Membrane Transport of Folate Compounds

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INTRODUCTION: FOLATE TRANSPORT IN CELL REPLICATION AND CANCER CHEMOTHERAPY

One-carbon (C1) derivatives of tetrahydrofolate, the coenzyme form of the vitamin folic acid, play a key role in cell replication through their involvement in the biosynthesis of purine nucleotides, the pyrimidine nucleotide thymidylate, and the amino acids methionine and serine (reviewed in [1]). All eukaryotic cells and some prokaryotes (e.g., Lactobacillus casei), however, lack the ability to construct the parent folate structure (pteridine-aminobenzoate-glutamate), even though this task would appear to be trivial in view of the formidable synthetic capabilities of these cells. As a result, these cells depend upon an exogenous source of the vitamin (or its C1 derivatives) and a mechanism for internalization of these compounds. The latter is accomplished by an active transport process, which is augmented by the intracellular conversion of folates to polyglutamate forms that are unable to efflux from cells. Folate transport systems are also responsible for the uptake of antifolates, such as Methotrexate (MTX), that are used extensively in cancer chemotherapy (reviewed in [2]). Indeed, studies of Kessel et al. [3] demonstrated that the efficacy of MTX against a series of malignant cell lines was directly proportional to their rate of uptake. For these reasons, folate transport has been the subject of numerous studies. Initial efforts were focused upon the kinetic characteristics of the transport systems in intact cells, but more recently attention has turned to components and mechanisms. The present report summarizes the current state of knowledge about the transport of folate and MTX in two well-studied models, L. casei and L1210 mouse leukemia cells.

Fig. 1. (Left) Structure of fluorescein methotrexate. In the structure shown, the fluorescein (FITC) is linked via a dianinopentane spacer (DAP) to the γ-carboxyl of methotrexate (MTX).

Fig. 2. (Right) Fluorescence microscopy of L. casei spheroplasts labeled with the NHS ester of F-MTX (from [6]).
FOLATE TRANSPORT IN LACTOBACILLUS CASEI

L. casei cells have an absolute growth requirement for folate compounds, a property that forms the basis of a microbiological assay for the vitamin. Since these cells possess an efficient system for uptake of the vitamin, achieving concentration ratios (C_in:C_out) of several hundred-fold, they provide a convenient model for examining folate transport in prokaryotes. Kinetic characteristics of the L. casei folate transport system have been studied extensively (reviewed in [4]). Folate is the optimal substrate (Kt = 16 nM), but other folate compounds including 5-methyltetrahydrofolate, 5-formyltetrahydrofolate and MTX also have Kt values in the nM range. V_max value with folate is 6 x 10^{-2} pmoles/min/mg protein, and the amount of membrane-associated folate transport protein, measured by binding of [3H]folate at 4°C, is ca. 3 x 10^{-2} pmoles/10^6 cells (2 x 10^4 molecules/cell). From these data, it can be calculated that the turnover number (molecules of substrate transported by each transporter molecule per min) is 10 min^{-1} and that the transit time (outside->inside) for each folate molecule is 6 s. ATP, derived from glycolysis, drives the transport process, although the energy-coupling mechanism is still obscure. Folate, biotin and thiamine, although utilizing separate transport proteins, appear to share some common component, possibly an energy-coupling factor. Electroneutrality of the transport process is accomplished by the cotransport of cations bound to the transporter. Expression of the transport protein is regulated by a repression/derepression mechanism, i.e., cells grown on levels of folate ranging from 10^{-9} to 10^{-6} M contain progressively lower amounts of the transporter. This may be due to a repressor protein containing bound folate that prevents transcription of the transporter gene.

Purification of the folate transport protein from L. casei involves extraction from membranes with Triton X-100, adsorption and elution from microgranular silica, and filtration through Sephadex G-25 [5]. The isolated protein has a molecular weight of 18 kDa based upon SDS-PAGE analysis. It is characterized by an unusually high content of hydrophobic amino acids (and methionine) and the absence of carbohydrate. The amino acid sequence is not yet available. Visualization of the L. casei folate transport protein [6] can be accomplished by covalent labeling with a fluorescein derivative of MTX (F-MTX), whose structure is shown in Fig. 1. Treatment of membrane fragments with the N-hydroxysuccinimide (NHS) ester of this probe, followed by SDS-PAGE of the detergent extract, yields a single fluorescent band (18 kDa). When intact cells are treated with the NHS-ester of F-MTX, however, no fluorescence is visible under the fluorescence microscope. This is attributed to the presence of cell walls that prevent photo-activation or -emission of the fluorophore, since spheroplasts devoid of cell walls are readily labeled by this procedure (Fig. 2). In this instance, the probe is not attached to the 18 kDa transporter but rather to a 33 kDa protein that appears to be located between the inner and outer membranes. The nature of this latter protein or its role, if any, in folate transport is unknown. Visualization of the L. casei folate transport protein has also been accomplished via electron microscopy, using a double-antibody technique. Spheroplasts labeled prior to sectioning reveal the presence of transporters on the membrane (Fig. 3, top). However, when
spheroplasts are sectioned first and then labeled, antibody-reactive material is also detected in the cytoplasm (Fig. 3, bottom). This observation suggests that *L. casei* cells express a membrane and a cytoplasmic form of the folate transporter [7]. Both forms, after isolation, have molecular weights of 18 kDa. The membrane form is more hydrophobic, as indicated by amino acid composition and by the fact that it can be extracted readily into *n*-butanol. Structural differences between the two forms, and the possible relationship between the cytoplasmic and membrane forms, remain to be investigated.

**FOLATE TRANSPORT IN L1210 CELLS: THE MICROMOLAR SYSTEM**

L1210 cells, passaged in mice or propagated *in vitro* on micromolar concentrations of folate, express a folate transport system (reviewed in [4]) in which 5-methyltetrahydrofolate is the primary substrate (*K*ₐ = 1 μM). 5-Formyltetrahydrofolate and MTX have *K*ₐ values of ca. 5 μM, but folate, curiously, is a relatively poor substrate (*K*ₐ > 100 μM). *V*ₘₐₓ with 5-methyltetrahydrofolate is 0.5 pmol/10⁶ cells (6 x 10⁴ molecules/cell), the turnover number is 5 min⁻¹, and the transit time is 12 s. Based upon these properties, this system has been termed the "reduced folate/MTX" or "high capacity/low affinity" folate transport system. In this report, it will be referred to as the "μM folate transport system".

Anion gradients (concentration inside > concentration outside) drive this transport system, but the identity and relative contributions of the various intracellular anions to this process are unclear. Anion exchange has been proposed [4] as the mechanism for the transport process. Activity of the μM folate transport system is decreased when the level of intracellular cAMP is raised [8]. It is possible that a cAMP-dependent protein kinase mediates phosphorylation (and inactivation) of the transport protein. The transporter can be visualized, via fluorescence microscopy, in individual L1210 cells labeled covalently with the NHS-ester of F-MTX (see below). This labeling technique may be useful for studying expression of the transporter as a function of cell growth or cell cycle. It may also have applicability in the evaluation of cells from cancer patients.

Isolation of the μM folate transport protein is hampered by its low level; a 10-liter culture of L1210 cells (ca. 10¹⁰ cells) contains only about 50 μg of the protein. To overcome this logistical problem, a highly efficient procedure for its purification has been developed [9]. The key reagent is a biotin derivative of MTX (biotin-SS-MTX) in which the two components are joined by a linker containing a disulfide bond (Fig. 4). L1210 cells are treated with the N-hydroxysulfosuccinimide (NHSS) ester of the probe, and a detergent extract of the membrane preparation is exposed to streptavidin-agarose beads. After extensive washing, the beads are treated with dithiothreitol to release the transporter, which migrates on SDS-PAGE gels as a 43 kDa protein. All operations are carried out in the presence of multiple protease inhibitors; when these are omitted, degradation of the transporter to a 36 kDa protein is observed. The physiological significance, if any, of this specific proteolytic transformation is not yet understood. The μM transporter contains no asparagine-linked carbohydrate, as indicated by the unchanged molecular weight after treatment with peptide:N-glycosidase F (N-glycanase). The amino acid composition is consistent with its hydrophobic character; the occurrence of a blocked N-terminus, however, prevents Edman sequencing. Cloning, sequencing and over-expression of the cDNA will provide the amino acid sequence and make available larger quantities of the transporter. All of the properties of the L1210 μM folate transport protein, including the absence of a glycosylphosphatidylinositol (GPI) anchor (as shown by the failure of phosphatidylinositol-specific phospholipase C [PI-PLC] to release the protein) indicate that it is an integral membrane protein.

The μM folate transport system in human malignant cell lines (e.g., K562, CCRF-CEM) has essentially the same kinetic characteristics as the L1210 counterpart. The transporter, however, has a much higher molecular weight (ca. 80 kDa), and it contains a considerable amount of carbohydrate [10]. A surprising, and potentially important, observation is that treatment of

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**Fig. 4. Structure of biotin-SS-MTX.**
K562 cells with DMSO, which induces transformation of these cells from the malignant to the normal state, is accompanied by down-regulation of this transporter [11]. This suggests that the μM folate transport protein may be a "marker" for malignant transformation. Acquisition of this transport system would be advantageous to a cancer cell by allowing it to take up folate compounds more efficiently than a normal cell. As a compensation, however, the cancer cell would also become more sensitive to MTX.

FOLATE TRANSPORT IN L1210 CELLS: THE NANOMOLAR SYSTEM

Evidence for the existence of a second folate transport system in eukaryotic cells was provided by the observation [12] that plasma membranes of KB cells (a human nasopharyngeal epidermoid carcinoma) were found to contain extremely large amounts (ca. 200 pmole/10⁶ cells) of a folate binding protein (KD ≈ 1 nM). A soluble form of this protein, released into the medium, closely resembled folate binders that had been identified previously in serum, milk and other fluids. Because of its abundance, the KB protein was readily isolated [12], and the amino acid sequence was deduced subsequently from its cDNA [13]. The protein contains a considerable amount of asparagine-linked carbohydrate [13] and it is anchored to the membrane by a GPI component [14]. Subsequent studies revealed that the KB membrane protein was able to transport folate compounds into the cells. Folate is the optimal substrate (K, < 1 nM) for this system, but K, values for 5-methyltetrahydrofolate and 5-formyltetrahydrofolate are also in the nM range; MTX is taken up less effectively. The rate of transport, however, is extremely slow which suggests that an unusual translocation mechanism is operative or that many of the transporters are non-functional. Because of these properties, the term "low capacity/high affinity" was applied to this system. In this report, it will be referred to as the "nM folate transport system". Counterparts of this system have also been described in MA104 monkey kidney cells, CaCo human intestinal carcinoma cells, and human placenta (see [9] for references).

L1210 cells grown on micromolar concentrations of folate do not express the nM folate transporter. However, since the KB, MA104 and CaCo cells had been propagated on nanomolar concentrations of folate, L1210 cells were subjected to a similar regimen. Under these conditions and with careful selection of cells at each stage, the nM folate transporter is gradually expressed [15]. In an L1210 subline (JF) developed in this laboratory over a six-month period, the level rose progressively to ca. 6 pmole/10⁶ cells. Possible mechanisms for this up-regulation include: (a) gene amplification; (b) loss of a repressor; and (c) selection of pre-existing mutant cells. The L1210 nM folate transport protein has been isolated by a procedure similar to that employed for the μM entity, except that biotin-SS-folate replaced the MTX derivative [9]. The protein migrates as a diffuse band (39 kDa) on SDS-PAGE gels, and no degradation is seen when protease inhibitors are omitted from the purification procedure. Amino acid analysis indicates that the nM transporter is somewhat less hydrophobic than the μM transporter, but it also has a blocked N-terminus. Treatment of the transporter with N-glycanase reduces the molecular weight to ca. 32 kDa, indicating that it contains a considerable amount of asparagine-linked carbohydrate. Anchorage of the nM transporter to the membrane by a GPI "tail" is demonstrated by its release when cells are treated with PI-PLC.

Fig. 5. Treatment of folate transporters with PI-PLC. L1210/JF cells (nM transporter) were labeled noncovalently with F-folate (A) and then treated with PI-PLC (B). Parental L1210 cells (μM transporter) were labeled covalently with the NHS ester of F-MTX (C) and then treated with the enzyme (D). From [9].
The latter experiment illustrates the use of fluorescein-folate to visualize this transporter; in this instance, activation of the probe is unnecessary because of its tight binding to the protein. The above properties of the L1210 nM folate transporter indicate that it is a heavily glycosylated protein, anchored exofacially to the plasma membrane. Since its characteristics are very similar to the counterparts from KB, MA104, and CaCo cells and from human placenta, it seems likely that nM folate transporters are relatively invariant in human and mouse cells and in normal and malignant cells. Still not understood, however, is how this protein, situated totally outside of the membrane, can translocate substrates through the membrane. Electron microscopy has provided some evidence that substrate internalization proceeds via an endocytosis mechanism, which utilizes "caveoli" instead of coated pits and does not involve lysosomes [16]. The recent demonstration [17] that proteins (e.g., serum albumin and peroxidase) attached to folate are taken up by KB cells further supports an endocytosis mechanism. But regardless of the uncertainty with regard to mechanism, the nM transporter does bring folate compounds and certain antifolates (at the nM level) into cells.

FUTURE DIRECTIONS
The above summary of three well-studied folate transport systems provides a view of the current status of this field. Considerable progress has been made, but much remains to be done. Future directions can be envisioned in three categories, listed in order of increasing difficulty. (I) Folate transport in normal mammalian tissues such as liver, kidney, intestine and choroid plexus is not well-understood, in part because of conflicting data from various laboratories on the general characteristics and kinetics of these systems. Isolation of the folate transport proteins from these sources, possibly through the use of biotin-MTX or -folate, may resolve some of these uncertainties; folate efflux proteins that are not identical with the transporters also need to be isolated from various sources and characterized. Visualization of folate transporters in individual cells and their localization on membranes, with the aid of fluorescein and biotin derivatives of MTX and folate (or comparable probes) in conjunction with fluorescence or electron microscopy, will augment the body of information about a wider spectrum of folate transport systems. The important question of whether the mammalian μM folate transporter can be used as a marker for the malignant state will certainly receive further attention; in this instance, too, the above probes may facilitate the rapid screening of a variety of cells, including those undergoing malignant transformation and vice versa. (II) Mechanisms for the induction of the L1210 nM folate transport protein and for the regulation of the L. casei and L1210 μM transporters by repression/depression and cAMP, respectively, are intriguing problems waiting to be solved. Likewise, the existence of a cytoplasmic form of the L. casei folate transport protein may provide a lead for investigating the processing of transporters en route to the membrane. And finally, additional studies on the 33 kDa periplasmic protein in L. casei cells and the proteolytic cleavage of the L1210 μM folate transport protein may reveal unsuspected facets in the mechanism of the transport process. (III) Cloning, sequencing and over-expression of the L. casei transporter and the L1210 μM transporter will certainly reveal structural features of these proteins and make them accessible in sufficient quantity for additional structural studies and for reconstitution experiments. The more difficult task of obtaining the 3-dimensional structures of these transporters by X-ray diffraction must await their crystallization. Another distant but prominent objective is elucidation of the mechanism (defined specifically in spatial and temporal terms) by which these integral membrane proteins actually translocate their folate substrates across the cell membrane. The energy-coupling mechanism associated with this process also represents a formidable challenge, although the occurrence of a factor shared by the folate, thiamine and biotin transporters in L. casei may offer a lead that could be exploited. An easier task, perhaps, will be to understand the mechanism by which the L1210 nM transporter, presumably located entirely external to the membrane, internalizes folate substrates.

SUMMARY
All eukaryotic cells and some prokaryotes that are unable to synthesize folic acid utilize membrane-associated transport systems for acquisition of the pre-formed vitamin or its coenzyme forms from external sources. These transport systems, in addition to providing folates essential for cell replication, are also important because of their role in the internalization of antifolates such as Methotrexate (MTX) that are used extensively in cancer chemotherapy.
Information about the components and mechanism of folate transport systems has been derived, in large part, from studies with Lactobacillus casei and L1210 mouse leukemia cells, which serve as convenient models for prokaryotes and eukaryotes, respectively. L. casei contain a single folate transport system whose $K_t$ value (i.e., concentration for half-maximum rate of uptake) for the preferred substrate folate is in the nanomolar range. The hydrophobic membrane-associated folate transport protein (18 kDa) has been purified to homogeneity and characterized. Expression of this transporter is repressed in cells grown on high concentrations ($\mu$M) of folate. L1210 cells contain two separate transport systems for folate compounds: (1) the low affinity system ($K_t$ values for the preferred substrates 5-methyl- and 5-formyltetrahydrofolate and MTX in the $\mu$M range); and (2) the high affinity system ($K_t$ for folate in the nM range). Fluorescein and biotin derivatives of MTX and folate, after conversion to N-hydroxysuccinimide esters, can be attached covalently to the transporters. These probes have been used for visualizing the transporters by fluorescence and electron microscopy and for their purification to homogeneity. The $\mu$M transporter (43 kDa) is a non-glycosylated, integral membrane protein, while the nM counterpart (39 kDa) is heavily glycosylated and anchored exofacially to the membrane by a glycosylphosphatidylinositol component.

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