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Role of N-glycosylation in the structure and function of the methotrexate membrane transporter from CCRF-CEM human lymphoblastic leukemia cells

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Abstract—The carrier protein for methotrexate and tetrahydrofolate cofactors (GP-MTX) in CCRF-CEM human lymphoblastic leukemia cells is a 117 kDa glycoprotein containing both N- and O-linked oligosaccharides (Matherly *et al.*, *J Biol Chem* **267**: 23253–23260, 1992). Tunicamycin, an inhibitor of N-glycosylation, was used to investigate the roles of asparagine-linked oligosaccharides in the structure, intracellular routing, and transport function of GP-MTX. Tunicamycin was growth inhibitory toward CCRF-CEM cells (IC_{50} = 0.80 μg/mL) and caused a potent suppression of [^{3}H]mannose incorporation into nascent glycoproteins. From 1–3 μg/mL, inhibition of [^{3}H]mannose incorporation was 66–87%, exceeding that for [^{35}S]methionine incorporation by 2 to 4-fold. Tunicamycin (1 and 2 μg/mL) exposures decreased the median molecular masses of GP-MTX on immunoblots (to 82 and 67 kDa, respectively) and were accompanied by reduced maximal rates of methotrexate uptake (31 and 37%, respectively, of control levels). Conversely, the K_t values for methotrexate binding to the transporter were unaffected by tunicamycin treatments. The effects of tunicamycin on methotrexate influx closely correlated with lower levels of immunoreactive GP-MTX in plasma membranes and specific [^{3}H]methotrexate binding to intact cells, suggesting that the transport effect was due to decreased numbers of carrier proteins at the membrane surface. The reduced molecular mass values for GP-MTX, which accompanied tunicamycin exposures, were further decreased (to 55 and 50 kDa at 1 and 2 μg/mL, respectively) by digestions with *N*-glycanase. Hence, despite the large loss of *N*-glycan from GP-MTX in tunicamycin-treated cells, residual core oligosccharides remained. The sizes of hypoglycosylated GP-MTX following both treatments were similar to that of the functionally homologous methotrexate membrane carrier previously identified in L1210 murine leukemia cells.

Key words: folate; methotrexate; antifolate; membrane transport; tunicamycin; glycoprotein

Membrane transport of MTX* is an important determinant of its antitumor activity [1, 2]. Likewise, impaired drug uptake is an established mechanism of MTX resistance [3–6]. MTX membrane transport has been characterized extensively in L1210 murine leukemia cells, and a 42–48 kDa carrier protein was identified in plasma membranes by radioaffinity labeling with NHS esters of [^{3}H]MTX or aminopterin [4, 7], or with APA[^{125}I]ASA-Lys [8].

In cultured human tumor cells, including both K562 erythroleukemia and CCRF-CEM lymphoblastic leukemia lines, the MTX carrier is somewhat larger (99 and 117 kDa respectively†) than its murine counterpart, in part due to its extensive glycosylation [3, 9, 11]. Although the majority of the carbohydrate appears to be attached to asparagine residues and is sensitive to cleavage by *N*-glycanase, low levels of O-linked oligosaccharides can also be detected [3]. The binding of the NHS[^{3}H]MTX labeled transporter to immobilized lectins was examined recently [3]. On this basis, fractionation in *Ricinus communis* agglutinin I-agarose, combined with preparative electrophoresis, was used to isolate the glycoprotein carrier (hereafter, designated GP-MTX) from transport-upregulated K562 cells in sufficient quantity and purity to prepare antiserum [3].

Since membrane transport in cultured L1210 and CCRF-CEM cells shows virtually identical specificities and transport kinetics for most folate and antifolate substrates [12–14], it is notable that only the MTX carrier from cultured human cells seems to be glycosylated [3, 9, 15]. This implies a low stringency requirement for this post-translational modification for carrier insertion into plasma membranes, or for conferring the optimal polypeptide conformation necessary to mediate MTX influx.

A well established approach for investigating the functional significance of protein glycosylation is to use specific inhibitors of intracellular glycoprotein processing enzymes. For the present study, TN, an inhibitor of core oligosaccharide addition to asparigine residues of nascent glycoproteins (at the level of the transfer of *N*-acetylglucosamine to dolichol phosphate to form dolichol pyrophosphate *N*-acetyl glucasamine [16]), has been used to directly assess the importance of N-glycosylation in the structure, intracellular routing, and function of the MTX/tetrahydrofolate cofactor carrier in CCRF-CEM cells.

Materials and Methods

Chemicals. [3′,5′,7-^{3}H]MTX (20 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). L-[^{35}S]-Methionine (2000 Ci/mmol) and *d*[2-^{3}H]mannose (30 Ci/mmol) were purchased from New England Nuclear (Boston, MA) and ICN Biochemicals (Costa Mesa, CA), respectively. [^{125}I]Protein A was purchased from Dupont/New England Nuclear (Boston, MA). Unlabeled MTX and TN were obtained from the Drug Development Branch, National Cancer Institute, Bethesda, MD. Both labeled and unlabeled MTX were purified prior to use by reversed-phase HPLC [17]. Genzyme (Boston, MA) was the source of *N*-glycanase. Bio-Rad (Richmond, CA) was the source of the majority of the electrophoresis reagents. GP-MTX-specific antiserum was prepared in a female New Zealand white rabbit as previously described [3].

* Abbreviations: APA[^{125}I]ASA-Lys, *N*-(4-amino-4-deoxy-10-methylpteroyl)-*N*-(4-azido-5-[^{125}I] iodosalicylyl)-L-lysine; DPBS, Dulbecco's phosphate-buffered saline; GP-MTX, methotrexate glycoprotein carrier; K_t, Michaelis–Menten constant for transport; MTX, methotrexate; NHS, *N*-hydroxysuccinimide and TN, tunicamycin.

† Electrophoresis was in the presence of SDS on 4–10% linear gradient polyacrylamide gels [9]. The molecular weights for highly glycosylated proteins, including GP-MTX, vary with the gel system used [9, 10].

Cell culture. The CCRF-CEM lymphoblastic leukemia line was a gift from Dr. Andre Rosowsky (Boston, MA). Cell maintenance and cytotoxicity assays were performed as detailed preivously [3].

Membrane transport methodology. For membrane transport measurements, logarithmically growing cells were washed with DPBS [18] and suspended into phosphate-buffered Hanks' balanced salts [19]. Transport experiments were performed as described previously [9]. K_t and V_{max} values for MTX uptake were calculated from Lineweaver–Burk plots.

Analyses of specific [^{3}H]MTX binding and radioaffinity labeling with NHS[^{3}H]MTX. Specific [^{3}H]MTX binding to intact cells was performed by a modification of the method of Henderson *et al.* [20], exactly as described earlier [3, 4]. The difference between total surface bound radiolabeled drug in the presence and absence of excess unlabeled MTX corresponds to MTX specifically bound to the membrane carrier [20].

NHS[^{3}H]MTX was prepared by the method of Henderson and Zevely [21], and surface MTX transporters were radiolabeled with 700 nM NHS[^{3}H]MTX as previously described [9]. The cell pellets were extracted with 1% peroxide-free Triton X-100 at room temperature for 30 min. The solubilized membrane proteins were precipitated with an equal volume of acetone at −20°. The precipitate was solubilized in 0.5 N NaOH for protein determination and scintillation counting. The specificity of radioligand incorporation in these assays was established in parallel incubations containing 500 μM unlabeled MTX.

Radioactive precursor incorporation into macromolecules. Following an overnight exposure to TN, cells (approx. 5×10^5/mL) were incubated for 24 hr at 37° in growth medium containing [^{35}S]methionine (1 μCi/mL) or [^{3}H]-mannose (45 μCi/mL). Cells were washed twice with 10 mL of ice-cold DPBS and suspended into 10% trichloroacetic acid at 0°. The acid-insoluble precipitates were washed twice with 10% trichloroacetic acid and solubilized in 1 mL of 0.5 N NaOH for quantitation of the levels of incorporated radioactivity. Radioactivity was measured with a Tracor Analytic liquid scintillation counter with Ready Value Scintillation fluid (Beckman). Relative incorporations were normalized to the protein contents of the trichloroacetic acid-insoluble fraction. Proteins were assayed by the method of Lowry *et al.* [22].

Immunoblot analyses of GP-MTX. Plasma membranes were prepared from CCRF-CEM cells as described previously [3] and solubilized in 10 mM Tris–HCl (pH 7.0) containing 2% SDS and assorted proteolytic inhibitors (0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin, 10 μg/mL antipain, 10 μg/mL bestatin, 3 μg/mL aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride). Proteins were electrophoresed on 4–10% linear gradient gels in the presence of SDS [23] and electrophoretically transferred to Immobilon P [24]. Immunoblot analysis were performed as previously described using rabbit antiserum prepared to GP-MTX from human erythroleukemia cells [3], or preimmune serum. Blots were developed with [^{125}I]Protein A followed by autoradiography. Unstained molecular weight standards were detected by staining the Immobilon P with Coomassie Brilliant Blue R250 [24]. The relative amounts of immunoreactive proteins were quantitated using a Molecular Dynamics computing densitometer.

For *N*-glycanase treatments, membranes were solubilized in 2% SDS and diluted 12-fold into 10 mM Tris (pH 7.2), 1.25% *N*-octyl glucoside, and 0.2 mM phenylmethylsulfonyl fluoride. Incubations were for 18 hr at 37° using 5–7 U/mL of *N*-glycanase. Controls were incubated in parallel without enzyme. At the end of the incubation, the samples were concentrated under vacuum, diluted into electrophoresis sample buffer, centrifuged in a microcentrifuge, and loaded onto polyacrylamide gels for electrophoresis.

Results and Discussion

TN was used to explore the relationships between N-linked glycosylation and GP-MTX structure, plasma membrane insertion, and carrier-mediated MTX transport in CCRF-CEM cells. Initial studies were performed to correlate the growth inhibitory effects of TN with inhibition of protein synthesis and glycoprotein processing. TN was somewhat growth inhibitory to CCRF-CEM cells (IC_{50} = 0.80 μg/mL). This was associated with an inhibition of [^{3}H]mannose incorporation into trichloroacetic acid-precipitable macromolecules (66–87% from 1–3 μg/mL TN). [^{35}S]Methionine incorporations were inhibited to a much reduced extent (18–45%) over the same range of TN concentrations, consistent with a primary drug effect at the level of N-glycosylation.

Our initial approach to assess the effects of TN on GP-MTX structure involved a 48-hr exposure to various concentrations of inhibitor (1–3 μg/mL). However, immunoblot analyses of plasma membrane proteins following these treatments (not shown) revealed that the electrophoretic migrations for GP-MTX were unchanged from the untreated controls. This result establishes that minimal levels of hypoglycosylated GP-MTX had accumulated and, presumably, reflects the slow turnover of GP-MTX under these experimental conditions.

To obviate this difficulty, cells were *grown continuously* for 7–10 generations in the presence of TN concentrations (1 and 2 μg/mL) that potently inhibited N-glycosylation (66 and 82%, respectively, based on [^{3}H]mannose incorporations), yet allowed for sustained, albeit diminished, rates of division (25 and 16%, respectively, of untreated controls). The data presented in Fig. 1 show that this experimental design had a dramatic effect on GP-MTX structure. The median molecular masses were decreased significantly, approaching values (82 and 67 kDa, respectively), similar to that resulting from *N*-glycanase treatment (calculated as 74 kDa in Fig. 1). The magnitude of these shifts in electrophoretic mobilities for GP-MTX accompanying the TN treatments indicated that a major loss of *N*-glycan had occurred.

The relative levels of plasma membrane GP-MTX on immunoblots, measured by densitometry, were also somewhat reduced in response to TN (Table 1), suggesting that intracellular routing of hypoglycosylated carriers to the membrane surface was hindered by the absence of N-linked oligosaccharides. Similar results were obtained when carrier numbers were quantitated by specific [^{3}H]MTX cell surface binding (Table 1). Decreased carrier contents were accompanied by nearly identical decreases in the maximal rates (i.e. V_{max}) for [^{3}H]MTX influx (Table 1); conversely, the K_t values for drug binding to the transporter were unaffected by the TN treatments. Interestingly, NHS[^{3}H]-MTX labeling of GP-MTX correlated poorly with [^{3}H]-MTX influx rates (53.7 and 9.7% of maximal incorporation at 1 and 2 μg/mL TN, respectively).

The immunoreactive GP-MTX bands in TN-treated cells were somewhat broadened compared with those resulting from *N*-glycanase; however, they could be sharpened by an additional digestion with this enzyme. In addition, the apparent molecular masses decreased further to values less than those for either treatment, alone (55 and 50 kDa for 1 and 2 μg/mL, respectively; Fig. 1). This result shows that, under our experimental conditions, *neither* *N*-glycanase nor TN, alone, effects the complete removal of the asparagine-linked oligosaccharides from GP-MTX. For *N*-glycanase, this may reflect the presence of N- or C-terminal oligosaccharides, which are largely insensitive to enzyme cleavage [25].

Although these results establish that an additional, residual fraction of N-linked oligosaccharides can be removed from GP-MTX by treatments with both TN and *N*-glycanase, the data with TN, alone, demonstrate that the loss of the major portion of the *N*-glycan has only a

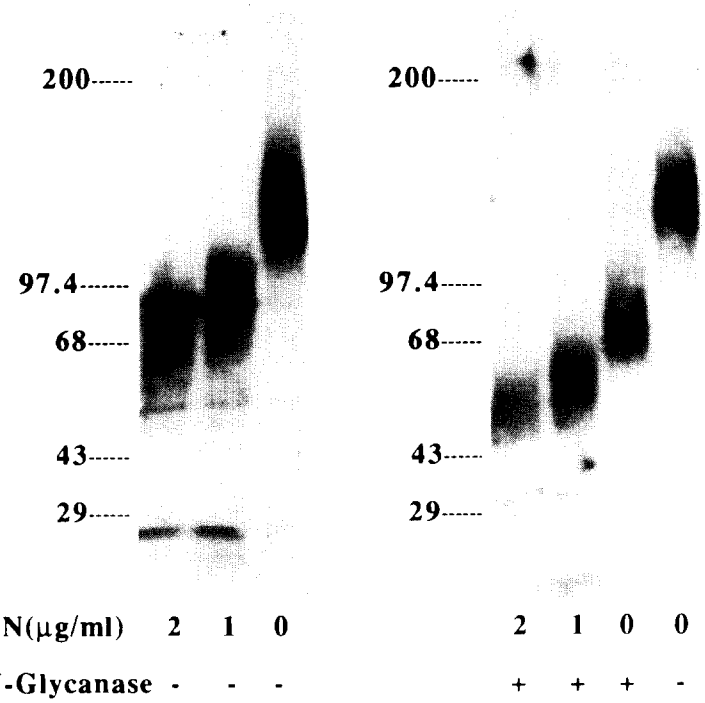

Fig. 1. Effects of TN and *N*-glycanase on GP-MTX in CCRF-CEM cells. *Left:* The results of an immunoblot analysis of plasma membrane proteins from untreated or TN-treated cells are illustrated. TN treatments were for 7–10 generations. *Right:* The effects of *N*-glycanase on immunoreactive membrane proteins from TN-treated cells were analyzed on immunoblots. In all experiments, samples were electrophoresed on 4–10% linear gradient gels in the presence of SDS. Immunoblot analyses were performed as described in Materials and Methods. Fifty micrograms of membrane proteins were used for all analyses. The molecular masses (in kDa) for standard proteins are shown. No significant immunoreactive bands were detected on identical blots probed with preimmune serum (not shown).

Table 1. Effects of TN on MTX transport parameters and GP-MTX content of CCRF-CEM cells

Treatment	MTX K_t*	MTX V_{max}* (pmol/min/mg)	Relative V_{max}	Relative GP-MTX	
				Immunoblot†	MTX binding‡
None	2.87	3.39	1	1	1
TN (1 μg/mL)	2.92	2.60	0.77	0.68	0.70
TN (2 μg/mL)	2.82	2.35	0.69	0.63	0.65

* Untreated cells or cells pretreated with TN for 7–10 generations were assayed for [^{3}H]MTX transport. Kinetic constants for MTX transport were calculated by Lineweaver–Burk analysis of initial rate data over a range of [^{3}H]MTX concentrations (0.5 to 5 μM). Data are calculated as the mean values from 2–6 experiments. SE did not exceed 10%.

† Relative GP-MTX levels were calculated by quantitative immunoblot analysis and computer densitometry.

‡ Specific [^{3}H]MTX binding to intact cells was measured in the presence of 750 nM [^{3}H]MTX, as described in Materials and Methods. Data for TN-treated cells were normalized to the [^{3}H]MTX binding capacity of 1.31 ± 0.28 pmol/10^7 cells (± SEM; N = 3) for untreated cells.

small effect on the routing of carrier molecules to the cell surface, and no effect on the capacity of GP-MTX to mediate MTX uptake. The disproportionate decrease in radioaffinity labeling of TN-treated cells with NHS[^{3}H]-MTX suggests that deglycosylated GP-MTX undergoes conformational changes that render it less reactive with NHS[^{3}H]MTX, independent of its transport activity.

The molecular masses of hypoglycosylated GP-MTX, resulting from *both* TN and *N*-glycanase treatments, approximate that for the functionally homologous L1210 MTX transporter [4, 7, 8], a 42–48 kDa form reported to be insensitive to *N*-glycanase [15]. This size similarity should be even closer if the minor O-linked oligosacchraride fraction [3] was absent from N-deglycosylated GP-MTX.

However, it is notable that these systems also differ in a manner unrelated to their degrees of glycosylation since the murine and human transporters can be distinguished in their rates of membrane translocation [26], immunoreactivities (Matherly LH and Angeles SM, unpublished observation), and, for at least one antifolate substrate (i.e. 1843U89), membrane transport [27]. Studies are underway to assess further the structural similarities between the MTX transporters from cultured human and murine cells.

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