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INCREASED EXPRESSION AND CHARACTERIZATION OF TWO DISTINCT FOLATE BINDING PROTEINS IN MURINE ERYTHROLEUKEMIA CELLS

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Abstract—We previously identified two membrane-bound folate binding proteins, FBP1 and FBP2, in murine L1210 leukemia cells. We now report on the development of two variant murine erythroleukemia cell lines that were used for direct comparison and biochemical characterization of the two murine folate binding proteins. Based on the results of northern analysis and the mobilities of affinity-labeled proteins on polyacrylamide gels, these cell lines exhibit specific up-regulated expression of FBP1 or FBP2. The affinities of the folate binding proteins for various (anti)folates were determined based upon the ability of the compounds to inhibit binding of [^{3}H]folic acid. The two proteins exhibited considerably different affinities and stereospecificities and, in general, FBP2 consistently bound each test compound with lesser affinity than FBP1. Both proteins displayed greatest affinity for folic acid, 5-methyltetrahydrofolate, and the antifolates CB3717 and 5,10-dideazatetrahydrofolate (DDATHF). Conversely, the proteins exhibited poor affinity for the dihydrofolate reductase inhibitors methotrexate and aminopterin. For 5-formyltetrahydrofolate, FBP1 had high affinity for the (6*S*) diastereoisomer, whereas FBP2 showed preference for the non-physiologic (6*R*) diastereoisomer. The binding properties of FBP1 and FBP2 overexpressed in these cell lines closely paralleled those of their respective human homologs. These lines provide a model system in which to examine the biochemical characteristics of the individual folate binding proteins without the potential problems associated with expression of proteins in dissimilar cell lines.

Key words: folate binding protein, erythroleukemia, binding affinity

Transport of natural folate compounds and their analogues is mediated by several systems. The reduced folate carrier, a rapidly cycling membrane transporter, preferentially transports tetrahydrofolate cofactors and their analogues such as methotrexate (MTX)† and aminopterin, but has a very low affinity for folic acid [1, 2]. Membrane-associated folate binding proteins, or folate receptors, represent another much slower transport route for (anti)folate accumulation and function via a receptor-mediated endocytotic mechanism [3]. These heavily glycosylated, glycosylphosphatidylinositol-linked proteins have a very high affinity for folic acid and 5-methyltetrahydrofolate but a relatively low affinity for the 4-amino antifolates [4]. Several of the novel antifolates, inhibitors of thymidylate synthase (TS) and glycinamide ribonucleotide (GAR) transformylase, bind tightly to the folate binding protein but some also have affinities for the reduced folate carrier comparable to its natural substrates [5–7].

Two distinct isoforms of the human folate binding protein are predicted from sequence analyses of clones isolated from cDNA libraries from cell lines and tissues: a form isolated from placenta [8] and a more common form identified in several carcinoma (epidermoid, colon, and ovarian) cell lines [9–13]. Our laboratory previously identified two cDNAs from L1210 murine leukemia cells, which encode the murine homologs of these two human forms, cFBP2 and cFBP1, respectively [14, 15]. To understand the role that each folate binding protein isoform plays in the process of folate accumulation, it is necessary to characterize their binding properties for (anti)folates and identify those cells and tissues that express the particular isoforms.

A recent study by Wang *et al.* [16] characterized the relative affinities of various (anti)folates for the two human folate binding protein isoforms obtained from different cell systems: The placental isoform was expressed in membrane preparations of COS cells transfected with a cDNA encoding this protein, while the other more common isoform was studied using membrane preparations from KB cells. These human folate binding proteins exhibited significant differences in their affinities and stereospecificities toward several (anti)folates.

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† Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide electrophoresis; nt, nucleotide(s); CB3717, N^{10}-propargyl-5,8-dideazafolic acid; MTX, methotrexate; TS, thymidylate synthase; GAR, glycinamide ribonucleotide; MEL, murine erythroleukemia; DDATHF, 5,10-dideazatetrahydrofolate; SSC, 0.15 M sodium chloride + 0.015 M sodium citrate; NHS, *N*-hydroxysuccinimide; and FBP, folate binding protein

In this paper, we report on the development of two variants of murine erythroleukemia (MEL) cells which were used for direct comparison and biochemical characterization of the two murine folate binding proteins. These model lines are derived from the same parent and, as such, they should be essentially identical with exception of the exclusive overexpression of either FBP1 or FBP2. Hence, variations observed between the properties of the two folate binding proteins would not be attributable to indirect effects of cell line specific physiologic or genetic characteristics. The results confirm and extend the previous study of Wang *et al.* [16] concerning the affinities and stereospecificities of the folate binding proteins for various (anti)folates and establish the biochemical similarities between the human and murine proteins.

MATERIALS AND METHODS

Chemicals. [3′,5′,7,9-^{3}H]Folic acid was obtained from Moravek Biochemicals and purified by HPLC [17] prior to use. Restriction enzymes were obtained from New England Biolaboratories or Bethesda Research Laboratories. All other reagents were obtained in the highest purity available from various commercial sources. The 6*S* and 6*R* isomers of 5-formyltetrahydrofolate and 5,10-dideazatetrahydrofolate (DDATHF) and (6*R*)-5-methyltetrahydrofolate were provided by Dr. Richard Moran.

Cell culture. Murine erythroleukemia cells were grown in RPMI 1640 medium supplemented with 10% dialyzed bovine serum (Hyclone), 2 mM glutamine, 20 μM 2-mercaptoethanol, penicillin (100 U/mL), and streptomycin (100 μg/mL). Cell lines were adapted to growth on low folate by culturing in folate-free RPMI 1640 medium supplemented with decreasing amounts of either (6*R*,*S*)-5-formyltetrahydrofolate or folic acid. Cells were passaged twice weekly with the folate concentration decreased stepwise to a level of 0.5 nM. At this point, when the developing cell lines had growth rates approximately 50% of the parental MEL line, individual clones were isolated in soft agar and maintained at 0.5 nM folate.

Northern analysis. RNA was isolated using the RNAzol B (Biotecx) procedure. Ten micrograms of total RNA was fractionated by electrophoresis on 1.0% formaldehyde-agarose gels, transferred to Nytran membrane (Schleicher & Schuell), and fixed by a combination of Stratalinking (Stratagene) followed by baking in a vacuum oven at 80° for 1 hr. Blots were prehybridized at 42° in 5 × SSC, 50 mM $NaPO_4$ (pH 7.0), 0.1% sodium dodecyl sulfate (SDS), 5 × Denhardt's solution, 250 μg/mL total yeast RNA (Boehringer Mannheim), and 50% formamide, followed by hybridization in fresh solution with nick-translated probes (1×10^6 cpm/mL) with 10% dextran sulfate added. The blots were washed three times with 0.1 × SSC/0.1% SDS at 65° prior to autoradiography for 18 hr at −80° with an intensifying screen.

Southern blots. Genomic DNA was digested for 18 hr with 3 U of *Bam*HI or *Xba*I restriction enzyme/μg DNA. Samples (10 μg) were fractionated on 0.7% agarose gels, denatured, neutralized, and blotted onto Nytran membrane. Fixing, hybridizations, washes, and autoradiography were done as described for northern analysis.

DNA probes. For northern and Southern analysis, the FBP1 and FBP2 probes were the 500 bp *Sst*I fragment from pKD18 and the 600 bp *Bam*HI fragments from pKD21, respectively [14]. Dual blots were hybridized initially with either the FBP1 or FBP2 probe, stripped, and rehybridized with the respective complementary secondary probe. In all cases, results were identical regardless of the order of probe addition.

Affinity labeling and glycanase treatment of cell membranes. The *N*-hydroxysuccinimide (NHS) ester of tritiated folic acid was prepared by a modification of the method of Henderson and Zevely [18] as previously described [19]. Membrane preparations, affinity labeling, *N*-Glycanase treatment, and processing of SDS–PAGE gels for liquid scintillation counting or autoradiography were performed exactly as detailed previously [14].

Binding assays. To determine folic acid binding capacity and affinity, cells in log phase growth were harvested, washed with acid HBS (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose, pH 4.5) to remove bound folate, and then washed twice in HBS, pH 7.4 (assay buffer). Cells (1×10^6) were suspended in 1 mL of assay buffer at 0° with [^{3}H]folic acid (0.2 pmol) in the presence of increasing nonlabeled folic acid, giving a final folic acid concentration range of 0.7 to 1000 nM. After 30 min the cells were collected by centrifugation (14,000 *g*, 4°, 5 min), and the supernatant was aspirated. Cell pellets were recentrifuged and aspirated to remove residual supernatant and resuspended in assay buffer; radioactivity was determined in a liquid scintillation spectrometer (Beckman Instruments). Nonspecific binding was measured in the presence of 5 μM nonlabeled folic acid. For determination of inhibition constants for folates and folate analogues, cells were added to assay buffer containing a range of concentrations of nonlabeled competitor and a fixed amount (50 pmol) of [^{3}H]folic acid. At this [^{3}H]folic acid concentration, less than 15% of the extracellular label was bound by the cells. All data points were in triplicate and represent the means of 3–6 experiments except for those with aminopterin which are the mean of two experiments. Cell numbers were measured by a Coulter counter and protein concentration was determined by the Bio-Rad assay. Computer-aided Scatchard analyses to determine values for the binding constants, binding capacities, and inhibition constants were performed using the software package LIGAND [20].

RESULTS AND DISCUSSION

Development of MEL cell lines with increased expression of folate binding proteins: Northern and Southern analysis. MEL cells were cultured in folate-free medium containing decreasing levels of either 5-formyltetrahydrofolate or folic acid. Following 2 months on selection, cells were cloned in soft agar and six clones from each selection strategy were characterized for expression of folate binding protein

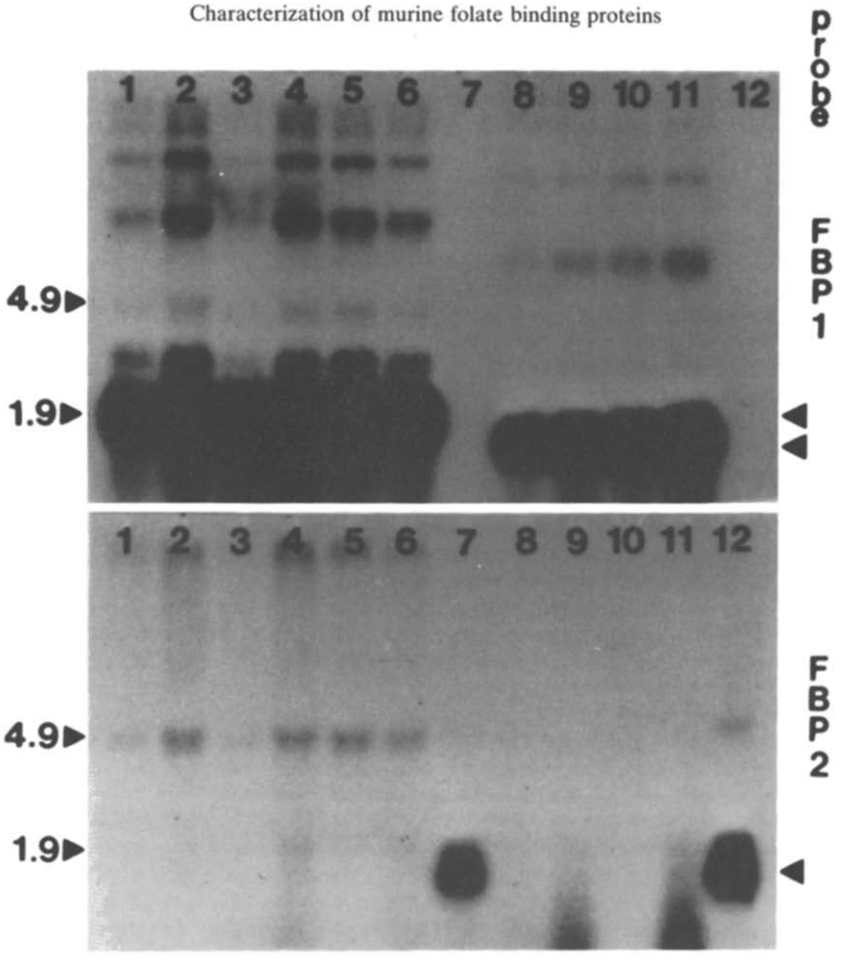

Fig. 1. Northern analysis of total RNA isolated from twelve clones selected for growth on low folate. Total RNA was isolated from clonal lines selected in low 5-formyltetrahydrofolate (lanes 1–6) or folic acid (lanes 7–12) and fractionated on a 1% agarose gel. Identical blots were hybridized with an FBP1 probe (upper panel) or an FBP2 probe (lower panel). Arrowheads on the right of the figure designate the major 1900 nt and 1700 nt FBP1-encoding transcripts in the upper panel and the major 1600 nt FBP2-encoding transcript in the lower panel. Clones shown in lanes 1 and 12 (La and Fa, respectively) were further characterized in this study.

by northern analysis. Two of the six clones selected in folic acid expressed FBP2, but the other four clones and all six of those selected in 5-formyltetrahydrofolate expressed FBP1 (Fig. 1). These results suggest that growth on low levels of 5-formyltetrahydrofolate favors the increased expression of FBP1, whereas growth on limiting folic acid shows no preference for which folate binding protein will ultimately be up-regulated. The clones shown in lanes 1 and 12 (designated La and Fa and selected in 5-formyltetrahydrofolate and folic acid, respectively) were further characterized in the studies that follow. As shown in Fig. 2, the FBP1-encoding transcript present in La was larger than the FBP2-encoding message expressed in Fa (1900 nt vs 1600 nt, respectively), but neither was detectable in parental MEL cells. This size pattern for the two transcripts is opposite to that observed for the FBP-encoding transcripts present in murine L1210 cells [14]. In addition, the FBP1-encoding transcripts present in the clones selected in 5-formyltetrahydrofolate were slightly larger than the FBP1-encoding transcripts from the four clones selected in folic acid (Fig. 1, upper panel). These size variations displayed by the FBP-encoding transcripts may reflect heterogeneity in the 5′ ends of these messages as demonstrated for FBP-encoding transcripts isolated from a variety of human carcinomas [12, 13]. Those heterogeneous 5′-noncoding regions were suggested to play a regulatory role by functioning as a determinant of message stability or translatability. Upon Southern analysis, the *Bam*HI and *Xba*I restriction patterns for the FBP1- and FBP2-genomic loci were identical between these cell lines and the parental MEL line (Fig. 3), which suggests that the increased expression observed for each of these proteins is due to a transcriptional or post-transcriptional mechanism. Throughout the course of this study, the La and Fa cell lines were monitored by northern analysis to confirm exclusive and sustained expression of the respective folate binding proteins.

In vivo *identification of each folate binding protein.* To correlate increased expression of the specific FBP1- and FBP2-encoding transcripts with increased

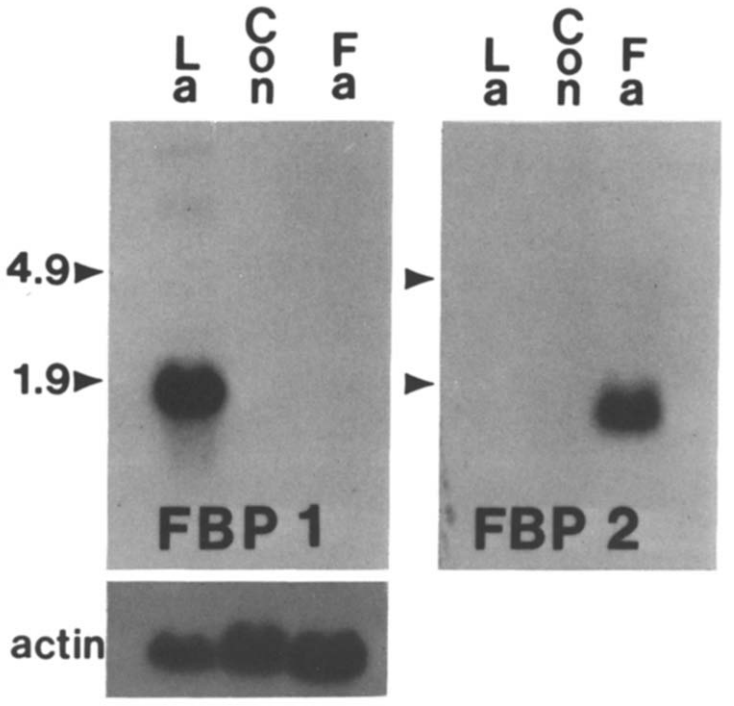

Fig. 2. Northern analysis of total RNA isolated from parental MEL cells (Con) and clones La and Fa. Total RNA was isolated, fractionated on a 1% agarose gel, and transferred to Nytran. The blot was hybridized successively with probes encoding FBP1, FBP2, and actin.

cell surface expression of each protein, cells were treated with the affinity agent NHS-[^{3}H]folic acid, and the labeled membranes were isolated and fractionated on denaturing polyacrylamide gels. As shown in Fig. 4A, the labeled polypeptides present in the membrane preparations migrated as a single broad band with a prominent peak of $M_r = 40{,}000$ and $M_r = 37{,}000$ in the La and Fa cell lines, respectively. This variation in size between the two folate binding proteins is shown in detail by the ^{3}H profile and is easily discernible upon autoradiography of the fractionated membrane proteins (Fig. 4, inset). Following treatment with *N*-Glycanase, the labeled polypeptide bands present in each membrane preparation were converted to a single sharp peak with an apparent M_r of 26,000 (Fig. 4, panel B and inset). The apparent M_r values of the glycosylated and non-glycosylated affinity-labeled proteins in these cells are consistent with those observed for folate binding proteins isolated from murine L1210 cells [14, 21]. Consistent with the northern analysis data presented above, no affinity-labeled protein was observed in membrane preparations from parental MEL cells either by liquid scintillation counting of gel slices or following extended autoradiography (not shown). Thus, the increased expression of the specific FBP1- and FBP2-encoding transcripts in these cell lines correlates with the enhanced expression of two distinct membrane-associated folate binding proteins of expected molecular mass. The Fa line characterized in the present study is the first reported cell line in which the placental folate binding protein has been overexpressed in response to growth on low folate.

The [^{3}H]folic acid binding capacity of the intact cells was quantitated following acid treatment to remove endogenous membrane-bound folates. While binding to the parent MEL line was essentially undetectable, the La line had both a receptor density and a folate binding capacity ~5-fold greater than the Fa line (Table 1).

Binding characteristics of FBP1 and FBP2. The affinities of each folate binding protein for a number of folates and antifolates were determined in whole cell assays by measuring inhibition of [^{3}H]folic acid binding. As shown in Table 2, the proteins exhibited considerably different affinities and stereospecificities; however, FBP2 consistently bound each test compound with lesser affinity than FBP1. The affinity of each folate binding protein for folic acid was very high and only the TS inhibitor CB3717 bound more tightly. This slightly increased affinity for CB3717 was also observed by Westerhof *et al.* [6] in L1210-B73 cells which overexpress one of the murine folate binding proteins. This cell line was very sensitive to growth inhibition by CB3717, a result also observed in human leukemic CCRF-CEM cells which overexpress a folate binding protein [5, 22]. While the specific protein overexpressed in the L1210-B73 cells was not identified, these binding data suggest that the presence of either folate binding protein would serve to enhance toxicity of this drug.

The GAR transformylase inhibitor DDATHF exists as two diastereoisomers, (6*S*)- and (6*R*)-

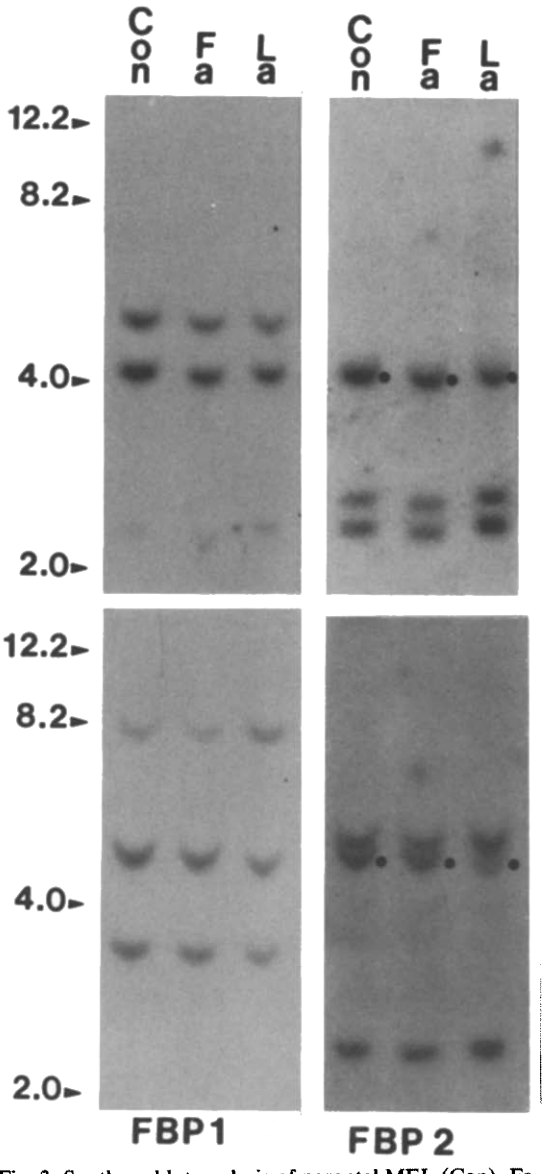

Fig. 3. Southern blot analysis of parental MEL (Con), Fa, and La genomic DNA. Genomic DNA was digested with *Bam*HI or *Xba*I, fractionated on 0.7% agarose and transferred to Nytran. The blots were hybridized with probes encoding FBP1 (left panels) or FBP2 (right panels). Bullets in right panels denote FBP1 bands which cross-hybridize with the FBP2 probe [14]. Evenness of loading was determined by ethidium bromide staining.

DDATHF; the latter is currently undergoing Phase I trials. This compound has been shown to be transported by both the reduced folate carrier and the folate binding protein endocytotic mechanism, with relative utilization of each dependent upon the concentration of the drug; the lower the concentration of DDATHF, the greater the dependence on the endocytotic mechanism for transport [23]. As shown in Table 2, FBP1 bound the DDATHF stereoisomers with very high and equal affinities. FBP2 also bound 6*R* and 6*S* with comparable affinities (6*R*:6*S* = 1.3), but at levels 12- and 16-fold less than for FBP1, respectively. Absence of stereospecificity for DDATHF has also been observed for the folate binding protein expressed on MA104 monkey kidney epithelial cells (6*R*:6*S* = 1.30) [23] and for the human placental protein produced in transiently transfected COS cells (6*R*:6*S* = 1.4) [16]. Only the human FBP1 homolog present in KB cells exhibited stereospecificity with a 3-fold greater binding to (6*S*)-DDATHF [16].

Stereospecificity by the reduced folate carrier has been suggested for 5-formyltetrahydrofolate [24] but not 5-methyltetrahydrofolate [25, 26]. The 6*S* isomer of 5-formyltetrahydrofolate is rapidly cleared from the plasma by biotransformation to 5-methyltetrahydrofolate [27], and studies have failed to demonstrate interference of its bioactivity by the 6*R* diastereoisomer [28]. As shown in Table 2, the murine folate binding proteins also demonstrated stereospecific binding for 5-formyltetrahydrofolate. FBP1 exhibited a 4-fold higher affinity for the 6*S* compared with 6*R* but, conversely, FBP2 bound the 6*R* with an approximately 5-fold greater affinity than the physiological 6*S*. In fact, the 6*R* had greater affinity for FBP1 (44 nM) than either stereoisomer exhibited for FBP2 (250 and 1188 nM). Similarly, the human folate binding protein isoforms exhibited this same pattern of stereospecific binding not only for 5-formyltetrahydrofolate, but also for the stereoisomers of 5-methyltetrahydrofolate [16]. While the bioactive (6*S*)-5-methyltetrahydrofolate was not tested in the present study, FBP1 bound the 6*R* isomer of this folate 4-fold better than FBP2. The nearly 100-fold higher affinity that FBP1 exhibits for the 6*S* diastereoisomer of 5-formyltetrahydrofolate may be the determining factor which results in the preferential increased expression of the FBP1 isoform when cells are grown on low levels of this folate (Fig. 1 and Ref. 14). Conversely, as the two folate binding proteins have similar affinities for folic acid, there is no apparent selective advantage for increased expression of one folate binding protein over the other when cells are grown on low levels of this folate (Fig. 1).

Each of the folate binding proteins exhibited low affinity for the two dihydrofolate reductase inhibitors, MTX and aminopterin. Compared to FBP2, FBP1 had an 11.6- and 3-fold greater affinity, respectively, for these compounds. Aminopterin, which lacks the N^{10} methyl group, has a 4-fold higher affinity for the reduced folate carrier compared with MTX [2]. In contrast, FBP1 had a 3.5-fold lesser affinity for aminopterin than MTX, but FBP2 affinity for both antifolates was the same. While the affinity of each folate binding protein for MTX is quite low, its increased expression in various tissues has been suggested to play a role in the adverse cytotoxic side-effects of MTX therapy [29]. However, murine and human cell lines, which have increased expression of folate binding protein either by adapting cells to growth on low folate or by transfecting with the encoding cDNA, show no correlation between the extent of folate binding protein expression and MTX cytotoxicity [6, 30, 31]. It is likely that the high affinity which these proteins have for the natural folates is sufficient to prevent MTX binding and

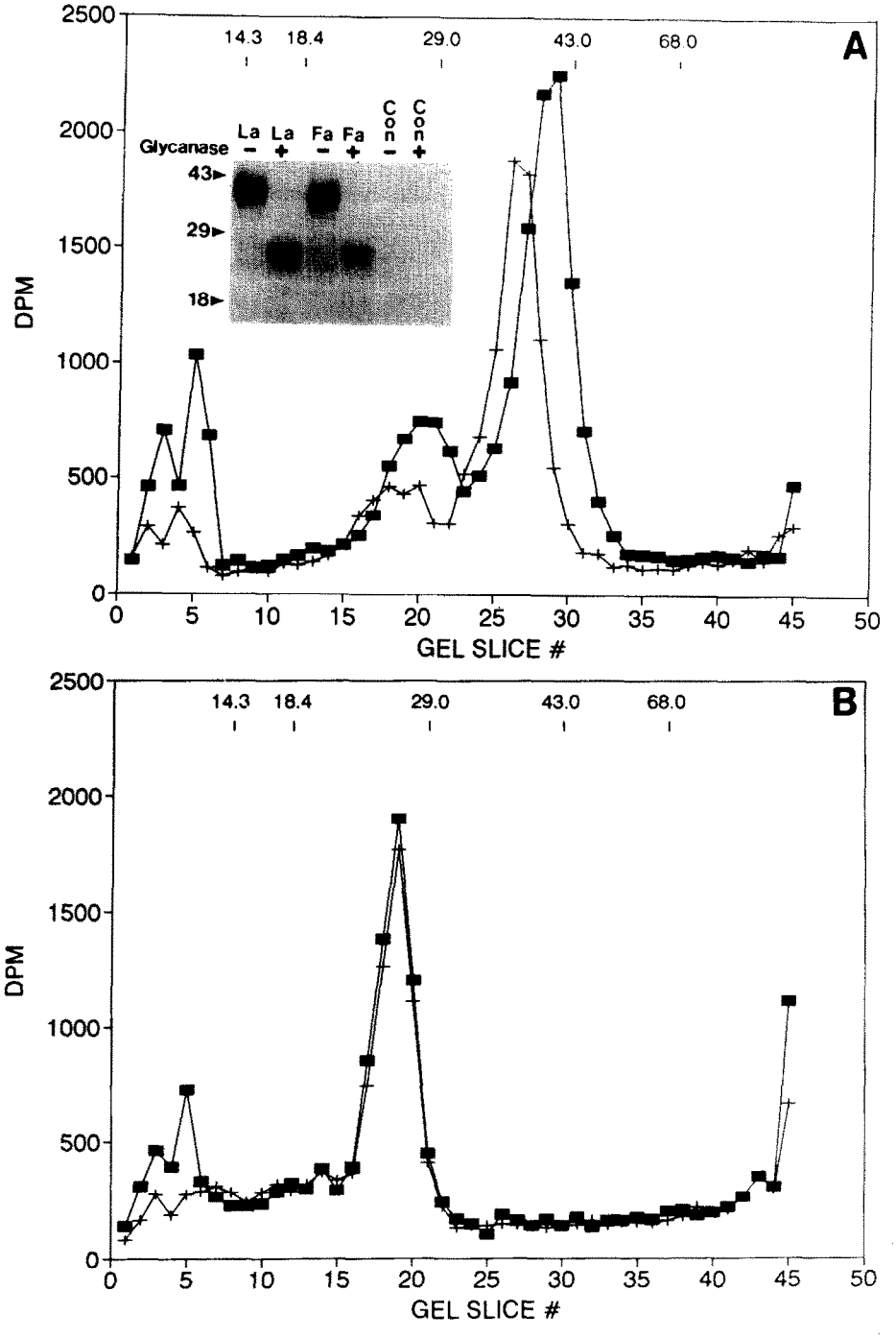

Fig. 4. Affinity labeling of folate binding protein in membrane preparations of La and Fa. NHS-[^{3}H]-folic acid affinity labeled membrane preparations (50 μg) of La (■) and Fa (+) were incubated in the absence (panel A) or presence (panel B) of *N*-Glycanase, electrophoresed, processed, and counted. The ^{3}H profiles and positions of the molecular weight markers are from the same gel. Inset: Affinity labeled membrane preparations of La, Fa and parental MEL (Con) were incubated in the presence (+) or absence (−) of *N*-Glycanase prior to electrophoresis and autoradiography.

uptake by this mechanism. Current available data suggest that the major determinant of the tissue specificity and cytotoxicity of MTX is related to the efficiency of transport via the reduced folate carrier and subsequent metabolism to its retained polyglutamyl forms [32, 33].

Results of this study confirm and extend the earlier report by Wang *et al.* [16] which demonstrated that

Table 1. Quantitation of folate binding protein and [^{3}H]-folic acid binding capacity of intact La and Fa cells

	Folate (pmol/mg protein)	FBP (molecules/cell)
La (FBP1)	185 ± 59	$8.8 \times 10^6 \pm 0.3$
Fa (FBP2)	38 ± 7.5	$1.6 \times 10^6 \pm 0.3$

Values are means ± SD. All data points were done in triplicate and represent the means of 3–6 experiments.

Table 2. Binding constants for folic acid* and inhibition of binding of [^{3}H]folic acid to the folate binding proteins expressed in the La (FBP1) and Fa (FBP2) cell lines

	K_i (nM)	
	La (FBP1)	Fa (FBP2)
Folic acid*	1.1 ± 0.1	1.7 ± 0.2
5-CHO_4folate (6*S*)	13 ± 2	1188 ± 115
5-CHO_4folate (6*R*)	44 ± 5	250 ± 12
5-CH_3H_4folate (6*R*)	7.9 ± 2.7	29.5 ± 7.5
Methotrexate	143 ± 15	1665 ± 525
Aminopterin	512 ± 25	1648 ± 111
CB3717	0.44 ± 0.50	1.40 ± 0.06
DDATHF (6*S*)	2.4 ± 0.4	28.4 ± 9.4
DDATHF (6R)	2.4 ± 0.8	37.7 ± 16.0

Values are means ± SD. All data points were done in triplicate and represent the means of 3–6 experiments except for those with aminopterin which are the mean of two experiments.

the two human folate binding proteins have distinct binding properties. The murine folate binding proteins exhibit comparable stereospecificities and binding affinities for (anti)folates. Compared to FBP2, murine FBP1 had a 2- to 91-fold greater affinity for the natural folates and a 3- to 16-fold greater affinity for the folate analogues tested in this study. Comparing the same compounds, the binding differences between the murine proteins are nearly identical to those observed for the corresponding human isoforms (4- to 114-fold and 5- to 17-fold, respectively). This implies that the biochemical characteristics of the murine folate binding proteins are very similar to, and representative of, the corresponding human isoforms. Previous assignments of murine FBP1 and FBP2 as homologs of the two respective human folate binding proteins were based on similarities of the primary sequences and structural features of the proteins [14]. Functional data from the present study support these assignments with the binding characteristics of FBP2 closely paralleling those of the protein identified from human placenta and the properties of FBP1 resembling those of the isoform commonly isolated from human carcinoma cell lines.

The MEL cell line variants developed in this study provide ideal models with which to directly compare the characteristics of the two proteins. This system excludes potential biochemical and functional differences that may result from expression of these proteins in dissimilar cell lines or from heterologous transfection and expression. Transfection of cDNA, which encodes the KB cell expressed-folate binding protein into human (ZR-75-1) and murine (NIH3T3 and PAM212) cells, has been shown to produce a functional protein as determined by the survival and growth of the resultant transfectants in medium containing low folate [31, 34, 35]. Compared with the folate binding protein normally expressed in KB cells, the protein expressed in the transfected NIH3T3 cells had a decreased apparent M_r of ~8%, which was attributed to differences in complex glycosylation. Glycosylation of proteins is cell type specific, and different glycoforms of the same protein have been shown to exhibit distinct physiological properties and biological activities [36, 37]. Luhrs [38] has shown that core glycosylation of folate binding protein during protein synthesis is necessary for ligand binding function. However, prevention of complex glycosylation during protein synthesis or complete deglycosylation of the native protein does not inhibit its ligand binding activity. It is unclear, however, if there are any specific alterations in the binding characteristics of the various differentially glycosylated forms of folate binding protein.

Using immunoblot analysis, Weitman *et al.* [39] demonstrated that folate binding protein is widely distributed throughout normal human tissues. However, it is not clear which isoform is expressed in the various tissues as qualitatively similar results were obtained in these immunoassays using either monoclonal antibodies (MOv18 and MOv19) developed to the folate binding protein present in ovarian carcinomas (murine FBP1 equivalent) or polyclonal sera developed to folate binding proteins isolated from human placenta (mixture of both murine FBP1 and FBP2 equivalents). Results of the present study indicate that it is important to identify which isoform(s) is expressed in the various tissues as each has distinct binding characteristics that could elicit different responses to new antifolate agents. In addition, it cannot be assumed that expression of folate binding protein in tumor cells represents only one protein isoform. Expression of the two proteins may vary during differentiation and maturation of tissues. The recognition of tissue and tumor specific isoforms may allow for the development of drugs that have high toxicity for cells expressing one isoform while having little adverse effect on cells that express the other. Likewise, isoform specific folate analogues may be developed that bind with high affinity and block folate uptake into tumor cells that use these proteins predominantly for acquiring folates.

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