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Characterization of Folate Transport Mediated by a Low pH Route in Mouse L1210 Leukemia Cells with Defective Reduced Folate Carrier Function

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ABSTRACT. Folate influx at low pH was characterized in MTX^{r}A cells, an L1210 mouse leukemia cell line with a functional defect in the reduced folate carrier. Folic acid influx in MTX^{r}A cells was negligible at pH 7.5, increased 13-fold as the pH was decreased to 6.0, and was indistinguishable from that in L1210 cells. In contrast, while methotrexate (MTX) influx in MTX^{r}A cells at pH 6.0 was 15-fold higher than at pH 7.5, in L1210 cells it was decreased by half. Influx of MTX, folic acid, 5-methyltetrahydrofolate and 5-formyltetrahydrofolate in MTX^{r}A cells was increased at pH < 7.0, but their pH optima and profile differed substantially. Influx of MTX and 5-methyltetrahydrofolate at pH 6.0 showed saturability, with a K_t of 2.65 and 0.56 μM, and a V_{max} of 0.45 and 0.083 nmol/g dry wt/min, respectively. MTX influx mediated by the low pH transporter was insensitive to the anionic composition of the transport buffer and affected minimally (~20%) by Na^+ substitution. The anion transport inhibitors sulfobromophthalein, diisothiocyanatostilbene disulfonic acid, and acetamidoisothiocyanatostilbene disulfonic acid were not effective inhibitors of the low pH route. MTX transport at low pH did not increase in MTX^{r}A-R16 cells, an MTX^{r}A derivative with 10-fold overexpression of the reduced folate carrier (RFC) due to transfection with RFC1 cDNA. Inhibition of reduced folate carrier activity with acetamidoisothiocyanatostilbene disulfonic acid resulted in identical MTX influx in L1210, MTX^{r}A, and MTX^{r}A-R16 cells at pH 5.5. Finally, low pH-mediated MTX influx was reduced by energy inhibitors and partially inhibited by ionophores (nigericin > monensin ≫ valinomycin). The data indicate that L1210 and MTX^{r}A cells express similar activities of a low pH folate transporter that has properties distinct from, and independent of, the reduced folate carrier. BIOCHEM PHARMACOL **55**;9:1505–1512, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. folate; methotrexate; transport; reduced folate carrier; pH

Folate cofactors provide the one-carbon units required for the synthesis of thymidine, purines, glycine, and methionine. Because mammals are unable to synthesize folates, their transport across the plasma membrane is essential for cellular metabolism. Several folate transport systems have been described in eukaryotic cells [1–4]. Murine L1210 leukemia cells have been used extensively as a model system to characterize the mechanism(s) of folate and antifolate transport. RFC1† is the major folate influx route in L1210 cells [1, 2, 5–7] with a high affinity for reduced folates and the antifolate MTX but very low affinity for folic acid [1, 8, 9]. RFC1-mediated folate influx is bidirectional, concentrative, sodium independent, and inhibited by anions [1, 6, 10, 11]. Uphill folate transport in L1210 cells has been proposed to occur by an anion exchange mechanism driven by intracellular organic anions [1, 12, 13]. Mouse, human, and hamster RFC1 cDNAs have been cloned and shown to encode a protein with 12 putative transmembrane domains [14–17].

When cultured under low folate selective pressure, L1210 cells can express a second folate transport mechanism, mediated by glycosylphosphatidylinositol-anchored FRs [18–20]. In contrast to RFC1, FRs exhibit much higher affinity for folic acid than for reduced folates and MTX, and mediate folate transport via an energy-requiring, endocytotic process [3, 21–23].

While both RFC1 and FRs operate efficiently at physiological pH, a third folate transport route that exhibits high activity at low pH and mediates influx of folic acid and reduced folates has been described [24, 25]. The present study was undertaken to further characterize folate transport mediated by the low pH transporter using MTX^{r}A cells, an L1210 subline resistant to MTX due to loss of RFC1 function [26], and to compare these properties with RFC1-mediated influx.

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† *Abbreviations:* RFC, reduced folate carrier; MTX, methotrexate; 5-CHO-THF, 5-formyltetrahydrofolate; 5-CH_3-THF, 5-methyltetrahydrofolate; MES,2-[*N*-morpholino]ethanesulfonic acid; BSP, sulfobromophthalein; SITS, 4-acetamido-4′-isothiocyanatostilbene-2′,2′-disulfonic acid; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; FR, folate receptor; and CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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MATERIALS AND METHODS

Chemicals

[3′,5′,7-^{3}H]MTX, [3′,5′,7′,9′-^{3}H]folic acid and, [3′,5′,7′,9′-^{3}H]leucovorin were obtained from Moravek Biochemicals. [^{3}H]5-Methyltetrahydrofolate was the gift of Dr. Barton Kamen (University of Texas Southwestern Medical Center). Monensin, nigericin, DIDS, SITS, folic acid and 5-CH_3-THF were from Sigma; Valinomycin and CCCP were from Calbiochem; 5-CHO-THF was from Lederle. Folate compounds were purified by high performance liquid chromatography prior to use [27].

Cell Culture

L1210, MTX^{r}A, and MTX^{r}A-R16 cells were grown in RPMI 1640 medium containing 2.2 μM of folic acid supplemented with 5% bovine calf serum (Hyclone), 2 mM of glutamine, 20 μM of 2-mercaptoethanol, penicillin (100 units/mL), and streptomycin (100 μg/mL). MTX^{r}A is an MTX-resistant L1210 subline developed from single-step selection in 50 nM drug [26]. MTX^{r}A-R16 was developed by transfecting MTX^{r}A with an expression vector encoding murine RFC1 [28].

[^{3}H]MTX Influx

[^{3}H]MTX influx was measured as previously described [24]. Briefly, cells were harvested and washed with unbuffered XBS (140 mM of NaCl, 5.3 mM of KCl, 1.9 mM of $CaCl_2$, 1 mM of $MgCl_2$, 7 mM of glucose). This was followed by a wash with buffer at the desired pH. Experiments at pH 5.0 to 6.5 were performed in MBS (20 mM of MES, 140 mM of NaCl, 5 mM of KCl, 2 mM of $MgCl_2$, 5 mM of glucose) [25] and those at pH 7.0 to 8.0 in HBS (20 mM of HEPES, 140 mM of NaCl, 5 mM of KCl, 2 mM of $MgCl_2$, 5 mM of glucose). Prior to influx measurements, cells were resuspended into the desired buffer at 1.5×10^7 cells/mL and then equilibrated for 20 min at 37°; uptake was initiated by the addition of the radiolabeled compound. Uptake was terminated by injecting 1.0 mL of the cell suspension into 9 mL of HBS at 0°. Cells were collected by centrifugation, washed twice with 0° HBS, and processed for determination of intracellular tritium as previously described [29]. All studies were performed with 0.2 μM of [^{3}H]MTX unless otherwise stated. Influx kinetics were determined over a range of MTX or 5-CH_3-THF concentrations (0.5 to 7.5 μM). Experiments utilizing metabolic poisons or ionophores were performed in MBS without glucose.

RESULTS

Folic Acid Transport in L1210 Cells at low pH

Since a folate transport route that operates optimally at low pH in L1210 cells has been described [24, 25], it was of interest to determine its status in MTX^{r}A cells in which endogenous RFC1 is not functional. As shown in Fig. 1A,

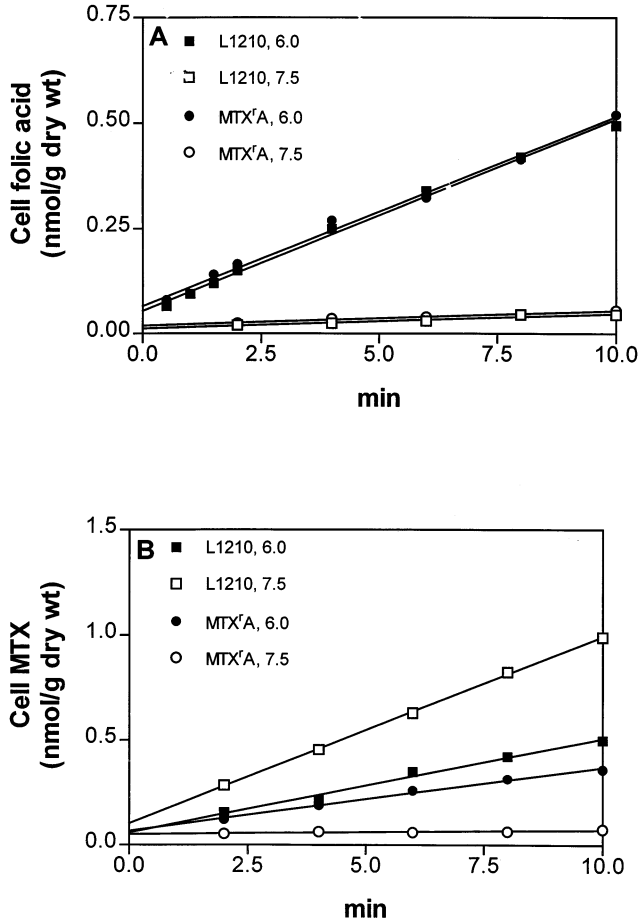

FIG. 1. Effects of pH on folic acid (A) and MTX (B) influx in L1210 and MTX^{r}A cells. Cells were harvested, washed, and resuspended in either HBS (pH 7.5) or MBS (pH 6.0) at 37°. At time zero, MTX or folic acid was added to achieve a concentration of 0.2 μM. The results are the averages of two experiments.

influx at 0.2 μM of [^{3}H]folic acid was very slow (3.5 pmol/g dry wt/min) and indistinguishable between L1210 and MTX^{r}A cells at pH 7.5. Influx increased 13-fold in both lines with a pH decrease to 6.0 (45 pmol/g dry wt/min). This contrasts with influx of 0.2 μM of [^{3}H]MTX, which decreased by a factor of two in L1210 cells and increased 15-fold in MTX^{r}A cells at pH 6.0 (Fig. 1B). MTX and folic acid influx in MTX^{r}A cells at pH 6.0 were comparable (30 and 45 pmol/g dry wt/min, respectively). Thus, the low pH route is present in MTX^{r}A cells at activity comparable to that of L1210 cells and, unlike RFC1, transports folic acid efficiently. Studies were undertaken to further characterize transport at low pH in MTX^{r}A cells.

pH Profiles of Folate Influx

The folate uptake mechanism operative at low pH has been shown previously to transport a variety of oxidized and reduced folates [24, 25]; thus, the effect of pH on influx of these substrates was studied in MTX^{r}A cells. While lowering the extracellular pH enhanced influx of all the sub-

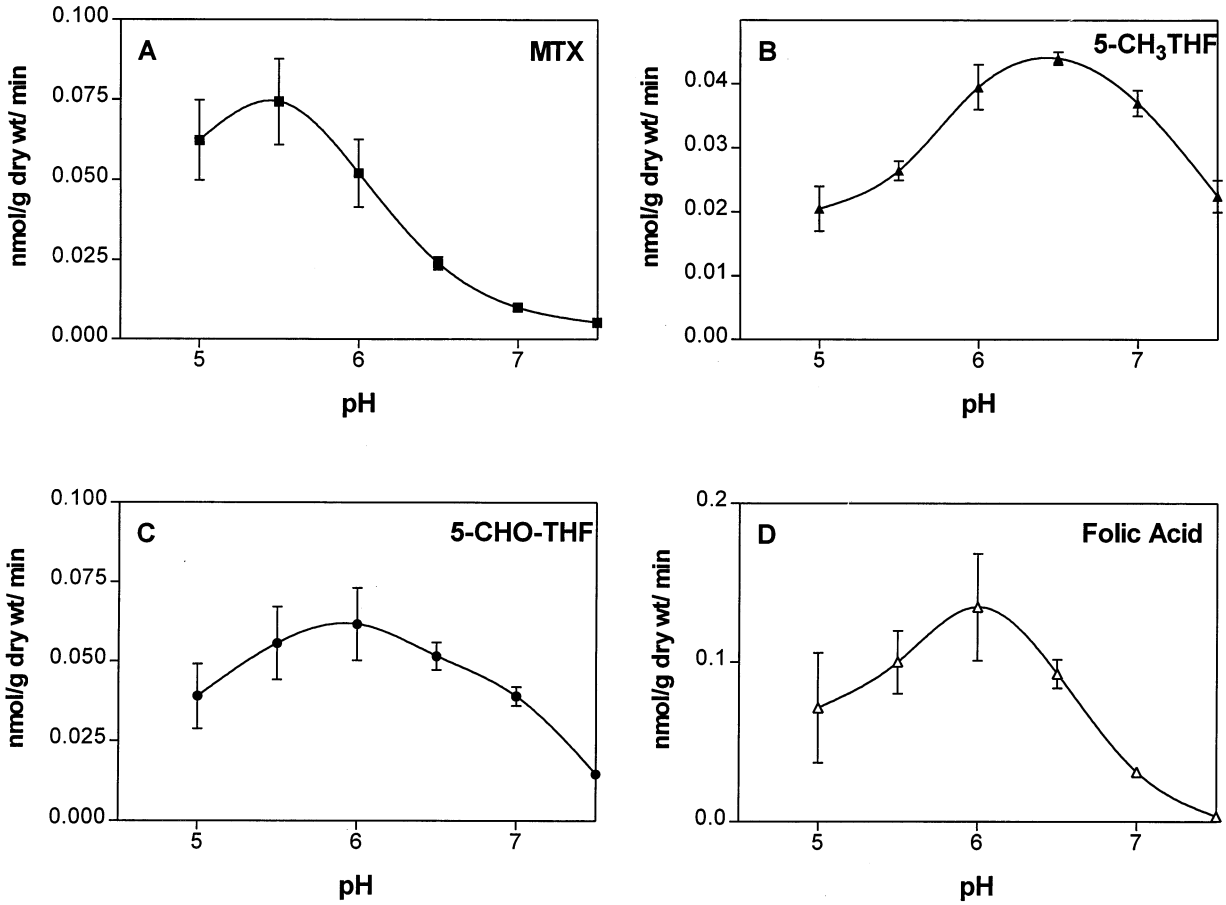

FIG. 2. pH dependence of MTX (A), 5-CH_3-THF (B), 5-CHO-THF (C), and folic acid (D) influx in MTX^{r}A cells. Cells were harvested, washed, and resuspended in buffer at the indicated pH as described in Materials and Methods. Cells were exposed to a 0.2-μM concentration of the indicated substrate, and influx was determined over 10 min. Results are averages of two (5-CH_3-THF) or three (MTX, 5-CHO-THF, folic acid) experiments.

strates studied, their profiles differed both in the pH of optimum activity and the rate changes observed with varying pH (Fig. 2). MTX exhibited the lowest extracellular pH optimum (5.5) followed by 5-CHO-THF (6.0), folic acid (6.0), and 5-CH_3-THF (6.5). There were considerable differences in transport rates at optimal pH versus 7.5; while folic acid influx was stimulated 27-fold at its optimum pH relative to pH 7.5, MTX influx increased 15-fold, 5-CHO-THF 4.4-fold, and 5-CH_3-THF only 2.2-fold.

Kinetics of Folate Transport via the Low pH Transporter

The kinetic parameters of 5-CH_3-THF influx in MTX^{r}A cells were determined at pH 6.0 and compared with those of MTX (Fig. 3). The K_m values for MTX (N = 4) and 5-CH_3-THF (N = 3) influx were 2.65 ± 1.65 and 0.56 ± 0.28 μM, and the V_{max} values were 0.45 ± 0.23 and 0.083 ± 0.037 nmol/g dry wt/min, respectively. Therefore, while 5-CH_3-THF appears to have 5-fold higher affinity than MTX for the low pH transporter, it is translocated at a slower rate at pH 6.0.

Ion Dependence and Effect of Organic Anion Transport Inhibitors on MTX Influx Mediated by the Low pH Transporter

Anions inhibit RFC1-mediated, and stimulate FR-mediated, MTX influx [22, 23], but the low pH transporter was affected minimally by the removal of anions from the transport buffer (Fig. 4). MTX influx (0.041 ± 0.01 nmol/g dry wt/min) was reduced by 24% when NaCl was replaced isosmotically with HEPES. Substitution of Na^+ by Li^+ resulted in a 17% reduction in influx. Thus, influx mediated by the low pH transporter appears to be insensitive to the anionic composition of the transport buffer and is affected minimally by Na^+.

The effects of several anion transporter inhibitors on MTX (0.2 μM) influx in MTX^{r}A cells at pH 6.0 are shown in Fig. 5. At 100 μM, BSP, DIDS, and SITS inhibited the low pH transporter by 55, 62, and 21%, respectively. In contrast, under these conditions, RFC1-mediated MTX influx was inhibited potently by all these agents (>80%; data not shown). Thus, of the stilbenedisulfonate derivatives, SITS was the weakest inhibitor and was evaluated further as a means of discriminating between RFC1-

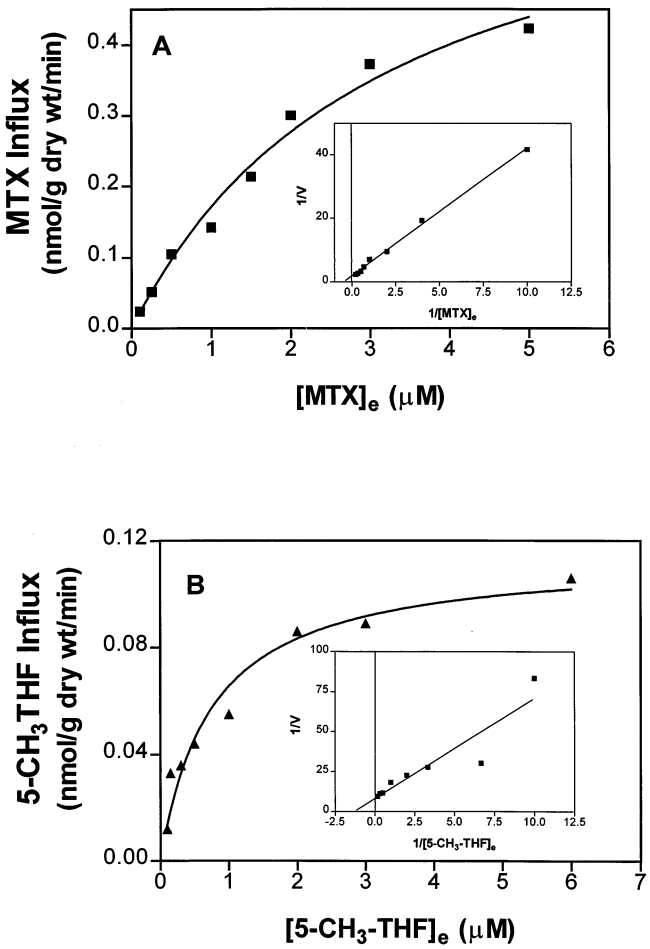

FIG. 3. (A) MTX and (B) 5-CH_3-THF influx as a function of the extracellular folate concentration. MTX^{r}A cells were harvested, washed as described, and resuspended in MBS, pH 6.0, at 37°. Substrate was added over a concentration range of 0.1 to 5 μM (MTX) or 0.1 to 6 μM (5-CH_3-THF). Results shown are from a representative experiment. Inset: Lineweaver-Burk representation of the data.

mediated influx and transport mediated by the low pH system.

Effects of Alterations in RFC1 Expression on MTX Transport at Low pH

To exclude the possibility that folate influx at low pH was mediated or influenced in some way by the mutated RFC1 present in MTX^{r}A cells, SITS was used to discriminate between RFC1-mediated MTX transport and that mediated by the low pH transporter. SITS (100 μM) strongly inhibited (83%) RFC1-mediated MTX influx at pH 7.5 in L1210 cells (Fig. 6A). In contrast, MTX influx at pH 5.5 was inhibited by only 29 and 39% in MTX^{r}A and L1210 cells, respectively. Furthermore, the pH profiles of MTX influx in both cell lines became virtually undistinguishable in the presence of 100 μM of SITS.

MTX^{r}A-R16 is an MTX^{r}A RFC1 transfectant with 10-fold overexpression of the carrier relative to L1210 cells

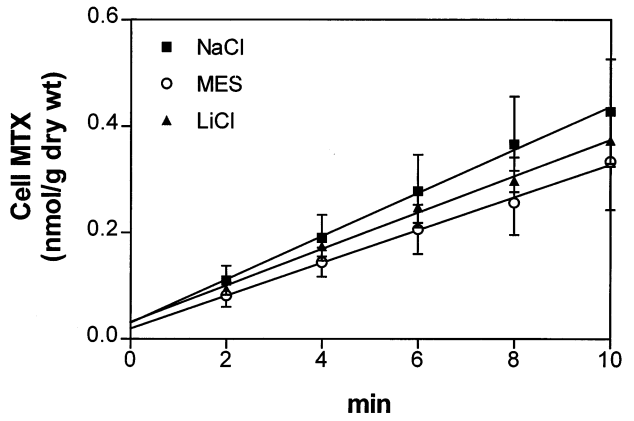

FIG. 4. Effect of buffer ionic composition on MTX influx at pH 6.0. MTX^{r}A cells were harvested, washed, and resuspended in MBS at pH 6.0, or MBS where NaCl was removed and replaced isosmotically with MES or LiCl. Results are the means ± SEM of five experiments.

[28]. The ratio of MTX (0.1 μM) influx at pH 7.5 to 5.5 was 3.6 in L1210 cells and 13.7 in MTX^{r}A-R16 cells (Fig. 6B). Hence, while influx at pH 7.5 in MTX^{r}A-R16 is ~8-fold greater than in L1210 and 100-fold greater than in MTX^{r}A cells, at pH 5.5 influx was comparable in the three cell lines and was identical in the presence of SITS. Thus, overexpression of RFC1 did not result in a concomitant appreciable increase in the low pH transport route.

Effects of Metabolic Inhibitors and Ionophores on MTX Influx at Low pH

The metabolic poisons azide (10 mM) and dinitrophenol (100 μM) inhibited MTX influx via the low pH transporter by 64 and 61%, respectively (N = 4, data not shown), while CCCP decreased influx by 52% (Table 1), consistent with an influx process that requires energy either directly or indirectly. Ionophores partially inhibited MTX influx (ni-

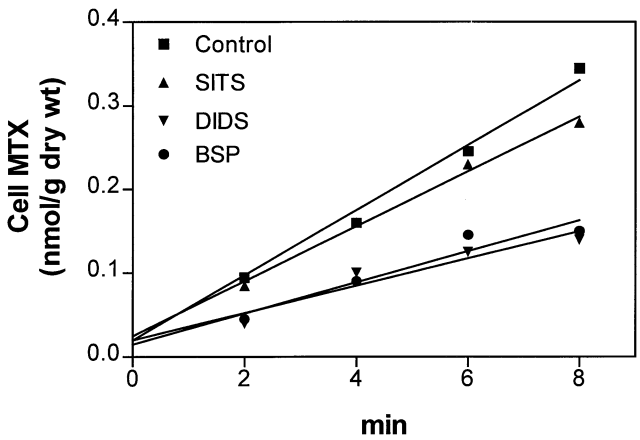

FIG. 5. Effect of anion transport inhibitors on MTX influx. MTX^{r}A cells were harvested, washed, and resuspended in MBS at pH 6.0 as described in Materials and Methods. Cells were exposed to 0.2 μM of MTX and 100 μM of the indicated inhibitor. Results are the averages of two experiments.

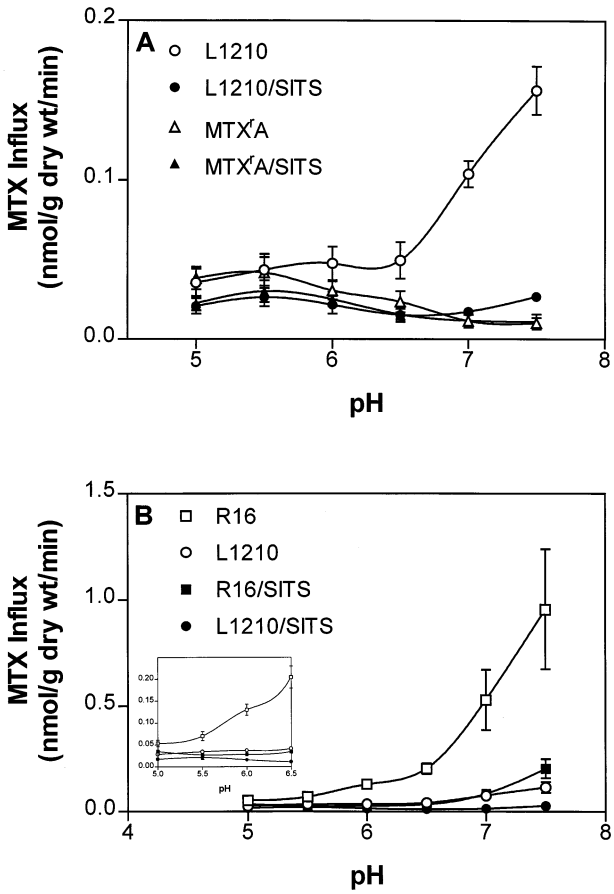

FIG. 6. Comparison of the effects of 100 μM of SITS on the pH profile of MTX influx in (A) L1210 and MTX^{r}A cells, and (B) L1210 and MTX^{r}A-R16 cells. Cells were harvested, washed, and resuspended in buffer at the indicated pH as described in Materials and Methods. At time zero, cells were exposed to 0.2 μM of MTX, and influx was determined over 10 min (A) or 3 min (B). Results are the means ± SEM of four (A) or three (B) experiments.

gericin > monensin ≫ valinomycin; Table 1). Alkalinization of intracellular acidic compartments with ammonium chloride did not affect MTX influx at low pH. The combination of nigericin and CCCP equalizes intra- and extracellular pH in L1210 cells [30] and resulted in 74% inhibition of MTX influx (Table 1).

TABLE 1. Effects of metabolic inhibitors and ionophores on MTX influx at low pH

Addition	Influx (nmol/g dry wt/min)	% Control
Control	0.023 ± 0.004	100
Valinomycin (5 μM)	0.021 ± 0.004	91
Monensin (5 μM)	0.015 ± 0.004	65
Nigericin (5 μM)	0.011 ± 0.002	48
CCCP (5 μM)	0.011 ± 0.002	48
Nigericin (5 μM) + CCCP (5 μM)	0.006 ± 0.001	26
NH_4Cl (10 mM)	0.021 ± 0.003	91

Influx of MTX (0.2 μM) at pH 6.0 was determined as described in Materials and Methods. Results are the means ± SD of four experiments.

DISCUSSION

In this report we used the MTX^{r}A line, an L1210 derivative lacking functional RFC1, to further characterize a folate transporter that operates optimally at low pH, and to contrast its properties with those of the RFC1. Loss of RFC1 function in MTX^{r}A cells is due to a mutation that substitutes a proline for an alanine in the fourth putative transmembrane domain [17]. Because MTX^{r}A cells lack functional RFC1, this cell line appears to be a suitable model to dissect folate transport via alternative routes. This laboratory has used this strategy previously with this cell line to characterize folate transport mediated by the folate receptors [22, 23] and to assess the impact of high-level RFC1 expression on concentrative MTX transport [28]. This report establishes that transport via RFC1 and at low pH are distinct from, and independent of, each other. Hence, 1) folic acid influx at pH 6.0 in MTX^{r}A cells was undistinguishable from that in L1210 cells, indicating that the low pH transporter activity in parental and MTX^{r}A cells is the same; 2) the functional properties of the two transporters (energetics, anions, specificity) were different; 3) in the presence of SITS, RFC1-mediated influx was inhibited markedly, while at low pH MTX influx became virtually the same in L1210 and MTX^{r}A cells; and 4) MTX^{r}A-R16 cells, transfected with carrier and with 10-fold RFC1 overexpression in comparison to L1210 cells, showed a minimal increase in transport at pH < 6.

MTX^{r}A cells exhibit a small increase in the expression of FR-β relative to L1210 cells. However, this low level of expression does not result in appreciable augmented transport of MTX, 5-CHO-THF, or folic acid [20]. A previous report from this laboratory [24] characterized the pH dependence of folate transport in an MTX^{r}A subline that overexpresses FR-β. While FR-β-mediated MTX influx was optimal at pH 6.5, due to increased binding affinity, a transport route clearly separate from FR-β became evident at pH levels below 7.5. This route was insensitive to low levels of folic acid that abolished FR-mediated influx and showed properties distinct from FR-β, similar to those reported here. Thus, the data do not support a direct role for FRs in low pH-mediated folate transport in MTX^{r}A cells.

The pH dependence of influx in MTX^{r}A cells varied with the folate substrate but was always optimal at pH ≤ 7.0. The marked differences in the pH profiles (Fig. 2) cannot be explained by changes in the α- and γ-carboxyl groups of folates that have pK_a values of 3.1 to 3.5 and 4.6 to 4.8, respectively [31, 32]. Hence, protonation of these groups is not predicted to change significantly over the pH range studied. Furthermore, although the pK_a of the N-1 nitrogen of the pteridine ring of MTX is 5.4, similar to its optimal pH for transport, that of reduced folates and folic acid is 1.2 to 2.4 [31, 32], suggesting that the ionization of this group is not related to the pH dependence. The broader

pH range for transport of 5-CH_3-THF and 5-CHO-THF compared with MTX and folic acid raises the possibility that protonation of the N-5 position of the folate substrate is important for the specificity of the low pH transporter. Beyond this, changes in transporter conformation are likely responsible for the observed differences among the folates. It is possible that the folate substrates are bound with slightly different orientations within the binding site as in the case of dihydrofolate reductase [33]. It is not clear how changes in pH alter the kinetic parameters of the low pH transporter. A previous report by Henderson and Strauss [25] using folic acid as a substrate for the low pH transporter indicated both decreased K_t and increased V_{max} as the pH decreased from 7.4 to 6.2, suggesting increases in both substrate affinity and transport activity at low pH. Thus, the resulting overall pH profiles could be a result of complex and different changes in K_t and V_{max} for each of the folates, and it is conceivable that the pH optimum for substrate binding and translocation may be different.

Initial studies in L1210 cells had found little or no 5-CH_3-THF transport at low pH [25]. In contrast, studies from this laboratory using F2-MTX^{r}A cells, which lack functional RFC1 and overexpress FR-β, suggested that 5-CH_3-THF is a substrate for the low pH transporter [24]. The results presented here confirm that the low pH folate transporter mediates influx of 5-CH_3-THF, although with very different kinetics from those of MTX. Thus, at pH 6.0, the K_t for 5-CH_3-THF was approximately 5-fold higher than for MTX, but the V_{max} of transport was 82% lower. However, the relative catalytic efficiency of transport (V_{max}/K_t) is essentially the same for both substrates, 0.17 and 0.15 for MTX and 5-CH_3-THF, respectively. Based on a K_t of 4.2 μM and a V_{max} of 2.9 nmol/g dry wt/min [22], the catalytic efficiency of RFC1 for MTX transport at pH 7.4 is 0.7, over four times higher than transport at pH 6.0. However, the values obtained for the low pH transporter may be underestimated since neither MTX nor 5-CH_3-THF transport is optimal at pH 6.0.

Interestingly while influx of MTX and folic acid was nearly abolished at pH 7.5, this was not the case with 5-CH_3-THF or 5-CHO-THF. Thus, at pH 7.5, influx was 24 and 60% that at pH 6.0 for 5-CHO-THF and 5-CH_3-THF, respectively. Therefore, the low pH transporter can mediate influx of certain folates at physiological pH, although at a rate significantly lower than that mediated by RFC1. MTX influx in L1210 cells at pH 7.4 is ~0.13 nmol/g dry wt/min when $[MTX]_e$ is 0.2 μM [23]. This is 6- and 9-fold higher than the rates of 5-CH_3-THF and 5-CHO-THF influx at pH 7.5 via the low pH transporter in MTX^{r}A cells.

MTX influx mediated by the low pH transporter was minimally sensitive to the ionic composition of the transport buffer. This contrasts with RFC1-mediated MTX influx, which is sodium independent but significantly stimulated by removal of anions from the transport buffer [1, 11, 12]. RFC1 appears to function as an anion exchanger, and uphill transport is driven by the transmembrane organic anion gradient [1, 12, 13]. Removal of Na^+ had a small (~20%) inhibitory effect on the low pH transporter, as reported earlier [25]. The reason for this is unclear; however, the magnitude of this effect is small and inconsistent with a $Na^{\pm}$ folate exchange mechanism.

RFC1-mediated MTX influx is strongly inhibited by a variety of organic anions. BSP is a potent RFC1 inhibitor (K_i ~ 3.9 μM [34]) that has been used to discriminate between RFC1 and other folate transporters [22, 23, 25]. SITS and DIDS are powerful anion exchange inhibitors that have been shown to inhibit RFC1 (K_i ~ 5 μM) as well as general anion transport in L1210 cells [34]. None of these compounds were effective inhibitors of the low pH folate transporter under conditions in which they inhibit RFC1-mediated influx >90%. While the K_i for SITS inhibition of RFC1-mediated MTX influx at pH 7.5 was ~11 μM, that for the low pH transporter at pH 6.0 was >200 μM (data not shown). These results indicate that, unlike RFC1, the anion binding site of the low pH transporter exhibits a high degree of specificity for folates. Taken together with the results obtained in anion substitution experiments, folate transport mediated by the low pH transporter must operate by a mechanism different from RFC1 and other anion exchangers.

Consistent with a previous report [25], we did not detect low pH-mediated folate transport in human leukemic CEM cells but have found significant levels in Chinese hamster ovary (CHO) cells.* Further studies are needed to evaluate the presence and role of low pH folate transport in a variety of cell lines of different tissue origins.

Other physiologically relevant folate transport systems that operate optimally at acidic pH have been described, including transporters in kidney [35, 36] and small intestine [37–39]. In addition, ATP-dependent folate efflux in L1210 cells has been shown to be optimal at pH <7.0 [40, 41]. Finally, a folate carrier active at low pH has been proposed to be involved in FR-mediated folate translocation [42]. The potential relationship of these systems to the low pH transporter described here is not known. Studies using membrane vesicles have shown that the driving force of the intestinal and kidney transporters is the transmembrane proton gradient, rather than the extracellular pH *per se* [36, 43]. Folate influx mediated by the intestinal transporter has been attributed to the involvement of either OH^-/folate exchange or H^+/folate cotransport [43, 44]. The results presented here suggest that the low pH transporter in L1210 cells may be driven by a similar mechanism. A collapse of the transmembrane pH gradient using CCCP/nigericin produced potent inhibition of MTX influx at low pH. However, CCCP, an uncoupler of oxidative phosphorylation, by itself was also a potent inhibitor. Monensin, a Na^+ ionophore that disrupts vesicular proton gradients, inhibited, but did not abolish, low pH transport. Hence, the driving force for the low pH folate transporter depends, in

* Assaraf Y and Goldman ID, unpublished observation.

part, upon energy metabolism and the proton gradient, but a full understanding will require further investigation.

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