Variant GPI Structure in Relation to Membrane-Associated Functions of a Murine Folate Receptor†

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Received May 8, 1996; Revised Manuscript Received September 20, 1996⊗

ABSTRACT: Two variant sublines of murine L1210 leukemia cells (L1210A and L1210JF) overexpress the cell surface folate receptor (FR). The membrane bound FR in L1210A cells exhibited significantly (up to 17-fold) greater relative affinities for (6*S*)-N^5-methyltetrahydrofolate, (6*S*)-N^5-formyltetrahydrofolate and methotrexate compared to the FR in L1210JF cells. Furthermore, receptor-mediated transport of [^{3}H]-(6*S*)-N^5-methyltetrahydrofolate was much more efficient in L1210A cells compared to L1210JF cells. When solubilized with Triton X-100, the ligand binding characteristics of FR from both sublines resembled those of the receptor associated with L1210 JF cell membranes. N-terminal amino acid sequence analysis as well as RT-PCR analysis of the entire coding region revealed a single species of FR in both cells, identical to murine FR-α. The FR in L1210JF cells was sensitive to phosphatidylinositol specific phospholipase C (PI-PLC) indicating the presence of a glycosyl-phosphatidylinositol (GPI) membrane anchor while the FR in L1210A cells was resistant to PI-PLC; however, the FR in L1210A cells was released from plasma membranes by nitrous acid, as expected for GPI and its PI-PLC resistant structural variants. Treatment of L1210A cell membranes with mild base rendered the protein PI-PLC sensitive as expected for GPI anchors acylated in the inositol ring and also decreased the affinities of the membrane associated FR for reduced folates. When the cDNA for murine FR-α was expressed in parental L1210 cells the protein was PI-PLC resistant but was sensitive to PI-PLC when the cDNA was expressed in human 293 fibroblasts. In L1210JF, L1210A, and parental L1210 cells, several cell surface proteins, including FR, incorporated [^{3}H]ethanolamine, a component of the GPI membrane anchor; however, the labeled proteins were released by PI-PLC only in L1210JF cells. The above results preclude any peculiarity of the FR polypeptide in either L1210 subline as the basis for the observed differences in PI-PLC sensitivity and membrane-associated functions of FR. Partial deglycosylation of membrane associated FR from either cell with *N*-glycanase did not influence its ligand binding characteristics. The results of this study lead to the hypothesis that variant GPI structures may modulate the function of a protein by influencing its conformation/topography in the membrane. Such effects may be identified by their disappearance/reduction upon detergent solubilization or mild base treatment of the membrane.

A variety of cell surface proteins with diverse functions are known to be attached to the plasma membrane by a glycosylphosphatidylinositol (GPI)[1] membrane anchor (Ferguson, 1988; Low, 1989; Lublin, 1992). Structurally, these proteins are characterized by a carboxyl-terminal signal peptide of moderate hydrophobicity which is cleaved in the ER prior to the addition of the GPI anchor to the new carboxyl terminus. GPI-anchored proteins are also characteristically released from the cell surface by phosphatidylinositol specific phospholipase C (PI-PLC). GPI-anchored proteins may be variably resistant to PI-PLC due to fatty acyl substitution in the inositol ring. Such PI-PLC resistant GPI anchors are invariably cleaved at a glycosidic bond by nitrous acid, and this constitutes another diagnostic test for the presence of a GPI membrane anchor. The functional significance of variant GPI structures has remained obscure. This question is addressed in the present study using the murine folate receptor as a model system.

The folate receptor (FR) is an N-glycosylated protein with a single polypeptide that binds one molecule of folic acid per receptor molecule (Antony, 1992). Multiple isoforms of the receptor, whose primary structures are 70−80% identical have been identified in human and mouse tissues by cDNA cloning (Lacey et al., 1989; Ratnam et al., 1989; Elwood, 1989; Brigle et al., 1991; Shen et al., 1994, 1995). Membrane-bound isoforms of FR (types α and β) are

† Supported by NCI Grant R29CA57598 and a Junior Faculty Award to M.R. from the American Cancer Society.

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⊗ Abstract published in *Advance ACS Abstracts,* December 1, 1996.

[1] Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; PCR, polymerase chain reaction; HBSS, Hank's balanced salt solution; FR, folate receptor; GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol specific phospholipase C; RFC, reduced folate carrier; FBS, fetal bovine serum; MTX, methotrexate; 5-CH_3H_4-folate, N^5-methyltetrahydrofolate; 5-$CHOH_4$folate, N^5-formyltetrahydrofolate; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

S0006-2960(96)01098-7 CCC: $12.00

attached to the cell surface by a GPI anchor (Lacey et al., 1989; Luhrs & Slomiany, 1989; Verma et al., 1992; Yan & Ratnam, 1995), while FR-γ is secreted due to the absence of GPI modification in this protein (Shen et al., 1995). The human receptor isoforms are tissue specific and are strikingly elevated in a variety of malignant tissues (Shen et al., 1994, 1995; Coney et al., 1991; Ross et al., 1994). Tissue specificities of the two known murine FR isoforms are not yet known. The FR isoforms are also distinguished by their folate binding characteristics (Wang et al., 1992; Brigle et al., 1994).

FR mediates energy dependent uptake of folate compounds and antifolate drugs into cells by an endocytic mechanism. Expression of high levels of the receptor confers a growth advantage on certain cells in media containing low (nanomolar) folate concentrations (Luhrs et al., 1992; Matsue et al., 1992). However, most normal tissues do not appear to require FR for physiologic folate transport and depend upon a low-affinity anion carrier protein known as the reduced folate carrier (RFC). The RFC does not bind folic acid and has a 1000-fold lower affinity for (6*S*)-N^5-methyltetrahydrofolate (5-CH_3H_4folate) ($K_d \simeq 1$ μM) compared to FR-α (Goldman & Matherly, 1985; Spinella et al., 1995).

L1210 murine leukemia cells express RFC but not FR. Because RFC cannot bind folic acid, by growing L1210 cells in media containing low (nanomolar) concentrations of folic acid as the only source of folate, it is possible to select for variant L1210 cells overexpressing FR (Henderson et al., 1988; Brigle et al., 1991). In this study, the FR in two such L1210 variants named L1210A and L1210JF are characterized on the basis of initial findings that the two sublines exhibited different folate binding and transport characteristics as well as differential susceptibility of their cell surface FR to PI-PLC. The results highlight a possible role for variant GPI structures in influencing membrane topography in a manner that influences protein function.

MATERIALS AND METHODS

Chemicals. [^{3}H]Folic acid and [^{3}H]-(6*S*)-5-CH_3H_4folate (specific activity 27 Ci/mmol) were purchased from Moravek Biochemicals, Brea, CA. Folate-free RPMI 1640 medium, Dulbecco's modified Eagle medium (D-MEM), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), and dialyzed fetal calf serum were obtained from Gibco BRL, Grand Island, NY. RPMI 1640 medium was purchased from Irvine Scientific, Santa Ana, CA. Methotrexate (MTX) was a gift from Dr. J. A. R. Mead, National Cancer Institute, NIH, Bethesda, MD. (6*S*) diastereoisomers of 5-CH_3H_4folate and N^5-formyltetrahydrofolate (5-$CHOH_4$folate) were gifts from Drs. A. Melera, F. Marazza, and F. Giovannini of SAPEC, S. A. Barbengo, Lugano, Switzerland. Folic acid was obtained from Sigma Chemical Company, St. Louis, MO. Triton-X 114 and PI-PLC were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. ScintiVerse II general-purpose scintillation fluid was purchased from Fisher Scientific, Pittsburgh, PA.

Cell Culture. All media were supplemented with FBS (10% v/v), penicillin (100 units/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM). L1210 (ATCC CCL 219, a mouse lymphocytic leukemia cell line) cells were grown in RPMI 1640. L1210A and L1210JF cells were grown in folate-free RPMI 1640 medium with FBS as the only source of folate. L1210A and L1210JF cells are variants obtained by long term growth of L1210 cells in media containing low (1−10 nM) folic acid followed by subcloning as described (Henderson et al., 1988; Fan et al., 1992). The cells expressed 2 pmol (L1210A) or 3 pmol (L1210JF) of [^{3}H]-folic acid binding protein per 10^6 cells. Human 293 fibroblasts were grown in Eagle's minimal essential medium (MEM) with 10% FBS.

Preparation of Plasma Membranes. Crude plasma membrane fractions were prepared as described previously (Wang et al., 1992).

Purification of FR from L1210A and L1210JF Cells. L1210A or L1210JF cells (2.5×10^9) were harvested from culture in the mid-log phase of growth and crude, acid washed plasma membranes were prepared as described above. The membranes were resuspended at 37 °C in 10 mM sodium phosphate buffer, pH 7.5, containing 1% Triton X-100 to dissolve the membrane followed by centrifugation at 2800*g* for 5 min to remove insoluble debris. FR in the supernatant was purified by affinity chromatography on folic acid coupled Sepharose 4B and assayed essentially as described earlier for human FR (Ratnam et al., 1989).

Western Blot Analysis. Western blotting was carried out as described (Wang et al., 1992). The primary antibody used was an affinity-purified fraction of rabbit anti-human FR prepared as described (Wang et al., 1992).

Amino Acid Sequencing. Amino-terminal protein sequencing was performed on an ABI 475A gas phase sequencer. Purified protein (100 pmol) was sequenced up to 19 or 25 cycles. The sequence analysis was carried out at the Rockefeller University Protein Sequencing Facility (New York, NY) and at the University of Michigan Protein and Carbohydrate Structure Facility (Ann Arbor, MI). The yield in the initial sequencing cycle was >70%.

RT-PCR and cDNA Sequence Analysis. Total RNA from L1210, L1210A, and L1210JF cells was isolated by using the guanidinium thiocyanate-phenol-chloroform single-step extraction method (Stratagene). The 10 μL reverse transcription reaction contained 0.05 μg/μL total RNA, 0.05 M KCl, 0.01 M Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 1 mM each of dATP, dCTP, dGTP, and dTTP (Gibco-BRL), 1 unit/μL RNasin RNase inhibitor (Promega), random hexamer primers (5×10^{-4} OD units/μL; United States Biochemical), and MMLV-reverse transcriptase (5 units/μL; Gibco-BRL). The reaction mixture was first incubated at room temperature for 10 min, and then held at 42 °C for 15 min. The reaction was stopped by heating at 99 °C for 6.5 min. For amplification of FR specific cDNA by polymerase chain reaction (PCR), *Taq* polymerase was used with an oligo dT primer (Perkin Elmer) and an upstream primer specific for either murine FR-α or murine FR-β (Brigle et al., 1991). The FR-specific primers (custom synthesized by Genosys Biotechnologies) corresponded to the 5′ end of the coding region in the cDNA sequence; the oligonucleotides are CTGCATG-GATGCCAAGC (for murine FR-α) and GGTCTACATG-GTCACAACAGGCAG (for murine FR-β). The entire reverse transcription product was combined with additional buffer (0.05 M KCl, 0.01 M Tris-HCl, pH 8.3, 2 mM $MgCl_2$), 0.15 μM of each primer, and 1.25 units of Taq polymerase (Perkin Elmer) in a 50.0 μL reaction volume. The reaction mix was heated for 2 min at 95 °C. The PCR cycle comprised 1 min at 95 °C followed by 0.5 min at 60 °C then 0.5 min at 72 °C. After 35 cycles, the reaction was

held at 72 °C for 6.5 min. The resulting PCR products were analyzed on a 1% agarose gel. The PCR products were purified from the agarose gel using the Geneclean kit (BIO 101 Inc., La Jolla, Ca) according to the manufacturer's instructions. The purified PCR products were sequenced using a modification of the standard dideoxy method using the AmpliCycle sequencing kit (Perkin Elmer).

Expression of FR cDNA in Parental L1210 Cells and 293 Human Fibroblasts. FR cDNA from L1210A cells was inserted in the expression vector pcDNAI/neo (Invitrogen) at the restriction sites *Hin*dIII and *Xba*I. The restriction sites were introduced 53 base pairs upstream from the start codon and 26 base pairs downstream from the stop codon respectively, by PCR using *Taq* polymerase. The vector containing the folate receptor cDNA in the correct orientation was amplified in *Escherichia coli* MV1061/p3. The plasmid was purified by using an anion-exchange column (Qiagen), sequenced, and used to transfect parental L1210 cells using lipofectin (Gibco BRL) according to the manufacturer's protocol. Selection with G418 (Gibco BRL) at 400 μg/mL (active concentration) was initiated 72 h after transfection (Spies & Demars, 1991). The cells were subcloned 5 days after transfection on soft agar in the presence of G418. Individual colonies were picked and the cells were grown in RPMI 1640 liquid culture medium. Expression levels of FR were measured from [^{3}H]folic acid binding to whole cells. The FR cDNA construct described above was also used to transiently transfect human 293 fibroblasts as described earlier (Shen et al., 1995).

Phospholipase C Treatment of Membranes. Membranes containing 30−50 μg of total protein were sedimented at 13000*g* for 15 min at 4 °C, and the pellet was finely resuspended in 0.2 mL of 50 mM Tris-HCl, pH 7.45/10 mM EDTA, using a 1 cc U-100 insulin syringe. The membranes were treated with 0.5 unit of PI-PLC for 4 h at 37 °C with agitation followed by incubation with 3 pmol of [^{3}H]folic acid for 30 min at 37 °C. Control samples were subjected to the same treatment but without the addition of PI-PLC. The membranes were sedimented at 13000*g* for 15 min. The radioactivity in the pellet was quantitated after dissolving in ScintiVerse II general-purpose scintillation fluid.

Detergent Partitioning. L1210A and L1210JF membranes (0.3 mg of total membrane protein) were incubated with 15 pmol of [^{3}H]folic acid in 1 mL of PBS at 37 °C for 30 min. The membranes were centrifuged at 13000*g* for 15 min at 4 °C and then resuspended at 3 mg/mL total membrane protein in 50 mM Tris-HCl, pH 7.35/10 mM EDTA. 50 μL of the membrane suspension was treated with 0.5 unit of PI-PLC at 37 °C with agitation for 4 h. Control samples were incubated under the same conditions without PI-PLC treatment. 5 μL of ice-cold precondensed Triton X-114 (Hooper & Bashir, 1991) was added to each sample and vortexed for 2 s followed by incubation on ice for 5 min. The samples were centrifuged at 13000*g* for 15 min at 4 °C. The supernatants were overlayed on 0.3 mL of 6% (w/v) sucrose, 10 mM Tris-HCl, pH 7.5/0.15 M NaCl/0.06% Triton X-114, and incubated at 30 °C for 3 min followed by centrifugation at 3000*g* for 10 min in a swinging bucket rotor. Two phases were observed, an upper aqueous phase and a lower detergent rich phase. The radioactivity in each phase was measured using ScintiVerse II scintillation fluid.

Treatment with Nitrous Acid. Membranes from L1210A, L1210JF, and KB cells were suspended (2 μg of membrane protein per μL) in 50 mM sodium acetate, pH 3.5, containing 0.165 M freshly dissolved $NaNO_2$ and incubated for 6 h at room temperature. Control samples were treated similarly but without $NaNO_2$. After centrifugation at 13000*g* for 15 min, the membrane pellets were resuspended in the appropriate buffer to estimate the residual [^{3}H]folic acid binding protein. Alternately, the membrane pellets were subjected to Western blot analysis.

Treatment with Mild Base. The treatment was carried out essentially as described by Hirose et al. (1995). Membranes were suspended in 50 μL of 0.1 M Tris, pH 7.4, and 200 μL of 1 M hydroxylamine/0.1 M diethylamine, pH 10.7, or with buffer alone (negative control) followed by incubation at 4 °C overnight on an end-over-end rotator. The membranes were then sedimented and resuspended in PBS. The treated membranes were subjected to PI-PLC treatment and [^{3}H]-folic acid binding analysis as described elsewhere in this section.

[^{3}H]Ethanolamine Labeling of Cells and PI-PLC Treatment. L1210 cells, L1210A cells and L1210JF cells were incubated with [1-^{3}H]ethan-1-ol-2-amine hydrochloride (26 Ci/mmol, Amersham, Arlington Heights, IL) at a concentration of 25 μCi per mL in OPTI-MEM serum-free medium (Gibco BRL). The cell membranes were isolated (as described above) after 13 h of incubation. The membranes were treated with PI-PLC as described above. Control samples were treated similarly but without PI-PLC. The membranes were dissolved in SDS sample buffer and electrophoresed on a 12% SDS−polyacrylamide gel. The gel was fixed in 10% acetic acid/30% methanol and soaked in Fluoro-Hance autoradiography enhancer (Amersham Life Science) followed by autoradiography.

Alternately, the labeled cells (1×10^6) were suspended in 100 μI of PL-PLC buffer (25 mM Tris-HCl, 250 mM sucrose, 10 mM glucose, 1% bovine serum albumin, pH 7.5) and incubated with or without 0.2 units of PI-PLC at 37 °C for 2 h. The cells were then washed once with PBS, once with acid buffer (137 mM NaCl, 20 mM HEPES, 5.3 mM KCl, 1.0 mM $MgCl_2$, 1.9 mM $CaCl_2$, 7 mM D-glucose, pH 3.5) and again with PBS. The cells were then resuspended in PBS containing 1% Triton X-100 at room temperature for 30 min on a rotary shaker. After removing the insoluble material by sedimentation at 13000*g* for 15 min, the supernatant was mixed with 100 μL of 1:1 (v/v) suspension of folic acid-Sepharose and placed on a rotary shaker overnight at 4 °C. The folic acid-Sepharose beads were washed with PBS containing 1% Triton X-100, pH 7.5, and the bound protein was dissolved in 50 μL of SDS sample buffer. SDS−polyacrylamide gel electrophoresis and autoradiography were carried out as described above.

[^{3}H]Folic Acid Binding and Competitive Binding Assay for Folate Compounds. Membranes from L1210, L1210A, and L1210JF cells containing 0.3 pmol of FR were incubated with 1.2 pmol of [^{3}H]folic acid in 0.5 mL of PBS at 37 °C for 30 min with constant agitation. The membranes were sedimented at 13000*g* for 15 min at 4 °C. The radioactivity in the pellet was measured using ScintiVerse II scintillation fluid. The inhibition of [^{3}H]folic acid binding to the membranes by (6*S*)-5-CH_3H_4folate, (6*S*)-5-$CHOH_4$folate, and MTX was determined by carrying out the above experiment in the presence of different concentrations of the inhibitors

and a fixed amount (1.2 pmol) of [^{3}H]folic acid. Alternately, FR in the membranes was quantitatively solubilized at 37 °C in PBS containing 1% Triton X-100 and the [^{3}H]folic acid binding and competition experiments were carried out in this buffer, using 0.3 pmol of FR and 1.2 pmol of [^{3}H]-folic acid in 0.5 mL at 37 °C, by a charcoal binding assay described previously (Wang et al., 1992). Total solubilization of FR from the membranes was ensured from [^{3}H]folic acid binding before and after solubilization. The specificity of [^{3}H]folic acid binding was confirmed by competition with unlabelled folic acid (100 nM). Parental (FR negative) L1210 cells were used as a negative control.

Transport Measurements of [^{3}H]Folic Acid and [^{3}H](6S)-5-CH_3H_4Folate. Throughout this procedure, washing of cells was carried out by resuspending in the desired solution followed by sedimentation at 1000*g*. Cells (2×10^6 per mL) were chilled on ice and washed successively at 4 °C with acid buffer (10 mM sodium acetate, pH 4/150 mM NaCl/7 mM glucose) and HBSS, pH 7.5, in order to remove endogenous receptor bound folate and resuspended in folate-free RPMI 1640 medium containing 5% fetal bovine serum that was previously treated with Norit A charcoal to remove endogenous folate. The cells were then incubated at 37 °C and 5% CO_2 in a cell culture incubator. At various intervals, 1 mL aliquots (2×10^6 cells) were removed, and the cells were sedimented and washed with acid buffer to remove externally bound folate. Radioactivity due to internalized folate was determined by liquid scintillation counting. Specificity of uptake of the labeled compounds via FR was ensured using negative controls in which the cells were incubated for 10 min with 100 nM unlabeled folic acid prior to the addition of the labeled compounds.

Deglysosylation with N-Glycanase. Membranes equivalent to 25 pmol of FR were suspended in 0.4 mL of PBS containing 8 units of *N*-glycanase (Boeringher-Mannheim) and incubated on an end-over-end rotator for 18 h at 37 °C. The membranes were then sedimented, resuspended in PBS, and used for either Western blot analysis or [^{3}H]folic acid binding and inhibition analysis as described above.

RESULTS AND DISCUSSION

Functional Differences between FRs in L1210A and L1210JF Cells. Human FR isoforms have previously been shown to vary considerably in their affinities and stereospecificities for reduced folate compounds and antifolates (Wang et al., 1992). Similar results were reported for the two known murine FRs (Brigle et al., 1994). All of the FR isoforms, however, have similar high affinities for [^{3}H]folic acid. Although folic acid is not the physiologic form of the vitamin, it can be utilized by cultured cells. On the other hand, the FR isoforms have vastly different affinities for the circulating folate coenzyme, the (6*S*) diastereoisomer of 5-CH_3H_4folate. The binding of (6*S*)-5-CH_3H_4folate, (6*S*)-5-$CHOH_4$folate, and MTX to the FRs in L1210A and L1210JF cells were compared in terms of their affinities relative to [^{3}H]folic acid (Table 1). Significant differences (up to 17-fold) in relative affinities were observed between the two variant cell lines with the L1210A cell receptor having the higher relative affinity for the compounds tested. At nanomolar concentrations, [^{3}H]-(6*S*)-5-CH_3H_4folate was transported by the FR in L1210A cells (Figure 1). On the other hand, while FR-mediated transport of [^{3}H]folic acid in L1210JF cells was similar to that in L1210A cells, the former cells were unable to efficiently transport [^{3}H]-(6*S*)-5-CH_3H_4folate (Figure 1). The poor transport of [^{3}H]-(6*S*)-5-CH_3H_4folate in L1210 JF cells is consistent with the relatively low affinity (Table 1) of the FR in L1210JF cells for this compound. The data in Table 1 and Figure 1 reveal significant functional differences between the FRs in L1210A and L1210JF cells. When L1210A cell membranes were quantitatively solubilized at 37 °C with 1% Triton X-100, the relative affinities of FR for (6*S*)-5-CH_3H_4folate, (6*S*)-5-$CHOH_4$folate and MTX markedly decreased (Table 1). In contrast, there was only a small decrease, if any, in relative affinities for the ligands when the receptor from L1210 JF membranes was solubilized and the values were comparable to those of the solubilized FR from L1210A cells (Table 1). These results demonstrate a marked influence of membrane association on the ligand binding characteristics of the FR in L1210A cells.

Table 1: Inhibition of Binding of [^{3}H]Folic Acid to FR[a]

		IC_{50} ($\times 10^{-9}$ M)		
inhibitor	cell line	membrane	soluble	membrane +mild base
(6*S*)-5-CH_3H_4folate	L1210A	2.9	50	24
	L1210JF	52.5	83	55
(6*S*)-5-$CHOH_4$folate	L1210A	175	800	470
	L1210JF	748	910	812
MTX	L1210A	161	760	ND
	L1210JF	725	710	ND

[a] Crude plasma membranes (0.3 pmol of FR) from L1210A and L1210JF cells in 0.5 mL of PBS were incubated with different amounts of (6*S*)-5-CH_3H_4folate, (6*S*)-5-$CHOH_4$folate, or methotrexate and 1.2 pmol of [^{3}H]folic acid at 37 °C for 30 min. The [^{3}H]folic acid bound to the membranes was measured as described under Materials and Methods. Alternatively, 0.3 pmol of FR quantitatively solubilized at 37 °C from the membranes in 0.5 mL of PBS containing 1% Triton X-100 was assayed in solution at 37 °C using 1.2 pmol of [^{3}H]folic acid and various concentrations of inhibitors by a standard charcoal binding assay (Wang et al., 1992). Percent error <10. ND, not determined.

Identification of the FR in L1210A and L1210JF Cells. The functional differences between the FRs in L1210A and L1210JF cells suggested that they may be different isoforms of murine FR. It was also possible that the FR expressed by either L1210A or L1210JF cells did not belong to the FR gene family identified in human and murine sources and was an unrelated folic acid binding protein. To identify the FR in both L1210A and L1210JF cells, the receptor was purified from both the sublines essentially by affinity chromatography on folic acid-Sepharose. The purified protein from both sublines displayed a diffuse protein band by SDS−polyacrylamide gel electrophoresis with an apparent molecular weight of 40 kilodalton (Figure 2). The protein cross reacted with polyclonal rabbit antibodies to human placental FR on Western blots (Figures 6 and 8, and results not shown). The sequence of the first 19 (for L1210A) or 25 (for L1210JF) amino-terminal amino acids of the purified protein was determined (Figure 3). From the direct protein sequence analysis, a single species of FR was identified in L1210A and L1210JF cells, the complete amino acid sequence of which is identical to that of murine FR type α (Brigle et al., 1991).

Further analysis was carried out at the mRNA level to test the possibility that the FR in either L1210A or L1210JF

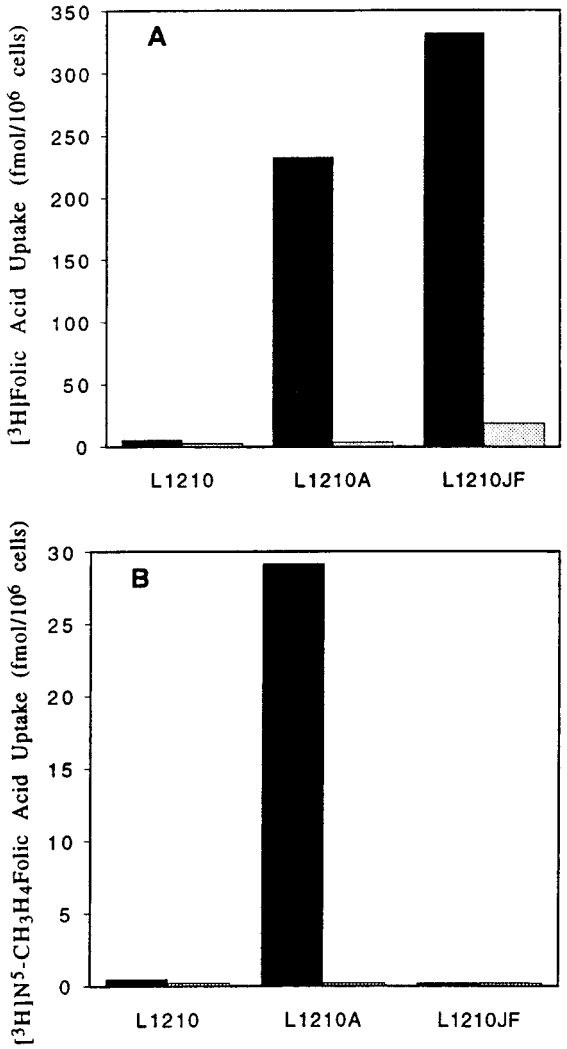

FIGURE 1: [^{3}H]Folic acid (panel A) or [^{3}H]5-CH_3H_4folate (panel B) uptake in murine leukemia cell lines L1210, L1210A, and L1210JF. The cells were incubated with 2 nM [^{3}H]folic acid (solid bars, panel A) or [^{3}H]-5-CH_3H_4folate (solid bars, panel B) in folic acid free RPMI 1640 medium with 5% charcoal-treated FBS at 37 °C, 5% CO_2 for 2 h. The cell surface bound [^{3}H]folic acid or [^{3}H]5-CH_3H_4folate was removed by treatment at low pH, and the radioactivity inside the cell was measured as described under Materials and Methods. The control samples were simultaneously incubated with 200 nM folic acid (stippled bars).

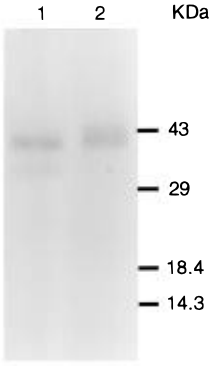

FIGURE 2: SDS−polyacrylamide gel electrophoresis of FR purified form L1210A cells (lane 1) or L1210JF cells (lane 2). The protein was purified as described under Materials and Methods.

cells contained downstream mutations that could account for the functional differences between these two proteins. Total RNA from each subline was reverse transcribed and amplified by PCR using synthetic oligonucleotides corresponding to either murine FR-α or FR-β. mRNA for FR-β was not detected in either subline (results not shown). On the other hand, by amplification of the cDNA using oligo dT and an upstream primer corresponding to murine FR-α, it was possible to obtain a FR cDNA containing the entire coding sequence (Figure 4). Direct sequence analysis of the amplified product from both L1210A and L1210JF cells revealed the cDNA sequence of murine FR-α and the absence of mutations. From these results it appears that the functional differences between the FRs from L1210A and L1210JF cells are not due to a difference in primary structure. It follows that the different properties of FR in the two sublines may be conferred post-translationally. Furthermore, since the two FRs appear to be identical in primary structure, it is likely that a fundamental difference exists in the machinery for post-translational modification in the two sublines rather than a difference that is peculiar to the FR polypeptide.

Mode of Membrane Anchoring of FR in the Variant L1210 Cells. The two known post-translational modifications in FR are N-linked glycosylation and attachment of the carboxyl-terminal GPI membrane anchor. Since GPI modification is involved in membrane association, preliminary observations in this laboratory on differences in the PI-PLC sensitivity of the membrane anchor for FR in the two sublines were investigated further.

When crude plasma membrane preparations from L1210JF cells were treated with PI-PLC, the FR was quantitatively released from the membranes (Table 2). Extraction of the JF membranes with Triton X-114 before PI-PLC treatment recovered the FR in the detergent-rich phase whereas after PI-PLC treatment, ~90 percent of the receptor was present in the aqueous phase (Figure 5). These results are clearly indicative of a GPI membrane anchor for the FR in L1210JF cells similar to observations in human FR isoforms (Lacey et al., 1989; Luhrs & Slomiany, 1989; Verma et al., 1992 Yan & Ratnam, 1995). No significant release of FR was observed upon treatment of L1210A cell membranes with PI-PLC (Table 2). Furthermore, the hydrophobicity of the FR in L1210A cells was retained after PI-PLC treatment as indicated by its partitioning into the detergent-rich phase after extraction with Triton X-114 (Figure 5). The detergent partitioning data for FR from L1210A cells after PI-PLC treatment also excludes the possibility of its association with the membrane due to weak non-specific interactions.

When parental L1210 cells were transfected with the cDNA for murine FR-α, the resulting cell surface FR was insensitive to PI-PLC similar to L1210A cells (Table 2). By contrast, when human 293 fibroblasts were transfected with the same cDNA the resulting protein was susceptible to PI-PLC (Table 2). These results demonstrate that the PI-PLC resistance of murine FR-α is a property conferred by the cell type in which it is expressed. It is thus very unlikely that the different PI-PLC sensitivities of the FR in L1210A vs L1210JF cells is due to any undetected mutant FR in either subline.

Although cleavage by PI-PLC is a common diagnostic test for a GPI membrane anchor, there are noted exceptions in certain cell types in which structural variations of the basic GPI anchor render it resistant to PI-PLC (Roberts et al., 1988; Walter et al., 1990; Wong & Low, 1992). Treatment with nitrous acid, which is known to cleave the GPI membrane anchor, released FR from L1210A cell membranes as seen

Table 2: Effect of PI-PLC, Nitrous Acid, and Mild Base Treatments on [^{3}H]Folic Acid Binding

cell line	specific [^{3}H]folic acid binding[a,b] (%)				
	− PI-PLC − nitrous acid − mild base	+ PI-PLC	+ nitrous acid	+ mild base	+ mild base + PI-PLC
KB cells	100[c]	0	3.0	ND	ND
L1210JF	100	0	29.0	50.0	0
L1210A	100	97.4	27.0	42.1	13.0
L1210 (transfected)[d]	100	99.9	ND[e]	ND	ND
293 fibroblasts (transfected)[d]	100	2.0	ND	ND	ND

[a] Specific [^{3}H]folic acid binding was determined by subtracting the amount of the radioligand bound after preincubation with unlabeled folic acid (100 nM). Nonspecific binding represented 4−15% of specific binding. [b] Percent error <10. The assays were repeated at least 3 times. [c] 100% binding represents 1 pmol of [^{3}H]folic acid bound. [d] The cells were transfected with the cDNA for murine FR-α. [e] ND, not determined.

FR (L1210JF cell)	R	A	T	R	A	R	T	E	L	L	N	V	C	M	D	A	K	H	H	K	E	K	P	G	P
FR (1210A cell)	R	A	T	R	A	R	T	E	L	L	N	V	C	M	D	A	K	H	H						
Murine FBP-1 cDNA	R	A	T	R	A	R	T	E	L	L	N	V	C	M	D	A	K	H	H	K	E	K	P	G	P

FIGURE 3: Amino-terminal protein sequence of the FR purified from L1210A and L1210JF cells and the amino terminal amino acid sequence deduced from the cDNA sequence for murine FR-α. Protein purification and amino acid sequencing are described under Materials and Methods.

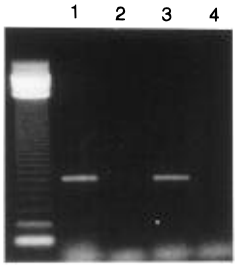

FIGURE 4: RT-PCR analysis of FR in L1210A (lane 1) and L1210JF (lane 3) cells. 0.5 μg of total RNA was reverse transcribed, and the resulting cDNA was amplified by PCR using oligo dT and an upstream primer corresponding to nucleotides 167−188 in a conserved region of the murine FRs (Brigle et al., 1991), as described under Materials and Methods. Lanes 2 and 4 represent controls corresponding to lanes 1 and 3, respectively, in which reverse transcriptase was excluded.

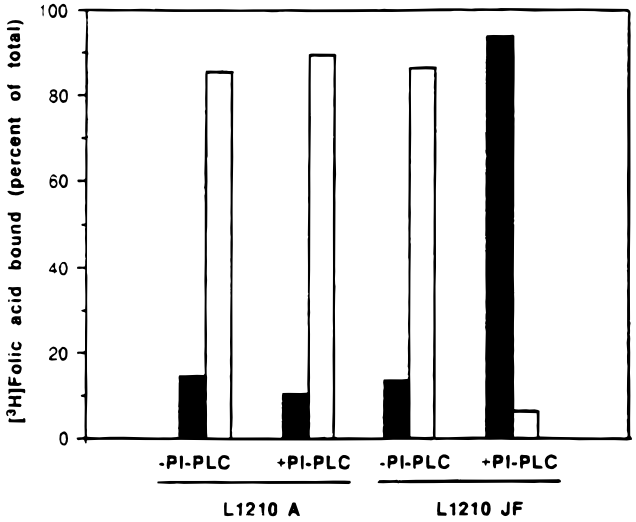

FIGURE 5: Detergent partitioning of FR in plasma membranes from L1210A and L1210JF cells. Plasma membranes with receptor-bound [^{3}H]folic acid were incubated with or without PI-PLC. They were then extracted with precondensed Triton X-114. Radioactivity in the aqueous phase (filled bars) and in the detergent rich phase (open bars) was measured. For experimental detail, please see Materials and Methods.

from both residual [^{3}H]folic acid binding protein on the membranes (Table 2) and Western blot analysis (Figure 6). This result suggests that the L1210A cell FR may be anchored by a modified form of GPI. An interesting observation from Table 2 is that while 97% of the KB cell FR was released by nitrous acid, nearly 30% of residual FR was observed in membranes from L1210A and L1210JF cells after 6 h. This does not reflect the maximum extent of FR that can be released by nitrous acid in the L1210 sublines but rather a slower time course of release of FR (results not shown). The observed differences in reaction rates may be related to steric factors affecting accessibility to nitrous acid.

When parental L1210 cells, L1210A cells and L1210JF cells were grown in media containing [^{3}H]ethanolamine followed by analysis of the membrane associated proteins by resolution on SDS−polyacrylamide gels and autoradiography, multiple radiolabeled protein bands were observed (Figure 7A). Thus all of these cells presumably incorporate the radiolabel in their GPI membrane anchors. Affinity purification of the labeled proteins followed by a similar analysis showed the occurrence of FR in L1210A and L1210JF cells as a major GPI modified protein (Figure 7B). As expected, the labeled FR band was absent in parental L1210 cells. The intensity of all of the labeled bands including FR diminished in L1210JF cells when the labeled cells were treated with PI-PLC (Figure 7A and 7B). On the other hand, all of the labeled bands in parental L1210 cells and L1210A cells were resistant to PI-PLC. These results are consistent with the existence of GPI membrane anchors for FR in both L1210A and L1210JF cells. The results confirm a fundamental difference in the nature of GPI modification between the two variant L1210 sublines. Thus, the PI-PLC insensitivity of the GPI anchor synthesized in parental L1210 cells is retained in the L1210A cell variant but not in L1210JF cells (Table 2 and Figure 7).

It is known that tissue specific structural variation in the GPI membrane anchor can lead to different degrees of resistance to PI-PLC. The known variant GPI structures show the presence of an additional fatty acyl (palmityl) substituent on the inositol ring (Roberts et al., 1988; Walter et al., 1990; Wong & Low, 1992). The different PI-PLC sensitivities of FR in L1210A vs. L1210JF cells may be explained on the basis of such a difference in their GPI structures. A diagnostic test for such a fatty acyl modifica-

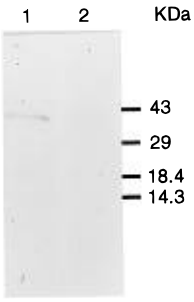

FIGURE 6: Effect of nitrous acid treatment on membrane anchoring of FR in L1210A cells. Membranes from L1210A cells were either untreated (lane 1) or treated (lane 2) with nitrous acid for 6 h at room temperature as described under Materials and Methods. Residual membrane proteins were then analyzed by Western blotting as described under Materials and Methods.

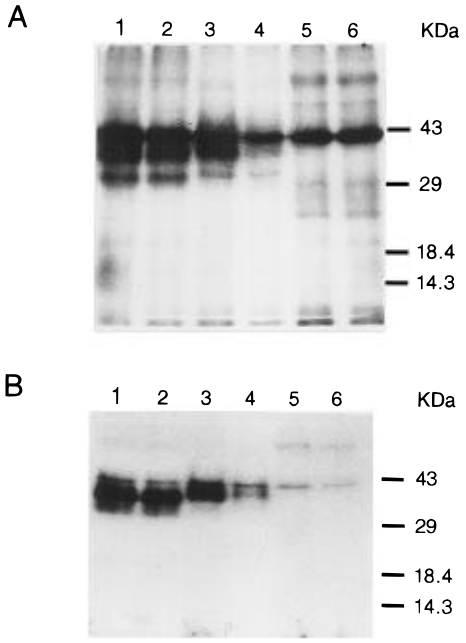

FIGURE 7: [^{3}H]Ethanolamine labeling of the cells and PI-PLC treatment. In panel A, L1210A (lanes 1 and 2), L1010JF (lanes 3 and 4), and parental L1210 (lanes 5 and 6) cells were incubated with [^{3}H]ethanolamine as described under Materials and Methods; the cell membranes were isolated and treated with PI-PLC (lanes 2, 4, and 6); control samples were treated under the same conditions but without PI-PLC (lanes 1, 3, 5); the cell membranes were dissolved in SDS−PAGE sample buffer and loaded on a 12% SDS−PAGE gel; the gel was fixed in 10% glacial acetic acid, 30% methanol for 20 min and then soaked in Fluoro-Hance autoradiography enhancer for 30 min; the gel was dried and exposed on X-ray film for 2 weeks. In panel B, the same samples were subjected to affinity purification using folic acid-Sepharose as described under Methods, prior to SDS−polyacrylamide gel electrophoresis and autoradiography as described above. Membranes from L1210A cells (lanes 1 and 2), L1210JF cells (lanes 3 and 4), and parental L1210 cells (lanes 5 and 6) were either untreated (lanes 1, 3, and 5) or treated (lanes 2, 4, and 6) with PI-PLC.

tion is its removal by mild base hydrolysis of the hydroxy-ester bonds (Hirose et al. 1995). As seen in Table 2, the FR in membranes from L1210A cells became PI-PLC sensitive after the mild base treatment, consistent with the occurence of a fatty acyl modification in the inositol ring of its GPI anchor. The mild base treatment also strikingly shifted the relative affinities of the membrane-associated L1210A cell FR for reduced folates to lower values but did not significantly alter the values for the FR in L1210JF cell membranes (Table 1). The data in Table 1 suggest a direct link between fatty acyl modification of the inositol ring of the GPI anchor and functional properties of membrane associated FR, although it may not be possible to rule out secondary effects of the mild base treatment on the structure of the FR polypeptide.

Possible Functional Significance of a Variant GPI Structure. The results of this study demonstrate that murine FR-α expressed in variants of a single cell type, i.e., L1210A and L1210JF cells displays significant functional differences. An obvious explanation for this observation is that differences in post-translational modifications of FR, may occur among these cells and they may influence the properties of the receptor. Since FR is extensively N-glycosylated, a necessary consideration is that possible differences in glycosylation may be responsible for the different properties of FR in L1210A vs L1210JF cells. Although no gross difference in N-glycosylation is apparent due to the similar electrophoretic mobilities of the glycosylated FRs purified from the two sublines (Figure 2), there may be subtle structural differences in their pattern of N-glycosylation.

The diverse functional roles of N-glycosylation have been studied for several decades and most cell surface and secretory proteins are glycosylated. Even so, we are not aware of any systematic study in which microheterogeneity in glycosylation has been shown to influence the ligand binding characteristics of a protein. It has also been our experience with human FRs that they may be expressed in various mammalian cell types from different species that have different N-linked carbohydrate compositions (e.g., KB cells, recombinant CHO cells, recombinant 293 cells, Cos cells, etc.) and even in insect cells (the bacculovirus system) without significantly altering their properties (unpublished observations). More importantly, in this study, detergent solubilization of the FR in L1210A and L1210JF membranes either greatly diminished or abolished the differences in their ligand binding characteristics. This observation indicates that the difference in functional properties conferred on FR by L1210A vs L1210JF cells must be a membrane associated phenomenon. When membranes from L1210A and L1210JF cells were incubated with a relatively high concentration of *N*-glycanase, partial deglycosylation was achieved, as seen by Western blot analysis (Figure 8). The lowest, relatively sharp bands presumably represent the fully deglycosylated protein. In both samples, the number of glycosylated species in Figure 8 is consistent with the three known potential sites of N-glycosylation in murine FR-α. Partial deglycosylation of membrane associated FR did not significantly alter its relative affinities for reduced folates and methotrexate (results not shown) either for L1210A cells or for L1210JF cells suggesting that glycosylation may not play a significant role in determining the ligand binding characteristics of murine FR-α.

The different functional properties and PI-PLC sensitivities of FR among the L1210 sublines may be explained on the basis of differences in extrinsic membrane associated factors such as a specific interaction of FR with some membrane protein or variability in membrane lipid composition. However, such possibilities are deemed less likely because the different ligand binding properties of FR isoforms are retained in their solubilized forms and these properties as well as PI-PLC sensitivity for each receptor isoform are similar in a diverse variety of cell types. In addition, the ligand binding characteristics of FR are not known to be

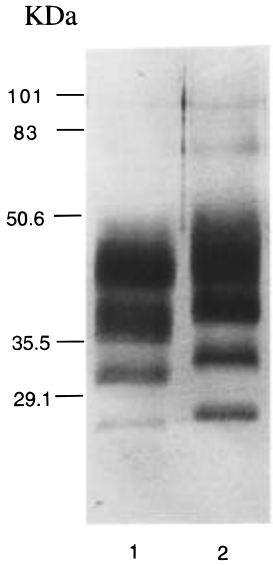

FIGURE 8: Deglycosylation of membrane associated FR in L1210A (lane 1) or L1210JF (lane 2) cell membranes with *N*-glycanase. The membranes were treated with the enzyme as described under Materials and Methods, sedimented, solubilized in 1% sodium dodecyl sulfate, and subjected to Western blot analysis using a cross-reacting affinity-purified antibody to human placental FR, as described under Materials and Methods.

modulated by interactions with other proteins and the PI-PLC resistance in L1210A cells is not a specific property of FR. Furthermore, the known examples of PI-PLC resistance are generally associated with modified GPI structures and not with membrane components.

Over one hundred proteins with diverse physiologic functions are known to be attached to the cell surface by a GPI anchor. There is compelling evidence that fatty acyl substitutions in the inositol moiety of the GPI anchor influence the ability of these proteins to be released from the membrane by the bacterial PI-PLC (Roberts et al., 1988; Walter et al., 1990; Wong & Low, 1992; Urakaze et al., 1992). It has been speculated (Roberts et al., 1988) that the differential PI-PLC sensitivities may reflect a regulatory mechanism inherent in the GPI membrane anchor that determines its susceptibility to endogenous phospholipase although there are many examples in which PI-PLC resistant anchors are sensitive to a known GPI specific serum phospholipase, PI-PLD (Wong & Low, 1992). It has also been speculated that structural differences in the GPI membrane anchor may underlie cell type specific differences in the function of decay accelerating factor (Walter et al., 1990).

Fatty acyl substitutions on the inositol ring could conceivably affect the function of a protein because of their bulk and hydrophobicity. The results in this study would suggest that such a substitution may influence protein function by imposing conformational constraints in the membrane bound protein and/or altering the topology of the protein in relation to the membrane. The large reduction of functional differences between FR-α in L1210A vs L1210JF cells upon solubilization, as well as the similarity in the properties of membrane associated and solubilized FR-α in the PI-PLC sensitive L1210JF cells is consistent with this concept. The concept is further supported by the effect of mild base treatment on the properties of membrane associated FR from L1210A cells. The results of this study lead to the testable hypothesis that tissue specific differences in the GPI structure may result in alterations in membrane conformation/membrane topography of GPI anchored proteins contributing to their functional diversity. The results also warrant detailed studies of possible functional correlates of variant GPI structures in other proteins.

ACKNOWLEDGMENT

The authors thank Jenny Zak and Marietta Wu for typing the manuscript.

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BI961098Q