Increased Activity of a Novel Low pH Folate Transporter Associated with Lipophilic Antifolate Resistance in Chinese Hamster Ovary Cells*

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Previous studies described a Chinese hamster ovary cell line, PyrR100, resistant to lipid-soluble antifolates due to the loss of an energy-coupled folate exporter resulting in a marked increase in intracellular folate cofactor accumulation. There was, in addition, an unexplained increase in folic acid influx in PyrR100 cells which is shown in this paper to be mediated by a transporter with a low pH optimum. The pH profile for folic acid influx in parental Chinese hamster ovary AA8 cells indicated peak activity at pH 6; this was increased >2-fold in PyrR100 cells. In contrast, methotrexate (MTX) influx in AA8 cells showed two peaks of comparable activities at pH 6 and 7.5; in PyrR100 cells, the component at pH 6 was increased 2-fold. Folic acid was a potent inhibitor of [3H]MTX or [3H]folic acid influx (1 μM) via the low pH route with IC50 values of ~1 μM. Prostaglandin A1 was a potent inhibitor of [3H]MTX influx via the reduced folate carrier 1 at pH 7.5 with only a small inhibitory effect on the low pH route. The addition of 10 μM folic acid to PyrR100 cells resulted in a MTX influx pH profile identical to that of AA8 cells, consistent with suppression of the low pH route. In contrast, addition of 25 μM prostaglandin A1 to PyrR100 cells resulted in a MTX influx pH profile comparable to that of folic acid, consistent with the loss of the reduced folate carrier-mediated component. Inhibition (~70%) of [3H]folic acid influx by ~10 μM unlabeled folic acid at pH 7.5 indicated that the low pH transporter accounts for the majority of folic acid transport at physiological pH.

This study demonstrates the functional importance of a low pH folate transporter that is increased when enhanced folic acid entry into cells is required as an adaptive response to antifolate selective pressure. This may represent a mechanism of resistance to new antifolate inhibitors of folate cofactor-dependent enzymes in which cytotoxic activity is limited by expanded cellular folate pools.

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MATERIALS AND METHODS

Chemicals—[3',5',7',9'-3H]folic acid, [3',5',7',9'-3H]MTX, and [6S]/[5-CH3]THF were obtained from Amersham. Folic acid and dihydrofolate reductase; RFC1, reduced folate carrier; MTX, methotrexate; TMQ, trimetrexate; 5-CH3,THF, 5-methyltetrahydrofolate; 5-CHO-THF, 5-formyltetrahydrofolate; PGA1, prostaglandin A1; PyrR100, pyrimethamine-resistant cell line; MES, 4-morpholineethanesulfonic acid.

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(GrAS)-5-CH0THF were from Lederle, Carolina, Puerto Rico. Folate and antifolate compounds were purified by high performance liquid chromatography prior to use (15). Trimetrexate (TMQ) glucuronate was kindly provided by Warner-Lambert, Ann Arbor, MI. Prostaglandin A1 (PGA1) was purchased from Cayman Chemical Co., Ann Arbor, MI. Stock solutions (20 mM) of PGA, were prepared before use in ethanol and stored at −20 °C; the final content of ethanol did not exceed 0.25% in all transport experiments, a level that did not have any effect on folate transport in AA8 or PyrR100 cells.

Cell Lines and Tissue Cultures—PyrR100 cells were obtained by multiple step selection of parental CHO AA8 cells in gradually increasing concentrations of the lipid-soluble antifolate pyrimethamine (1). This stepwise selection was initiated at 100 μM pyrimethamine (10 μM lethal dose, LD100) and terminated at 100 μM. The resultant 1000-fold pyrimethamine-resistant cells, termed PyrR100, retained wild-type dihydrofolate reductase (DHFR) gene copy number and mRNA levels, as well as parental levels of DHFR enzyme activity (1). The affinity of DHFR for various antifolates was preserved in PyrR100 cells, along with parental sensitivity to MTX but these cells were cross-resistant to the lipophilic antifolate trimetrexate and piritrexim (1, 16). AA8 cells and their PyrR100 subline were maintained in monolayer or suspension culture conditions in RPMI 1640 medium containing 2.3 μM folic acid (HyClone, Logan, UT), supplemented with 5% dialyzed fetal bovine serum, Gemini (Bio-Laboratories Inc., Calabasas, CA), 1 mM sodium pyruvate, (Mediatech, Herndon, VA), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.2 mM pyruvate, (Mediatech, Herndon, VA), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.2 mM pyruvate, (Mediatech, Herndon, VA), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.2 mM pyruvate, (Mediatech, Herndon, VA), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.2 mM pyruvate, and 5% dialyzed fetal bovine serum. The growth medium of PyrR100 cells influ was always observed at physiological pH (13, 14), in PyrR100 cells, the profile of pH dependence for MTX influx was different (Fig. 1B). There was a gradual increase in influx in AA8 cells as the pH was increased from 5.0 to 8.0 with a small peak at pH 6.0 and maximal activity at physiological pH. In contrast to other mammalian cell lines in which maximal MTX influx was observed at physiological pH (13, 14), in PyrR100 cells, the profile of pH dependence for MTX influx was different (Fig. 1B). Hence, transport of folic acid and MTX is mediated by two processes with different pH optima and the activity of the transporter that is optimal at low pH is substantially increased in PyrR100 cells.

Discriminating between Transport Mediated by the Low pH Route and RFC1—Further studies were undertaken to better discriminate between transport mediated by the low pH pathway and RFC1. Folic acid is a very poor substrate for RFC1 (3−5), but a very good substrate for the low pH route in murine leukemia cells and hence has properties that should permit the separation of these processes (13, 14). As illustrated in Fig. 2A, when influx of [3H]folic acid (1 μM) was monitored in PyrR100 cells as a function of increasing concentrations of unlabeled folic acid at pH 6.0, about 80% of influx was blocked by ≤10 μM folic acid. The remaining 20% was not inhibited by concentrations of folic acid in excess of 100 μM. The IC50 for the folic acid inhibitable component was ≤1 μM. This established that the major component of influx at pH 6.0 was mediated by a process with a high affinity for folic acid, clearly different from RFC1 which has an affinity for this folate which is 2 orders of magnitude lower (3−5). Similarly, at physiological pH (Fig. 2B), the component of [3H]folic acid influx inhibited by low concentrations of folic acid (IC50 ~ 2 μM) in PyrR100 cells was ~70% and the residual influx (~30%) was not inhibited by folic acid concentrations of up to 200 μM. Hence, the major component of folic acid influx at physiological pH appears to be mediated by the low pH transporter.

The components of [3H]MTX influx mediated by RFC1 and the low pH transporter were further distinguished by studies in which folic acid inhibition was evaluated over a broad pH range in PyrR100 and AA8 cells. It can be seen in Fig. 3 that in the presence of 10 μM unlabeled folic acid, the pH profiles of [3H]MTX influx in both PyrR100 (Fig. 3A) and AA8 cells (Fig. 3B) are essentially identical with a single pH optimum at physiological pH. Hence, the contribution of the low pH transport...
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Fig. 1. The pH dependence of folic acid (A) and MTX (B) influx in parental AA8 (triangles) and PyrR100 cells (squares). Exponentially growing cells in suspension cultures were harvested by centrifugation, washed three times at 0 °C and resuspended in buffer at the appropriate pH as detailed under "Materials and Methods." Following 20 min equilibration at the various pH levels, cells were exposed to labeled folate or MTX and initial rates measured over 60 and 120 s, respectively. In folic acid transport experiments, 5 μM TMQ was added to ensure complete blockade of DHFR activity. Results presented are the mean ± S.E. of five to six experiments.

Fig. 2. Effects of unlabeled folic acid on influx of [3H]folic acid in PyrR100 cells. Influx at pH 6.0 (A, solid squares) and pH 7.4 (B, open squares) of [3H]folic acid was measured in PyrR100 cells over 60 s after the simultaneous addition of unlabeled folic acid. Triplicate samples were obtained for cells to which only [3H]folic acid was added, representing the uninhibited controls (100%).

detectable folate receptor transcript in either cell line (data not shown). (b) Using a sensitive, high-affinity [3H]folic acid (high performance liquid chromatography purified immediately prior to use) binding assay in the presence or absence of competing unlabeled folic acid, no folate receptor expression was detected. (c) Binding of [3H]folic acid via the folate receptor is typically accompanied by a "high intercept" when folic acid uptake is analyzed as a function of time. This was not observed in PyrR100 cells. (d) Monensin, a potent vesicular transport inhibitor which blocks folate receptor-mediated endocytosis, enhanced rather than inhibited influx of folic acid.

The pH Dependence of Net MTX and Folic Acid Transport—To evaluate whether the pH dependence of folic acid uptake is accompanied by changes in net concentrations achieved within the cell, studies evaluated the full time course of uptake and steady-state levels for folic acid and MTX. As indicated in Fig. 6, a clear increase in the steady-state levels of folic acid was observed in both AA8 (Fig. 6A) and PyrR100 cells (Fig. 6B) at pH 6.0 relative to pH 7.5 under conditions in which folic acid reduction was blocked with TMQ. While the percentage increase was greater in AA8 cells, it should be noted that the absolute levels of folic acid accumulation in PyrR100 cells were 10-fold higher. Steady-state levels of MTX accumulation were comparable in AA8 cells (Fig. 6C) with a small increase in PyrR100 cells (Fig. 6D). Hence, enhanced influx of folic acid and MTX is accompanied by steady-state levels that are the same or greater at pH 6.0 than at pH 7.4.

Determination of Monolayer Culture Medium pH during Cell Growth—Since the activity of a low pH transporter was augmented in PyrR100 cells during selection with pyrimethamine in the presence of folic acid (2), studies were undertaken to evaluate the extent of acidification of the medium during growth of CHO cell monolayers under similar conditions. It can be seen in Fig. 7 that after plating, the pH of the growth medium falls...
steadily to a value of 7.0 by day 4. When AA8 and PyrR100 cells begin to reach confluence at days 6 and 7, respectively, the culture medium reaches a pH of 6.8 and 6.7, respectively. In contrast, the pH of the cell-free growth medium, alone, was stable at about pH 7.3.

**DISCUSSION**

In a prior report (2), resistance to the lipid-soluble antifolate pyrimethamine was associated with a marked decrease in folate growth requirement in a CHO cell line, PyrR100. Resistance was shown to be due primarily to the loss of an energy-dependent efflux pump resulting in marked augmentation of folate accumulation (2). There was a smaller increase (2-fold) in folic acid influx in PyrR100 cells. This paper clarifies the basis for this increase in folic acid influx within the context of the known routes of transport for folate compounds: RFC1, folate receptor-mediated endocytosis, and a process that functions optimally at low pH. The data indicate that the major change in folic acid influx in PyrR100 cells can be attributed to a substantial increase in transport mediated by the low pH route and that the increase in folic acid influx observed at physiological pH is due largely to the residual activity of this process. Beyond this, the findings represent the first demonstration of the functional overexpression of a low pH transporter as the result of suppression of DHFR activity by a lipophilic antifolate. This is apparently one of several stable functional alterations which take place to meet the need for high intracellular folate levels to overcome the pharmacologic activity of this class of antifolates in the PyrR100 cell line (16).

Several lines of evidence exclude the possibility that the overexpressed folate transporter in PyrR100 cells is a mutated RFC1 with increased affinity for folic acid and an optimal activity at pH 6.0. (i) The pH optimum of a single transporter cannot be acidic for one substrate, folic acid, but with an unchanged physiological pH optimum for another cognate substrate such as MTX. Furthermore, as with AA8 cells, PyrR100 cells displayed a single low pH optimum for folic acid transport but two pH optima for MTX transport, consistent with two distinct transport entities. (ii) The low pH folic acid transport activity is increased 3-fold in PyrR100 cells, whereas wild-type MTX transport activity is retained at physiological pH. A single transport entity (i.e. even a mutated RFC1) cannot at the same time be overexpressed for one substrate (folic acid) without a change in its cognate substrate (MTX). (iii) Folic acid blocks [3H]MTX influx via the overex-
pressed low pH transport route but leaves the RFC-mediated component of transport unchanged at physiological pH at the same level as in parental AA8 cells. IV PGA1, which was found here to be a potent and selective inhibitor of RFC1 in both PyrR100 and AA8 cells. But PyrR100 cells retain wild-type sensitivity to MTX.

In previous studies lipophilic antifolate-resistant clonal variants exposed to nanomolar concentrations of the lipid-soluble antifolate TMQ displayed marked resistance to the selecting agent and prominent cross-resistance to other lipophilic antifolates, but retained wild-type sensitivity to MTX without quantitative or qualitative changes in DHFR. This resistance was genetically stable and the frequency of emergence of resistant clones was enhanced 100-fold when cells were treated with a mutagen such as γ-irradiation prior to drug selection (17, 22). Thus, it is likely that during the early stages of exposure to low concentrations of lipophilic antifolates such as trimetrexate or pyrimethamine, stable mutations occur which abolish folate exporter function, while other mutations may increase the expression of a low pH folate transporter.

PyrR100 cells also display 31-fold cross-resistance to the folate-based glycaminide ribonucleotide transformylase inhibitor, 5,10-dideaza-5,6,7,8-tetrahydrofolic acid, and 27-fold cross-
resistance to the lipid-soluble thymidylate synthase inhibitor AG377 data not shown. High folate cofactor pools would be a basis for resistance to these agents by competition at the tetrahydrofolate cofactor-dependent enzyme sites. Alternatively, high folate pools could inhibit antifolate polyglutamylation at the level of folypoly-γ-glutamyl synthetase thereby blocking the formation of the active polyglutamate congeners. For instance, an antifolate-resistant CCRF-CEM cell line with high tetrahydrofolate cofactor pools and wild-type folylpoly-γ-glutamyl synthetase activity, has very low levels of antifolate polyglutamylation (23). This mechanism of resistance to antifolates that require polyglutamylation for activity also has features similar to what was recently reported for 5,10-dideaza-5,6,7,8-tetrahydrofolic acid-resistant L1210 cells in which folate cofactor accumulation was markedly enhanced (24). In this case, however, augmented cellular folate levels were due to mutations in RFC1 that resulted in a marked increase in the affinity of carrier for folic acid with resultant enhanced transport.

Folate transport systems that operate optimally at acidic pH have been identified in rat and human small intestine (25–27), and in rat kidney brush-border membrane vesicles (28, 29). The driving force for intestinal and kidney folate transport in membrane vesicles is the transmembrane proton gradient and not the increase in the affinity of carrier for folic acid with resultant enhanced transport.

Folate transport into mammalian low pH intestinal transporter has been identified in rat and human small intestine (25–27), and in rat kidney brush-border membrane vesicles (28, 29). The H+ transmembrane gradient is also the driving force for intestinal and renal PEPT1 and PEPT2 H+-coupled peptide transporters (32, 33). The basis for enhanced influx and increased transmembrane gradients for folic acid at low pH in CHO cells is not clear. This could be due to a proton-driven folate cotransport energized by a transmembrane H+ gradient. Alternatively, the low pH folate transporter could have maximal activity at acidic pH without H+/folate cotransport. The acidic pH optimum of the low pH transport route cannot be attributed to the protonation state of the α- and γ-carboxyl residues of folates that have pKa values of 3.1–3.5 and 4.6–4.8, respectively (34), since protonation would not change significantly over the pH range of 6.0–8.0 studied. Nor, for the same reason, could the ionization of the N-1 nitrogen of the pteridine ring (pKa = 2.4) be relevant to the pH dependence of transport (34, 35).

In summary, this paper provides evidence, for the first time, of the functional importance of a folic acid transport route distinct from RFC1 and the folate receptors, that has a low pH optimum. This route accounts for the majority of folic acid transport at physiological pH in CHO cells and is concentrative; the low pH mechanism generates higher transmembrane gradients for folic acid than are achieved by RFC1. While RFC1 is thought to be an anion exchanger the mechanism by which the low pH route achieves uphill transport is not clear and is currently under study. The relationship between this transporter and other low pH folate transport processes in kidney and intestine remains to be clarified.

REFERENCES

1. Assaraf, Y. G., and Slotky, J. I. (1993) J. Biol. Chem. 268, 4556–4566
2. Assaraf, Y. G., and Goldman, I. D. (1997) J. Biol. Chem. 272, 17460–17466
3. Yang, C., Dembo, M., and Sirotnak, F. M. (1983) J. Membr. Biol. 73, 11–20
4. Henderson, G. B., Suress, M. R., Vitais, K. S., and Hueneke, F. M. (1986) Cancer Res. 46, 1639–1643
5. Goldman, I. D. (1971) Ann. N. Y. Acad. Sci. 186, 400–422
6. Sirotnak, F. M. (1986) in Membrane Transport of Antineoplastic Agents (Goldman, I. D., ed) pp. 241–281, Pergamon Press, Oxford
7. Sirotnak, F. M. (1985) Cancer Res. 45, 3992–4000
8. Henderson, G. B. (1986) in Folates and Pterins: Nutritional, Pharmacological and Physiological Aspects (Blakley, R. L., ed) Vol. 3, pp. 297–250, John Wiley & Sons, Inc., New York
9. Spinella, M. J., Brigle, K. E., Sierra, E. E., and Goldman, I. D. (1995) J. Biol. Chem. 270, 7642–7649
10. Henderson, G. B. (1990) Annu. Rev. Nutr. 10, 319–335
11. Kamen, B. A., Wang, M. T., Streckfuss, A. J., Peryea, X., and Anderson, R. G. W. (1988) J. Biol. Chem. 263, 13602–13609
12. Sierra, E. E., Brigle, K. E., Spinella, M. J., and Goldman, I. D. (1995) Biochem. Pharmacol. 50, 1287–1294
13. Henderson, G. B., and Strauss, B. P. (1990) Cancer Res. 50, 1709–1714
14. Sierra, E. E., Brigle, K. E., Spinella, M. J., and Goldman, I. D. (1997) Biochem. Pharmacol. 53, 223–231
15. Matherly, L. H., Barlowe, C. K., Phillips, V. M., and Goldman, I. D. (1987) J. Biol. Chem. 262, 710–717
16. Sprecher, H., Barr, H. M., Slotky, J. I., Tzukerman, M., Eytan, G. D., and Assaraf, Y. G. (1995) J. Biol. Chem. 270, 20668–20676
17. Assaraf, Y. G., Molina, A., and Schimke, R. T. (1989) J. Biol. Chem. 264, 18226–18334
18. Fry, D. W., and Goldman, I. D. (1982) J. Membr. Biol. 66, 87–95
19. Zhao, R., Seither, R. L., Brigle, K. E., Sharina, I. G., Wang, P. J., and Goldman, I. D. (1997) J. Biol. Chem. 272, 21207–21212
20. Jansen, G., Barr, H. M., Kathmann, I., Peter, G. H., Noordhuis, P., Bunni, M., Priest, D. G., and Assaraf, Y. G. (1997) Pteridines 8, 116–117
21. Seither, R. L., Trent, D. F., Mikulecky, D. C., Rabe, T. J., and Goldman, I. D. (1989) J. Biol. Chem. 264, 17016–17023
22. Sharma, R. C., Assaraf, Y. G., and Schimke, R. T. (1991) Cancer Res. 51, 2949–2959
23. Mauritz, R., Assaraf, Y. G., Driwi, S., Schornagel, J. H., Priest, D. G., Bunni, M., Pinedo, H. M., Peters, G. J., and Jansen, G. (1997) Pteridines 8, 92–93
24. Tse, A., Brigle, K., and Moran, R. G. (1997) Proc. Am. Assoc. Cancer Res. 38, 162 (abstr.)
25. Selhub, J., and Rosenberg, I. H. (1981) J. Biol. Chem. 256, 4489–4493
26. Smith, M. E., Matty, A. J., and Blair, J. A. (1970) Biochim. Biophys. Acta 219, 37–46
27. Zimmerman, J. (1990) Gastroenterology 99, 964–972
28. Smith, M. E., Matty, A. J., and Blair, J. A. (1970) Biochim. Biophys. Acta 219, 37–46
29. Bhandari, S. D., Fortney, T., and McMartin, K. E. (1991) Proc. Soc. Exp. Biol. Med. 196, 451–456
30. Schorn, C. M., Washington, C., and Blitzer, B. L. (1985) J. Clin. Invest. 76, 2030–2033
31. Schorn, C. M. (1990) J. Membr. Biol. 120, 192–200
32. Fei, Y. J., Liu, W., Prasad, P. D., Kekuda, R., Oblak, T. G., Ganapathy, V., and Leibach, F. H. (1997) Biochemistry 36, 452–460
33. Leibach, F. H., and Ganapathy, V. (1996) Ann. Rev. Nutr. 16, 99–119
34. Poe, M. (1977) J. Biol. Chem. 252, 3724–3728
35. Temple, C., and Montgomery, J. A. (1984) in Chemistry and Biochemistry of Folates (Blakley, R. L., and Benkovic, S. J., eds) pp. 61–120, Wiley, New York
