kDa peptides were identified on SDS–PAGE in loading buffer containing DTT heated to only 60°C [15]. It is of interest that heating was omitted in preparation of samples for SDS–PAGE after affinity labeling of RFC1 in L1210 cells and its variant with NHS-[^{3}H]aminopterin [17]. Hence, it is highly likely that the 38-kDa protein represents a degradation product of RFC1 associated with DTT treatment, probably derived from the 46-kDa breakdown product and not from the intact 58-kDa RFC1. Hence, treatment of the total lysate, in which RFC1 was intact (Fig. 2), with DTT without heating did not result in the appearance of 38-kDa peptide (data not shown).

These findings resolve the discrepancies in observed molecular weights of RFC1 protein and suggest that the proteins detected in murine L1210 leukemia and small intestinal cells, which share a molecular mass of 58 kDa, are identical. However, RFC1-mediated influx in L1210 cells has an affinity for folic acid that is two orders of magnitude lower than that of folate transport in intestinal cells with an influx optimum at neutral pH as opposed to a low-pH optimum in intestine [12,13,20]. The basis for the very different transport properties in these tissues remains to be determined.

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