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Correlation of Altered Tyrosine Phosphorylation with Methotrexate Resistance in a Cisplatin-Resistant Subline of L1210 cells

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ABSTRACT. Collateral resistance to cisplatin and methotrexate has been reported in several cell lines. A murine leukemia cell line (L1210/DDP) selected for cisplatin resistance also has been shown to be highly resistant to methotrexate. Of the mechanisms proposed for methotrexate resistance, only changes in methotrexate transport into the cells were found in an earlier report. Methotrexate enters mammalian cells via an active transport system. In the present study, we demonstrated that the transport into the cell may be impaired in the resistant cells due to altered tyrosine phosphorylation of a membrane protein with a molecular mass of 66 kDa. This alteration was manifested by altered tyrosine phosphorylation of the 66 kDa protein and may be an underlying modification that renders the cells resistant to methotrexate. These results suggest involvement of tyrosine phosphorylation in folate transport and methotrexate resistance in L1210/DDP cells. BIOCHEM PHARMACOL 51;4:477–482, 1996.

KEY WORDS. methotrexate; cisplatin; tyrosine phosphorylation; drug transport; drug resistance; L1210 cells

MTX†, an antifolate oncolytic agent, competitively inhibits the binding of dihydrofolate to the enzyme DHFR. Consequently, the cell is incapable of maintaining the necessary stores of reduced folates, and the enzymatic pathways requiring these cofactors cease to function. DDP, a metal based oncolytic agent, binds to nucleophilic sites on DNA, such as the N-7 and O-6 positions of guanine. Consequently, DNA structure and synthesis are altered, and cell death ensues.

Despite the apparent disparity in the putative mechanisms of action between these two anticancer drugs, we, as well as others, have reported a collateral resistance to MTX in cells expressing DDP resistance [1–5]. Recently [6], we demonstrated that resistance to MTX in L1210 cells made resistant to DDP (L1210/DDP) is not related to alterations in DHFR levels or function. Rather, the only significant difference noted between wild-type L1210 (L1210/0) and L1210/DDP cells was a 2-fold decrease in cell-associated MTX in the latter cell type. While this difference is significant, it would be surprising if a 2-fold difference in drug association can account for the magnitude of resistance seen in the L1210/DDP cells (>25,000-fold), when comparing IC_{50} values of the two cell lines.

The 2-fold uptake difference may be deceiving, however, as we only measured cell-associated MTX. Unlike most anticancer drugs, MTX enters the cell via an active transport system that includes at least a 48 kDa membrane protein, a 38 kDa cytosolic protein that shuttles folates from the membrane to DHFR, and other cytosolic folate binding proteins [7]. Others have reported resistance to MTX resulting from alterations in this highly coordinated transport system that ultimately prevents MTX from reaching DHFR [8–10]. Simple membrane association of [^{3}H]MTX does not necessarily correlate with drug binding to DHFR, the ultimate target of MTX. Without measuring cellular drug distribution and protein-drug interactions, one cannot be certain that MTX binding to DHFR has occurred. In this paper, we have more carefully assessed intracellular drug distribution, using a photoaffinity probe.

The present report provides evidence, using a photoaffinity labeling technique, that MTX is not transported intracellularly in L1210/DDP cells. We further demonstrated a significant difference in protein tyrosine phosphorylation patterns between L1210/0 and L1210/DDP cells, which may be functionally linked to the observed differences in MTX transport.

MATERIALS AND METHODS

Cell Culture

Wild-type (L1210/0) and cisplatin-resistant (L1210/DDP) murine leukemia cells were grown in McCoy's 5A medium

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† *Abbreviations:* DDP, cisplatin, *cis*-diamminedichloroplatinum(II): MTX, methotrexate; DHFR, dihydrofolate reductase; APA-ASA-Lys, N^{α}-(4-amino-deoxy-10-methylpteroyl)-N^{ϵ}-(4-azidosalicylyl)-L-lysine; APA-[I^{125}I]ASA-Lys, N^{α}-(4-amino-deoxy-10-methylpteroyl)-N^{ϵ}-(4-azido-5-[I^{125}]iodosalicylyl)-L-lysine; and PMSF, phenylmethylsulfonyl fluoride.

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(Gibco), supplemented with 5% donor horse serum (Hazelton), and 5% fetal bovine serum (Hyclone), respectively, in a 10% CO_2, 37° humidified atmosphere. The cell cultures were free of mycoplasma contamination.

Preparation of Whole Cell Lysates for Western Blot Analysis

Cells were centrifuged (450 *g*, 10 min, 4°) in an IEC Centra-8R centrifuge and washed three times with cold PBS. The conditions were normalized based on equal cell numbers. The cell pellet was suspended in Buffer A (1% Triton X-100, 10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 2 mM PMSF, 0.1 mM sodium orthovanadate, 0.02 mg/mL leupeptin and 0.1% sodium azide) and sonicated for three 30-sec bursts with a Sonicator Cell Disrupter (Ultrasonics Inc.) and left on ice for 10 min. The suspension was centrifuged at 12,000 *g* for 10 min at 4°, and the supernatant was saved for electrophoresis and western blotting.

Isolation of Membrane and Cytosolic Fractions

Cells were washed three times with PBS as described above and suspended in Buffer B (1 mM dithiothreitol, 20 mM HEPES, 1 mM PMSF, 0.02 mg/mL leupeptin, 0.1 mM sodium orthovanadate and 50 mM sodium fluoride), followed by homogenization with 20 strokes of a Dounce homogenizer. When >90% of the cells were lysed, as determined by light microscopy, the homogenate was centrifuged at 3000 *g* for 5 min at 4°. The supernatant was then ultracentrifuged at 100,000 *g* for 60 min at 4°. The membrane pellet was suspended in Buffer A, and the supernatant (cytosolic fraction) was concentrated utilizing a Centricon 10 ultrafiltration device (Amicon Inc.). The concentrated fraction was suspended in Buffer A containing 1% Triton X-100.

Iodination

PY69 antibody (ICN) to phosphotyrosine was iodinated by the Chloramine-T method [11]. A photoaffinity analogue of methotrexate (APA-ASA-Lys, a gift from Dr. J. Freisheim, Medical College of Ohio) was iodinated using Iodobeads (Pierce) in red subdued light. The iodinated product (APA-[^{125}I]ASA-Lys) was purified on a silica gel thin-layer chromatographic plate [12].

Photoaffinity Labeling

Pulse labeling experiments were performed as described by Price and Freisheim [12]. All procedures were performed in subdued red light. Briefly, the iodinated photoaffinity analogue of methotrexate was added to a cell suspension (45×10^6 cells/mL) in ice-cold Buffer C (160 mM HEPES, 2 mM $MgCl_2$ buffer, pH 7.4). The cells were incubated with the photoprobe for 5 min on ice, washed twice with ice-cold Buffer C and centrifuged (450 *g*, 10 min, 4°C) to remove unincorporated APA-[^{125}I]ASA-Lys. The cells were suspended in Buffer C, (prewarmed to 37°), and 1-mL aliquots were removed from the cell suspension at various time points followed by UV irradiation for 2 min on ice. 2-Mercaptoethanol was added immediately to all irradiated samples at a final concentration of 50 μg/mL. Cells were washed with ice-cold PBS, centrifuged (450 *g*, 10 min, 4°), and resuspended in ice-cold Buffer A, and lysates were prepared as described above. Proteins were separated by SDS-PAGE, fixed for 3 hr in 25% isopropanol and 10% acetic acid in water, dried, and autoradiographed.

Western Blot Analysis with Antiphosphotyrosine Antibody

Membrane and cytosolic fractions and total cell homogenates were suspended in Laemmli buffer [13] and boiled for 3 min. Samples containing 100–350 μg of protein were separated by 12% SDS-PAGE and transferred to an Immobilon-P membrane (Millipore Corp.), which was blocked with 4% bovine serum albumin and 1% ovalbumin in PBS for 36 h at 4°. The blots were incubated with ^{125}I-labeled antibody to phosphotyrosine PY69 for 2 hr at 4° with shaking, washed twice for 1 hr with PBS, and autoradiographed [14].

Western Blot Analysis with Antibody to Bovine Serum Albumin

Whole cell lysates from L1210/0 and L1210/DDP cells were separated on 12% SDS-PAGE, transferred to a nitrocellulose membrane (Bio-Rad), and blocked in 5% nonfat milk. Western blotting of bovine serum albumin was carried out by a horseradish peroxidase kit (Bio-Rad) according to the directions of the supplier.

Immunoprecipitation of Photoaffinity Labeled Cells with Antiphosphotyrosine Antibody

The cells were labeled with APA-[^{125}I]ASA-Lys in the presence and absence of MTX as described above. The cells were lysed in lysis buffer and immunoprecipitated with PY20 antibody for 4 hr. The mixture was incubated with protein A agarose for an additional 2 hr and the beads were washed with lysis buffer four times by centrifuging in a microfuge for 30 sec at 3000 g. The beads were suspended in SDS buffer, boiled for 3 min, and centrifuged. The supernatant was applied to SDS-PAGE, and proteins were separated. The gel was dried and autoradiographed.

Other Methods

Protein determinations were carried out with Bio-Rad Reagent using bovine serum albumin as a standard [15]. Molecular weights were determined by the method of Weber and Osborn [16] using Bio-Rad and BRL molecular weight markers.

RESULTS

Photoaffinity Studies

Photoaffinity techniques have been used by others to discern the folate shuttle system in L1210 cells and to identify lesions

in this system related to MTX resistance. To determine whether similar lesions were induced in L1210 cells exposed to increasing concentrations of DDP, we utilized APA-[^{125}I]-ASA-Lys, an analogue of MTX, which covalently binds to proteins when activated upon irradiation with UV light. L1210/0 and L1210/DDP cells were treated with APA-[^{125}I]-ASA-Lys for 5 min at 4° and incubated in drug-free medium at 37° for 0, 1, 2, 5, 10 or 20 min prior to UV irradiation. Upon exposure of L1210/0 cells to the photoprobe (Fig. 1), bands were detected immediately at a 66 kDa protein and proteins in the 40–48 kDa molecular mass range. The intensity of these bands diminished within 5 min, whereas the intensity of a band detected at a 38 kDa protein increased or remained constant during the first 5 min and then decreased between 5 and 20 min. During this time, increased amounts of photoprobe at 21 kDa, a molecular mass corresponding to DHFR, were observed. In contrast to L1210/0 cells, where the photoprobe was initially associated with a 66 kDa protein, APA-[^{125}I]ASA-Lys bound to a 68 kDa protein in L1210/DDP cells. The intensity of the band at the 68 kDa protein diminished over time. However, unlike in L1210/0 cells where the MTX analogue was transported ultimately to a 21 kDa protein, no transport was observed in L1210/DDP cells at any time during the entire experiment. Three experiments gave the same result.

Western Blot Analysis with Antibody to BSA

Photoaffinity labeling of a 66 kDa protein has been reported previously [12] but this band was identified tentatively as BSA non-specifically bound to the surface of cells grown in BSA containing medium. To determine whether the protein labeled in these experiments was BSA, cell lysates from both L1210/0 and L1210/DDP cells were analyzed for BSA by western blotting. Protein lysate (600 μg) was loaded in each lane. As controls, 2 ng, 5 μg, and 10 μg of BSA were also loaded in separate lanes. BSA was not detected in either cell lysate as can be seen in Fig. 2, whereas as little as 2 ng of BSA was clearly detected by western blot analysis. These results conclude that if any BSA were present in the lysate, it represented less than 0.0003% of the total protein loaded for each cell lysate.

Tyrosine Phosphorylation

The importance of protein kinase and phosphatase activity in regulating protein function is becoming increasingly evident. To determine whether the state of protein phosphorylation could be correlated to protein function in the L1210/0 and L1210/DDP cells, lysates of both cell types were analyzed by western blot using an antibody [14] that recognizes phosphotyrosine epitopes (PY69). Two hundred micrograms of total cell lysate, 100 μg of membrane fraction, and 350 μg of cytosol were loaded for each cell line. The protein antibody labeling patterns of whole cell lysates were quite different between the L1210/0 and L1210/DDP cells (Fig. 3). The most intensely labeled protein in the wild-type cells had a molecular mass of 66–68 kDa, whereas no such protein was recognized in the resistant cells. Rather, two proteins of molecular mass 46–52

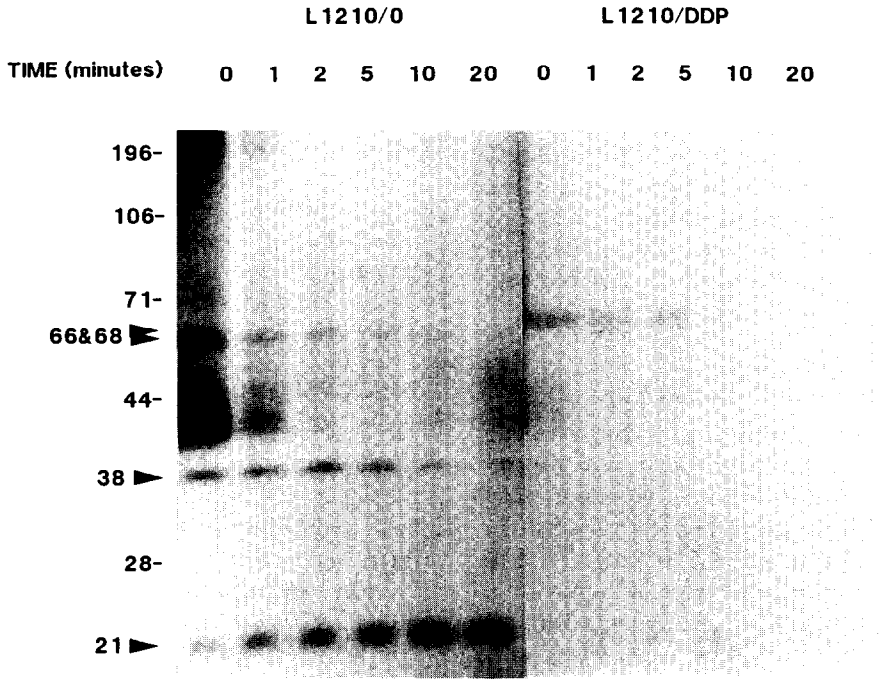

FIG. 1. Photoaffinity labeling of L1210/0 and L1210/DDP cells. Equal numbers of cells were treated with a photoaffinity analogue of MTX, APA-[^{125}I]ASA-Lys, for various time intervals and exposed to UV light. Cells were washed, and lysates were analyzed by SDS–PAGE and autoradiographed.

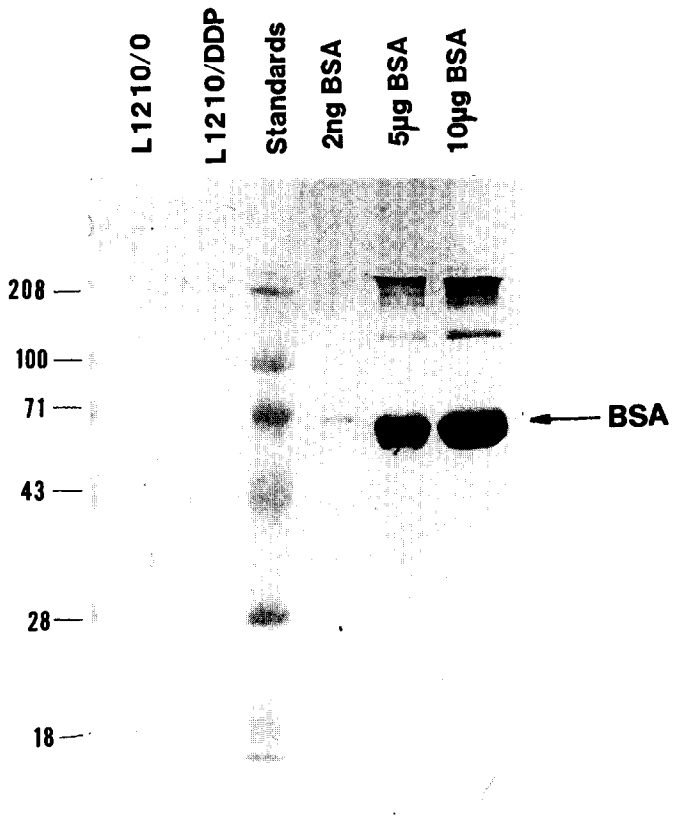

FIG. 2. Western blot analysis of cell lysates from L1210/0 and L1210/DDP cells using an antibody directed to BSA. BSA was used as a positive control.

kDa were apparent in the resistant cells. Similar changes were observed in four experiments.

Analysis of the membrane and cytosolic fractions of cell lysates demonstrated that the 66–68 kDa phosphoprotein identified in the L1210/0 cells was localized in the membrane. The 46–52 kDa phosphoproteins noted in the L1210/DDP cells were present in both the membrane and cytosolic fractions. The fact that a number of phosphoproteins were detected in the cytosolic fractions of both cell types that were not observed in the whole cell extracts is not surprising given that the cytosolic fraction was significantly concentrated prior to western blotting.

Immunoprecipitation of Photoaffinity Labeled Lysates with Antibody to Phosphotyrosine

The results obtained thus far are intriguing as they correlate MTX transport with the state of phosphorylation of a membrane-bound protein. To ascertain whether the protein band bound to the photoaffinity probe was indeed the same protein that was phosphorylated as detected by the western blot, cells were first labeled with the radiolabeled photoaffinity analog, lysed as before, and the lysate immunoprecipitated with an antibody to phosphotyrosine, PY20. These experiments were done at 0° for cell surface labeling. Results shown in Fig. 4 clearly demonstrate that the 66 kDa band immunoprecipitated with the PY20 antibody to phosphotyrosine was labeled with the photoaffinity analog (lane 4). The same results were obtained in three different experiments. Immunoprecipitation

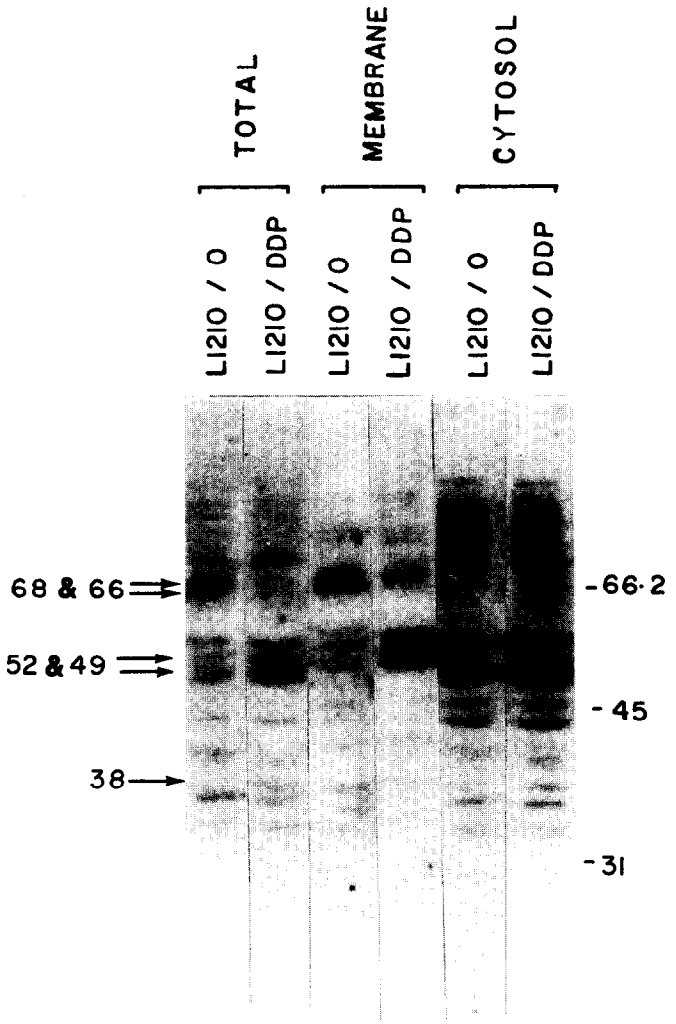

FIG. 3. Western blot analysis of cell lysates, membranes, and cytosolic fractions from L1210/0 and L1210/DDP cells using an antibody to phosphotyrosine (PY69).

with preimmune serum did not show any bands on photoaffinity labeling. These results are further strengthened by the fact that non-radiolabeled methotrexate effectively competed for the binding of the photoaffinity analog (lane 1 vs 2 for whole cell lysate; lane 3 vs 4 for immunoprecipitate).

DISCUSSION

The observed collateral resistance to MTX in L1210 cells selected for DDP resistance was an unexpected finding. Although several tumor systems known to be resistant to DDP have been reported to be collaterally resistant to MTX [1–4], there is no *a priori* reason to assume this cross-resistance pattern. Previous work from our laboratory provided evidence that the resistance was not related to altered intracellular folate metabolism [6]. The present studies focused on an alternative mechanism of resistance, i.e. altered drug uptake, by the resistant cell.

The folate transport systems in normal and neoplastic cells are widely divergent with respect to the proteins involved. Many tumor cells rely on a single transport pathway of high affinity to shuttle reduced folates and MTX across the mem-

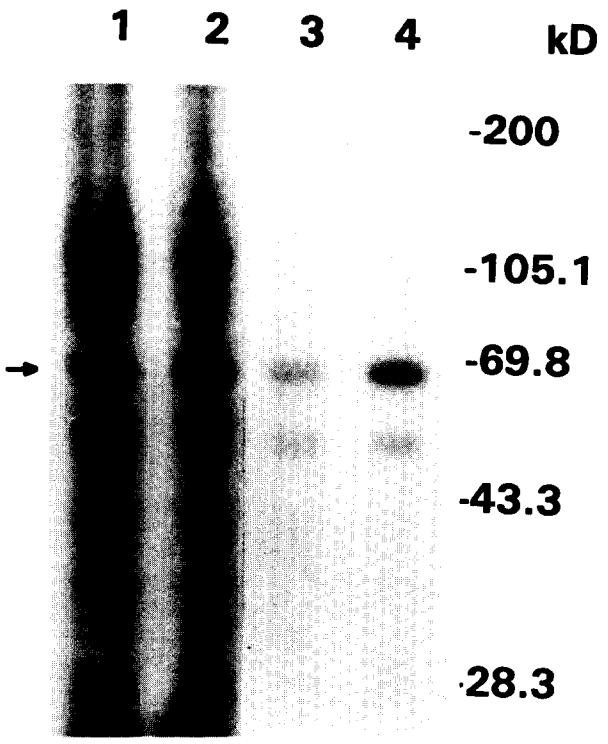

FIG. 4. Immunoprecipitation of photoaffinity radiolabeled cells with antiphosphotyrosine antibody. L1210/0 cells were labeled with APA-[^{125}I]ASA-Lys, and cell lysates were prepared in the presence and absence of MTX. The cell lysates from equal numbers of cells were immunoprecipitated with PY20 antibody, and proteins were separated on SDS–PAGE and autoradiographed. Lane 1, whole cell lysate in the presence of MTX (200 μM); lane 2, whole cell lysate in the absence of MTX; lane 3, immunoprecipitate in the presence of MTX (200 μM); and lane 4, immunoprecipitate in the absence of MTX.

brane [8, 17–19]. Much work has gone into delineating the folate transport system in L1210 cells. Using the same photoaffinity analog employed here, Price and Freisheim [12] have demonstrated that a membrane-bound 48 kDa protein and a 38 kDa cytosolic or peripheral membrane protein act in coordination to shuttle MTX from the extracellular medium into the cytoplasmic compartment of the L1210 cells. While these investigators observed a 66–68 kDa protein similar to ours, they ascribed this labeling to the presence of BSA in the lysate. Fan *et al.* [20] used biotin derivatives of MTX to affinity purify folate transport proteins and identified two proteins of 43 kDa and 39 kDa molecular mass important in MTX transport in L1210 cells. They also reported that the 43 kDa protein was a non-glycosylated integral membrane protein, whereas the 39 kDa protein was highly glycosylated and loosely associated exofacially to the membrane for cytoplasmic transport.

Resistance to MTX can arise from altered intracellular folate metabolism or impaired MTX uptake. Various cell lines have been shown to have impaired MTX uptake, and in those cells in which the impairment has been investigated there is an alteration in membrane proteins [21–24]. For example, Price *et al.* [7] have shown that in the transport-deficient L1210/R81 cells, the photoaffinity MTX analog does not bind to the 48 kDa membrane protein. Also, CEM cells resistant to MTX also have impaired membrane protein binding to MTX and consequently transport less MTX [21]. In another study, Bernal *et al.* [25] demonstrated that a squamous cell carcinoma resistant to either MTX or DDP has impaired MTX uptake due to a decreased amount and function of a 48 kDa membrane protein. Also, Goldman and coworkers [26] have isolated two membrane-bound folate binding proteins with molecular mass 29.4 kDa (FBP1) and 28.8 kDa (FBP2) from L1210 cells. It should be pointed out here that in each instance the investigators are describing membrane proteins with significantly lower molecular mass than the 66–68 kDa proteins. Matherly and coworkers [27, 28] have isolated a glycosylated membrane transporter (GP-MTX) of molecular mass 99 kDa from human erythroleukemia K562 cells.

The results of our studies clearly demonstrate that L1210/DDP cells were collaterally resistant to MTX and that this resistance was accompanied by impaired transport of MTX. In L1210/DDP cells, transport ceased at the membrane level with the photoaffinity analog being bound only to a 68 kDa protein. In the L1210/0 cells, MTX transport appeared to initiate at a 66 kDa membrane protein followed by transfer to 48 kDa and 38 kDa proteins and ultimately to a 21 kDa protein.

It is of interest that early studies done with the photoaffinity analog to MTX clearly demonstrated MTX binding to a 66 kDa protein in the L1210 lysate [12]. This MTX binding was attributed to non-specific binding of MTX to albumin adhering to the surface of the L1210 cells. Western blot analyses indicate, however, that neither the 66 kDa membrane protein of L1210/0 cells nor the 68 kDa membrane protein of the L1210/DDP cells was antigenically related to albumin. Hence, one must surmise that the MTX binding observed by ourselves and other investigators represents binding of MTX with a specific binding protein. The actual protein involved is currently under investigation in our laboratory.

Silver staining of whole extracts of both L1210/0 and L1210/DDP cells provided no grossly observable differences in protein staining patterns (data not shown). However, a clear difference between the two cells was observed in the tyrosine phosphorylation patterns. Whereas 66 kDa protein in the L1210/0 cells was phosphorylated markedly, the 68 kDa component of the L1210/DDP cells was not phosphorylated significantly. The fact that the 66 kDa protein labeled by the photoaffinity analog was immunoprecipitated from L1210/0 whole cell lysates using an antiphosphotyrosine antibody provides strong support for this protein as a member of the folate transport family. Although the preponderance of data accumulated to data relates to the role of tyrosine kinases in cell growth and differentiation, proteins known to be crucially involved in cell-substratum adhesion have also been identified as substrate for this enzyme system [29, 30]. Our work provides yet another potential role for tyrosine phosphorylation. To our knowledge, this is the first functional association between protein tyrosine phosphorylation and membrane shuttle systems. Information gained from these results could lead to a new therapeutic design for cancer chemotherapy. If tyrosine phosphorylation proves to be a mechanism of resistance, modulation

of phosphorylation by using inhibitors of tyrosine kinases or phosphatases should modulate transport and overcome resistance.

The fact that MTX apparently bound to the 68 kDa protein of the transport-deficient L1210/DDP cells but did not progress further in the folate shuttle pathway suggests that protein phosphorylation may not be required for ligand binding but may be necessary for ligand transfer. These results suggest that modulation of the state of shuttle protein phosphorylation could be vital in the regulation of the sensitivity or resistance of cells to classical antifolates. Also, whether the difference in protein phosphorylation between the two cells is related to decreased kinase activity or increased phosphatase activity is an important question. These corollaries are currently under investigation in our laboratory.

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