Identification of a Novel Folate Receptor, a Truncated Receptor, and Receptor Type β in Hematopoietic Cells: cDNA Cloning, Expression, Immunoreactivity, and Tissue Specificity[†,‡]

F. Shen, J. F. Ross, X. Wang, and M. Ratnam*

Department of Biochemistry and Molecular Biology, Medical College of Ohio, 3000 Arlington Avenue, Toledo, Ohio 43614

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ABSTRACT: The expression of a membrane-associated folate receptor (FR) was elevated in spleen samples from patients with chronic (CML) and acute (AML) myelogenous leukemias compared with normal spleen. Contrary to earlier reports, antibodies to a purified FR from placenta cross-reacted quantitatively with this protein in solution radioimmunoassays. Similar to FR-α (KB cells) and FR-β (placenta), the protein was released from the membrane by phosphatidylinositol-specific phospholipase C, indicating a glycosylphosphatidylinositol (GPI) membrane anchor. Screening of a cDNA library from CML spleen with a heterologous murine FR cDNA and also amplification of FR cDNAs from spleen and bone marrow in CML, AML, chronic lymphocytic leukemia (CLL), and acute lymphocytic leukemia (ALL) by polymerase chain reaction (PCR) using degenerate oligonucleotides yielded cDNA clones representing FR-β, a novel FR (type γ), and an aberrant transcript of FR-γ with a 2 base pair deletion resulting in a truncated 104-residue polypeptide; FR-α was not detected in these tissues. The cDNA for FR-γ predicts a 243-residue polypeptide with an amino acid sequence homology of 71% and 79% with FR-α and FR-β, respectively, a 23-residue amino-terminal signal peptide, and 3 potential sites for N-linked glycosylation. Transfection of COS-1 cells with the cDNA for FR-γ resulted in low expression of a [^{3}H]folic acid binding protein on the cell surface that was GPI-anchored. PCR analysis of total RNA from a number of normal and malignant tissues and cell lines indicated a limited tissue specificity of FR-γ. FR-γ and FR-β could thus be potential differentiation markers in hematopoiesis and potential therapeutic targets in certain malignancies.

The two known isoforms of the human cell surface-associated high-affinity folate receptor (FR) are homologous polypeptides of M_r ~28 000 that are N-glycosylated and attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor (Ratnam et al., 1989; Sadasivan & Rothenberg, 1989; Elwood, 1989; Lacey et al., 1989; Luhrs & Sloliamy, 1989; Verma et al., 1992). Although a dual functional role has been proposed for FR, i.e., cellular transport of folate compounds/anti-folates [reviewed in Antony (1992); Jansen et al., 1990; Westerhof et al., 1991; Luhrs et al., 1992; Matsue et al., 1992] and stimulation of cell growth in primary bone marrow cultures (Antony et al., 1991), the physiological significance of FR expression in various tissues is still unclear. Nevertheless, FR levels are greatly elevated in a variety of malignant tissues compared with normal tissues, suggesting a potential role for FR in tumor targeting either with FR-specific antibodies or with novel anti-folate drugs (Campbell et al., 1991; Coney et al., 1991; Weitman et al., 1992; Ross et al., 1994).

FR type α (KB cells) and type β (placenta) have opposite stereospecificities and quite different (2-fold to 100-fold) affinities for folate coenzymes and anti-folates (Wang et al., 1992). Furthermore, the two isoforms are differentially regulated in various tissues, showing a general tendency toward elevation of FR-α in malignant tissues of epithelial origin and elevation of FR-β in maligancies of nonepithelial origin (Ross & Ratnam, 1992; Ross et al., 1994).

Our interest in identifying folate binding proteins in leukemic cells was based on earlier reports that spleen tissue in chronic myelogenous leukemia (CML) expressed relatively high levels of a membrane-associated [^{3}H]folic acid binding protein that did not cross-react with polyclonal antibodies raised against purified placental FR (Sadasivan et al., 1986, 1987). Herein, we present our results on the molecular and immunochemical characterization of FR in spleen and bone marrow samples obtained from normal individuals and from patients with CML, acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL), and acute lymphocytic leukemia (ALL). We also characterize a novel FR isoform and its alternatively spliced product and examine its tissue specificity.

MATERIALS AND METHODS

Crude Plasma Membranes. Approximately 0.5 g of frozen tissue was pulverized and homogenized in 5 mL of ice-cold 20 mM sodium phosphate buffer, pH 7.5, 10 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride. The samples were centrifuged at approximately 1000*g* for 5 min at 4 °C to sediment nuclei and debris, and the supernatant was centrifuged at 30000*g* for 45 min at 4 °C. The pellet was resuspended in 5 mL of ice-cold 10 mM sodium acetate buffer (pH 3.5)/150 mM NaCl using a syringe and a 21-gauge needle with the orifice opposed to the wall of the glass container in order to obtain a fine suspension. The samples were centrifuged at 30000*g* for 20 min at 4 °C, and the supernatants were discarded. This step removed endogenously bound folates in the samples. (Additional acid washes did not expose any more [^{3}H]folic acid binding sites.) The membranes were washed with 5 mL of 10 mM sodium phosphate buffer (pH 7.5)/150 mM NaCl by resuspending and centrifuging as above. The resulting membrane pellets were dissolved in 1 mL of 10

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* To whom correspondence should be addressed.

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mM sodium phosphate buffer, pH 7.5, 150 mM sodium chloride, and 0.5% Triton X-100, and the insoluble material was removed by centrifugation.

[^{3}H]Folic Acid Binding Assay. The [^{3}H]folic acid binding assay was performed both by direct binding of [^{3}H]folic acid to membranes and by a solution-phase assay (Wang et al., 1992). In the direct binding assay, membrane samples (10–100 μg of protein) were incubated with 3 pmol of [^{3}H]folic acid (Moravek) (40 Ci/mmol) in 10 mM sodium phosphate buffer (pH 7.5)/150 mM NaCl/10 mM EDTA for 30 min at 37 °C with constant agitation. The membranes were sedimented at 12000*g* for 15 min, washed once with the same buffer, dissolved in 10 mM sodium phosphate buffer (pH 7.5)/150 mM NaCl/1% Triton X-100, and subjected liquid scintillation counting. Nonspecific binding of [^{3}H]folic acid was determined for both the assays by performing the assay in each case simultaneously with controls in which the addition of [^{3}H]folic acid was preceded by incubation for 5 min at room temperature with 100 pmol of unlabeled folic acid. The assays are quantitative for both FR-α and FR-β.

Protein and Antibodies. FR was purified from human placenta as described (Ratnam et al., 1989). Rabbit antisera against the placental FR were produced and FR-specific antibodies were affinity-purified using placental FR coupled to Sepharose as described (Ratnam et al., 1989).

Immunoprecipitation. Three picomoles of either purified FR or FR in the form of a plasma membrane extract in 10 mM sodium phosphate buffer (pH 7.5)/150 mM NaCl/1% Triton X-100 was incubated in this buffer (0.3 mL) with a stoichiometric amount of [^{3}H]folic acid for 30 min at 37 °C followed by the addition of various amounts of the primary antibody. After incubating for 1 h at 37 °C, 30 μL of goat anti-rat IgG-coupled Sepharose 4B (Cappel) was added. The suspension was agitated overnight at 4 °C. The Sepharose beads were then sedimented by centrifuging for 2 min at 10000*g*, and the radioactivity in the supernatant was estimated by liquid scintillation counting. The decrease in the radioactivity in the supernatant was monitored as a measure of antibody binding to FR. In control samples, either the primary antibody or the receptor was excluded.

Immunoblotting. Total membrane protein (10 μg) from FR-γ-transfected COS-1 cells was electrophoresed on a sodium dodecyl sulfate–polyacrylamide gel followed by electroblotting onto nitrocellulose and probing with affinity-purified antibodies to placental FR as described previously (Wang et al., 1992). Total protein from 1 mL of cell culture media, collected at the time of harvesting FR-γ-transfected COS-1 cells, was precipitated by treatment with 10% trichloroacetic acid followed by sedimentation for 15 min at 12000*g*. The precipitate was washed with 1 mL of acetone, dried, and subjected to electrophoresis and Western blotting as described for the membrane proteins.

Treatment with Phosphatidylinositol-Specific Phospholipase C (PI-PLC). FR in the plasma membrane preparation was saturated with [^{3}H]folic acid by incubating with a 2-fold molar excess of the radioligand in 50 mM Tris-HCl (pH 7.5)/10 mM EDTA (buffer B) at 4 °C for 30 min with constant agitation. The membranes were sedimented at 10000*g* in a microfuge at 4 °C. The membranes were then resuspended in the same buffer and sedimented again at 4 °C. The washed pellets were resuspended again in the above buffer, and 10-μL aliquots were incubated in the absence or in the presence of 0.5 unit of PI-PLC in a final volume of 50 μL of buffer B for 3.5 h at 37 °C with intermittent agitation. The membranes were sedimented as above, and 30 μL of the supernatant was counted for radioactivity due to released FR-bound [^{3}H]folic acid by liquid scintillation counting.

Oligonucleotides. The following oligonucleotides, used as primers for PCR analysis, were custom-made from Genosys except for β-actin 1 and β-actin 2 which were purchased from Stratagene:

CML2	AGCGCATTCTGAACGTGCCCCTG
CML4	CAGGAATCAATAATCCCACGAGACGG
SEARCH 1	TTCTCAATGTCTGCATGAACGCCAAGCANN
SEARCH 2	AGAACCTCGCCACCTCCTCATTGGGGTTNN
β-actin 1	TGACGGGGTCACCCACACTGTGCCCATCTA
β-actin 2	CTAGAAGCATTGCGGTGGACGATGGAGGG
MF1	CATGGCTGCAGCATAGAACCTCGC
M2	GTAGTAGGGGAGGCTCAGACAAGG
F1	CACAGCCAGCACCAGCCAGGAGCTG

RNA and PCR Analysis. Total RNA from 0.5–1.0 g of tissue was isolated using the guanidinium thiocyanate/phenol/chloroform single-step extraction method (Stratagene). Total RNA from the bone marrow samples was kindly provided by Curt I. Civin, M.D. When Taq polymerase was used for the PCR reaction, 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; USB), and MMLV-reverse transcriptase (5 units/μL; Gibco—BRL). When Vent polymerase was used for the PCR reaction, the reverse transcription reaction (10 μL) consisted of 0.05 μg/μL total RNA, 0.01 M KCl, 0.01 M $(NH_4)_2SO_4$, 0.02 M Tris-HCl, pH 8.6, 5 mM $MgSO_4$, 0.01% Triton X-100, 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; USB), and MMLV-reverse transcriptase (5 units/μL; Gibco—BRL). In either case, the reaction mixture was first incubated at room temperature for 10 min to allow the random hexamer primers to anneal. The temperature was raised to 42 °C and held at this temperature for 15 min, and then stopped by heating at 99 °C for 6.5 min.

For PCR using Taq polymerase, the entire reverse transcription product was combined with additional KCl/Tris buffer (the same concentration as in the reverse transcription reaction except for $MgCl_2$, which was 2 mM), 0.15 μM of each primer, and 1.25 units of Taq polymerase in 50 μL. The reaction mix was heated for 2 min at 95 °C. The PCR cycle comprised 1 min at 95 °C followed by 1 min at 60 °C. After 25–35 cycles, the reaction was held at 60 °C for 6.5 min.

For PCR using Vent polymerase, the entire reverse transcription product was combined with additional KCl/$(NH_4)_2SO_4$/Tris/$MgSO_4$/Triton buffer (the same concentration as in the reverse transcription reaction except for $MgSO_4$ which was 2.6 mM), 1 unit of Vent polymerase, and 0.15 μM of each primer in 50 μL. In both cases, the reaction mix was heated for 2 min at 95 °C. The PCR cycle comprised three steps: 1 min at 95 °C, 0.5 min at 60 °C, and 0.5 min at 72 °C. After 35 cycles, the reaction was held at 72 °C for 6.5 min; 10–20 μL of each reaction was electrophoresed through a 1.0% agarose gel containing 0.05 μg/mL ethidium bromide. The DNA was visualized by UV illumination.

For tissue distribution studies of FR-γ, the sensitivity of the PCR was enhanced by using ^{32}P-labeled primer (CML4); in the same PCR reaction tube, the cDNA for β-actin was amplified using ^{32}P-labeled primers (β-actin 1 and β-actin 2) as an internal control. The expected PCR products for FR-γ and β-actin were 361 base pairs and 661 base pairs, respectively. In order to detect FR-α and FR-β, a common primer (MF1) was labeled and used in combination with primer M2 (for FR-α) or F1 (for FR-β). The expected PCR products for FR-α and FR-β were 648 base pairs and 502 base pairs, respectively. The labeling reaction was carried out in 50 mM Tris-HCl (pH 7.6)/10 mM $MgCl_2$/5 mM dithiothreitol/0.1 mM spermidine hydrochloride/0.1 mM EDTA/30 units of T-4 polynucleotide kinase (Gibco–BRL)/0.37 nmol of the [γ-^{32}P]ATP (6000 Ci/mmol; New England Nuclear) and 0.3 nmol of the CML4 or MF1 primer or 0.16 nmol of each β-actin primer in a volume of 50 μL for 45 min at 37 °C followed by heating at 68 °C for 10 min to inactivate the enzyme. The product was purified on a Sephadex G-25 (Sigma) column.

The PCR conditions were the same as stated above for Taq polymerase. Unlabeled primers were added to the PCR reactions to ensure the primers were not limiting in the reaction due to any loss of the primers in the labeling and purification procedure. For FR-γ, the reaction was allowed to proceed for 5 cycles with the unlabeled CML2 primer and the mixture of labeled and unlabeled CML4 primers before adding the β-actin primers (the mixture of labeled and unlabeled). A further 25 cycles were carried out. The entire 50 μL of each reaction product was electrophoresed through a 6% polyacrylamide gel. The gel was fixed, dried, and autoradiographed. FR-α and FR-β were similarly detected using the appropriate primers described above.

Subcloning PCR Products. PCR products generated by Vent polymerase were directly subcloned into the *Sma*I site in the polylinker of pBluescript by blunt-end ligation for DNA sequence analysis.

cDNA Cloning and Sequencing. A human CML cDNA library constructed in the expression vector λgt11 was obtained from Clontech, Inc., and plated on *Escherichia coli R Y1090*. Approximately 400 000 recombinant plaques were screened using a radiolabeled DNA probe. The DNA probe was prepared by labeling with [^{32}P]dATP a 722 bp *Ava*I and *Hae*II fragment of murine FR using the Prime A Gene labeling system from Promega. The cDNA inserts were cut out with *Eco*RI and subcloned into puC19 or pBluescript. Sequencing of the plasmids was carried out by the dideoxy sequencing method using Sequenase version 2.0 (USB).

Expression in COS-1 Cells. The full-length cDNA for FR-γ was placed in the polylinker region of the expression vector pCDNA1 Neo (Invitrogen), and the plasmid was amplified in *E. coli* MC1061/P3. The resulting plasmid was used to transfect COS-1 cells using lipofectamine (Bethesda Research Laboratories) according to the manufacturer's protocol. COS-1 cells were harvested 48 h after transfection. Untransfected COS-1 were used as the negative control in all experiments.

RESULTS AND DISCUSSION

Immunoreactivity of Membrane-Associated [^{3}H]Folic Acid Binding Protein in CML and AML with Anti-Placental FR. Consistent with earlier reports on CML, when acid-washed membrane preparations from normal spleen samples were compared with those from CML and AML spleen, the level of specific [^{3}H]folic acid binding was significantly elevated in the leukemic spleens (Table 1). Affinity-purified rabbit polyclonal antibodies raised against purified human placental FR cross-reacted quantitatively with the [^{3}H]folic acid bound membrane protein in CML and AML (Figure 1). In fact, the immunoprecipitation curves for the leukemic samples overlapped closely both with placental FR (FR-α plus FR-β) and with the FR in KB cells (FR-α) (Figure 1). This result suggests that the protein that is elevated in the leukemic spleens is identical either to a placental FR (FR-α or FR-β) or to a homologous FR isoform.

Table 1: Membrane-Associated [^{3}H]Folic Acid Binding Protein in Normal and Leukemic Spleen[a]

spleen sample	pmol of [^{3}H]folic acid bound/mg of membrane protein
normal	0.3
normal	1.3
CML	11.3
CML	9.7
AML	4.8
AML	13.4

[a] Standard deviation <0.1. The assays were repeated at least 3 times.

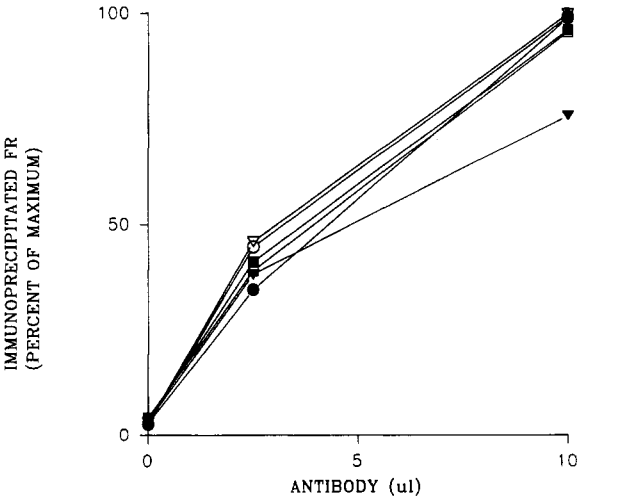

FIGURE 1: Radioimmunoprecipitation of membrane-associated FR from tissue explants. [^{3}H]Folic acid was bound to FR solubilized from crude membrane preparations and immunoprecipitated using an affinity-purified polyclonal antibody to purified placental FR (FR-α plus FR-β) as described under Materials and Methods. The tissues used were CML spleen (●), AML spleen (□), myeloproliferative disorder spleen (■), KB cells (FR-α) (▽), ovarian carcinoma (FR-α) (○), and meningioma (FR-β) (▼). In control experiments, either the primary antibody or the receptor was excluded.

The result in Figure 1 is at variance with earlier reports (Sadasivan et al., 1986, 1987) that the membrane-associated folate binding protein in CML spleen did not cross-react with antibodies to placental FR. One explanation for this discrepancy could be that the particular sample of leukemic spleen used in those studies did express a folate binding protein that was structurally unrelated to FR. However, we did not encounter such a protein in any of the leukemic spleens in this study, although they invariably showed elevated FR levels. A second possibility is a difference in the quality of the antibodies used in the two studies, such as a difference in the fraction of the antibody directed against tissue-specific carbohydrate epitopes on the protein.

Membrane-Anchoring of [^{3}H]Folic Acid Binding Protein in CML and AML. Table 2 shows that the [^{3}H]folic acid binding protein associated with membrane preparations from CML and AML is quantitatively released by the action of GPI-specific phospholipase C, indicating a GPI membrane anchor for the protein. It has been previously established

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CG CAG GAA TAG ATG GAC ATG GCC TGG CAG ATG ATG CAG CTG CTG CTT CTG        50
                       M   A   W   Q   M   M   Q   L   L   L   L

GCT TTG GTG ACT GCT GCG GGG AGT GCC CAG CCC AGG AGT GCG CGG GCC AG       100
A   L   V   T   A   A   G   S   A   Q   P   R ↑ S   A   R   A   R

G ACG GAC CTG CTC AAT GTC TGC ATG AAC GCC AAG CAC CAC AAG ACA CAG C      150
  T   D   L   L   N   V   C   M   N   A   K   H   H   K   T   Q   P

CC AGC CCC GAG GAC GAG CTG TAT GGC CAG TGC AGT CCC TGG AAG AAG AAT      200
   S   P   E   D   E   L   Y   G   Q   C   S   P   W   K   K   N

GCC TGC TGC ACG GCC AGC ACC AGC CAG GAG CTG CAC AAG GAC ACC TCC CG      250
A   C   C   T   A   S   T   S   Q   E   L   H   K   D   T   S   R

C CTG TAC AAC TTT AAC TGG GAT CAC TGT GGT AAG ATG GAA CCC ACC TGC A      300
  L   Y   N   F   N   W   D   H   C   G   K   M   E   P   T   C   K

AG CGC CAC TTT ATC CAG GAC AGC TGT CTC [TA]T GAG TGC TCA CCC AAC CTG     350
   R   H   F   I   Q   D   S   C   L   Y   E   C   S   P   N   L

GGG CCC TGG ATC CGG CAG GTC AAC CAG AGC TGG CGC AAA GAG CGC ATT CT       400
G   P   W   I   R   Q   V   N   Q   S   W   R   K   E   R   I   L

G AAC GTG CCC CTG TGC AAA GAG GAC TGT GAG CGC TGG TGG GAG GAC TGT C      450
  N   V   P   L   C   K   E   D   C   E   R   W   W   E   D   C   R

GC ACC TCC TAC ACC TGC AAA AGC AAC TGG CAC AAA GGC TGG AAT TGG ACC      500
   T   S   Y   T   C   K   S   N   W   H   K   G   W   N   W   T

TCA GGG ATT AAT GAG TGT CCG GCC GGG GCC CTC TGC AGC ACC TTT GAG TC      550
S   G   I   N   E   C   P   A   G   A   L   C   S   T   F   E   S

C TAC TTC CCC ACT CCA GCC GCC CTT TGT GAA GGC CTC TGG AGC CAC TCC T      600
  Y   F   P   T   P   A   A   L   C   E   G   L   W   S   H   S   F

TC AAG GTC AGC AAC TAT AGT CGA GGG AGC GGC CGC TGC ATC CAG ATG TGG      650
   K   V   S   N   Y   S   R   G   S   G   R   C   I   Q   M   W

TTT GAC TCA GCC CAG GGC AAC CCC AAT GAG GAG GTG GCC AAG TTC TAT GC      700
F   D   S   A   Q   G   N   P   N   E   E   V   A   K   F   Y   A

T GCG GCC ATG AAT GCT GGG GCC CCG TCT CGT GGG ATT ATT GAT TCC TGA T     750
  A   A   M   N   A   G   A   P   S   R   G   I   I   D   S   *

CC AAG AAG GGT CCT CTG GGG TTC TTC CAA CAA CCT ATT CTA ATA GAC AAA      800

TCC ACA TGA AAA AAA AAA A                                              819
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FIGURE 2: Complete cDNA sequence and deduced amino acid sequence of FR-γ and FR-γ'. The initiator methionine was deduced on the basis of the closest match to the consensus Kozak sequence. The upward arrow indicates the putative site of cleavage of the signal peptide. N denotes potential sites of N-linked glycosylation. Boxed TA indicates the deleted bases in FR-γ'. The asterisk indicates the stop codon for FR-γ.

Table 2: Effect of PI-PLC Treatment on [^{3}H]Folic Acid Binding by Plasma Membranes

	sp [^{3}H]folic acid binding (%)[a]	
tissue	-PI-PLC	+PI-PLC
KB cells	100	0
CML spleen	100	21.2
AML spleen	100	0
AML spleen	100	19.1
FR-γ-transfected COS-1 cells	100	16.3

[a] Standard deviation <0.1. The assays were repeated at least 3 times. COS-1 cells transfected with the cDNA for FR-γ expressed 5–20 pmol of [^{3}H]folic acid binding sites/mg of membrane protein.

that FR is a GPI-anchored protein. This result is further evidence that the elevated membrane folate binder in CML and AML spleen is a member of the FR family and not a structurally unrelated protein.

Identification of the FR in Leukemic Spleen and Bone Marrow. We undertook two complementary approaches to identify the FR in normal and various pathologic specimens of spleen including CML, AML, CLL, and lymphoproliferative and myeloproliferative disorders. We also examined bone marrow samples from CML, AML, and ALL.

One approach was to amplify all FR cDNAs in the samples using RNA–PCR with two degenerate oligonucleotide primers. The PCR primers (SEARCH 1 and SEARCH 2, see Materials and Methods) were designed so as to correspond to 30 bases in conserved regions of FR-α, FR-β, and the two murine FR sequences (Brigle et al., 1991) with complete degeneracy in the ultimate and penultimate bases at the 3′ ends. The primers spanned a 588 base pair fragment of FR-α, FR-β, and murine FRs. In control experiments, the primers amplified the expected fragment from total RNA samples containing mRNAs for FR-α, FR-β, and murine FR. Vent polymerase was used in these experiments in order to minimize potential "PCR errors". The amplified cDNA fragments were subcloned into the vector pBluescript, and the partial cDNA clones were sequenced.

In the second approach, a cDNA library prepared from a CML spleen sample in λgt11 was screened using a 722 base pair cDNA fragment in the coding region of a mouse FR (Brigle et al., 1991). The mouse FR cDNA was chosen as a probe rather than a human FR cDNA in order to minimize bias for the selection of any one type of human FR in the screening procedure. Forty-five positive clones were obtained, and the cDNA inserts were subcloned into pBluescript for sequencing.

By both the above methods, two types of FR were identified in the spleen and bone marrow samples. One of the cDNAs corresponded to FR-β. A second cDNA did not correspond to the known FR isoforms and represented a novel FR isoform, designated herein as FR-γ (Figure 2). A truncated form of

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                                                    *       *   * *
FRα  M A Q R M T T Q L L L L L V W V A V V G E A Q T R I A W A R T
FRβ            M V W K W M P L L L L L V C V A T M C S A Q D R T
FRγ      M A W Q M M Q L L L L A L V T A A G S A Q P R S A R A R T

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FRα  E L L N V C M N A K H H K E K P G P E D K L H E Q C R P W R K
FRβ  D L L N V C M D A K H H K T K P G P E D K L H D Q C S P W K K
FRγ  D L L N V C M N A K H H K T Q P S P E D E L Y G Q C S P W K K

       * * * *      * * * *  * * *  *  * *  * * *  * * *  *
FRα  N A C C S T N T S Q E A H K D V S Y L Y R F N W N H C G E M A
FRβ  N A C C T A S T S Q E L H K D T S R L Y N F N W D H C G K M E
FRγ  N A C C T A S T S Q E L H K D T S R L Y N F N W D H C G K M E

       *  * * * * * * * *  * * * * * * * * * * * * * *  * *  * * *
FRα  P A C K R H F I Q D T C L Y E C S P N L G P W I Q Q V D Q S W
FRβ  P A C K R H F I Q D T C L Y E C S P N L G P W I Q Q V N Q S W
FRγ  P T C K R H F I Q D S C L Y E C S P N L G P W I R Q V N Q S W

       * * * *  *  * * * * * * *    * * * * *  * *  * * * * *
FRα  R K E R V L N V P L C K E D C E Q W W E D C R T S Y T C K S N
FRβ  R K E R F L D V P L C K E D C Q R W W E D C H T S H T C K S N
FRγ  R K E R I L N V P L C K E D C E R W W E D C R T S Y T C K S N

       * *  * *  * * * *  *  *    * *  *    *    * * * * *
FRα  W H K G W N W T S G F N K C A V G A A C Q P F H F Y F P T P T
FRβ  W H R G W D W T S G V N K C P A G A L C R T F E S Y F P T P A
FRγ  W H K G W N W T S G I N E C P A G A L C S T F E S Y F P T P A

         * *      *  * *  * * * * * * * * * * * * * * * * * * *  *
FRα  V L C N E I W T H S Y K V S N Y S R G S G R C I Q M W F D P A
FRβ  A L C E G L W S H S Y K V S N Y S R G S G R C I Q M W F D S A
FRγ  A L C E G L W S H S F K V S N Y S R G S G R C I Q M W F D S A

       * * * * * * * * *  * * * * * *
FRα  Q G N P N E E V A R F Y A A A M S G A G P W A A W P F L L S L
FRβ  Q G N P N E E V A R F Y A A A M H V N A G E M L H G T G G L L
FRγ  Q G N P N E E V A K F Y A A A M N A G A P S R G I I D S

FRα  A L M L L W L L S
FRβ  L S L A L M L Q L W L L G
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FIGURE 3: Aligned amino acid sequences of FR-α, FR-β, and FR-γ. Positions marked by an asterisk indicate amino acids conserved among all the FR isoforms.

FR-γ (FR-γ') that is possibly the result of alternative splicing was also found (Figure 2). FR-γ' was the result of a 2-base (TA) deletion resulting in a stop codon and a 139 amino acid carboxyl-terminal deletion (Figure 2). Since the cDNA for FR-γ' is identical in sequence to FR-γ (including the 3'- and 5'-untranslated regions) except for the two-base deletion, FR-γ' is in all likelihood an alternatively spliced product of the gene for FR-γ and is not encoded by a separate gene. FR-α was not detected in the above experiments, suggesting either its complete absence or the occurrence of relatively insignificant amounts of FR-α.

Structure of FR-γ. The cDNA for FR-γ predicts a 243-residue polypeptide with a 23-residue amino-terminal signal peptide (Figure 2). FR-γ has an amino acid sequence homology of 71% and 79%, respectively, with FR-α and FR-β, 16 conserved cysteine residues, and 3 potential sites for N-linked glycosylation (Figures 2 and 3). The carboxyl-terminal sequences of FR-α and FR-β are represented by 30 and 19 unconserved hydrophobic amino acids, respectively; the carboxyl-terminal sequence of FR-γ is unusual in that it is represented by 16 hydrophobic amino acids in a sequence that is interrupted by charged amino acid residues (aspartic acid at the second position and arginine at the sixth position from the carboxyl terminus), perhaps lowering GPI signal efficiency (Figure 3).

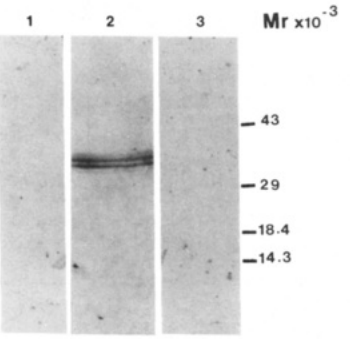

FIGURE 4: Western blot analysis of membranes and media from transfected COS-1 cells expressing FR-γ. Membrane proteins (10 μg) from COS-1 cells transfected with the vector pCDNA1 Neo (lane 1) or from COS-1 cells transfected with pCDNA1 Neo containing the cDNA for FR-γ (lane 2) and proteins precipitated from 1 mL of FR-γ-transfected COS-1 cell culture media (lane 3) were subjected to Western blotting and probing with affinity-purified polyclonal antibodies to placental FR as described under Materials and Methods.

Expression and Membrane-Anchoring of FR-γ. Transient expression of FR-γ in COS-1 cells resulted in relatively low [^{3}H]folic acid binding activity in the cell membrane (Table 2). Treatment with PI-PLC released FR-γ quantitatively from the membranes (Table 2), indicating a GPI membrane anchor for FR-γ. Expression of FR-γ in the transfected COS cell membranes was observed by Western blot analysis using affinity-purified polyclonal anti-placental FR antibody as probe (Figure 4). The protein expressed in COS-1 cells appeared as closely spaced bands of M_r ~32 000 (Figure 4); the multiple bands in the Western blot in all likelihood represent microheterogeneity in the N-linked glycosylation as seen for other FR isoforms (Ratnam et al., 1989). FR-γ was not detected in the cell culture medium from the short-term-transfected COS-1 cells (Figure 4).

Tissue Distribution of FR-γ. Using PCR primers (CML2 and CML4, see Materials and Methods) specific for FR-γ (Figure 5), total RNA samples (0.5 μg) from a number of normal and malignant tissues and established tumor cell lines were analyzed for the presence of the mRNA for FR-γ (Table 3). The sensitivity of the PCR method was enhanced by using a ^{32}P-labeled oligonucleotide primer followed by autoradiography of a polyacrylamide electrophoretic gel of the PCR products (Figure 5). The cDNA for β-actin in each sample was amplified as an internal control (Figure 5). Due to the overabundance of the transcript for β-actin, the primers for its amplification were added after the first five PCR cycles (Material and Methods). As seen in Table 3, the mRNA for FR-γ (or FR-γ') was detected in certain carcinomas (ovarian, cervical, uterine) and in tissue sources of hematopoietic cells such as normal or malignant spleen, bone marrow, and thymus. Furthermore, when PCR primers specific for FR-α (MF1 and M2) and FR-β (MF1 and F1) were used, FR-β but not FR-α was detected in the samples of spleen, bone marrow, and thymus (results not shown). Thus, FR-β and FR-γ were the only receptor isoforms expressed in the normal and leukemic hematopoietic cells in this study. A variety of other normal and malignant tissues tested did not express significant amounts of FR-γ (Table 3). Further studies should reveal the relative levels of FR-γ in various tissues and its cell-type specificity.

The foregoing results clearly show that the high-affinity [^{3}H]folic acid binding protein in normal and leukemic spleen and bone marrow is represented by FR type β, which was one of the receptor isoforms originally identified in placenta, and/or a novel homologous protein (FR-γ). The soluble folate

Table 3: Tissue Distribution of FR-γ[a]

positive[b] cell lines	negative[c] cell lines
ovarian adenocarcinoma (SKOV-3), cervical carcinoma (HT-3), whole embryo (FHS 173We)	nasopharyngeal epidermoid carcinoma (KB), placental choriocarcinoma (JEG-3, JAR), colon adenocarcinoma (HT-29), breast adenocarcinoma (SKBR-3), lung squamous cell carcinoma (Nci H520)
positive tissue explants	**negative tissue explants**
ovarian carcinoma, uterine carcinoma, spleen, thymus, spleen (AML, CML, CLL), bone marrow (AML, CML, ALL)	breast, brain, liver, ovary, kidney, astrocytoma, meningioma, renal cell carcinoma, testicular choriocarcinoma, osteosarcoma, lung adenocarcinoma, Wilm's tumor, uterine sarcoma

[a] The tissues were tested by RNA–PCR analysis for the presence of the mRNA for FR-γ (or FR-γ') as described under Materials and Methods and Figure 3. [b] Tissues in which the mRNA for FR-γ (or FR-γ') was detected. [c] Tissues in which FR-γ (or FR-γ') was not detected.

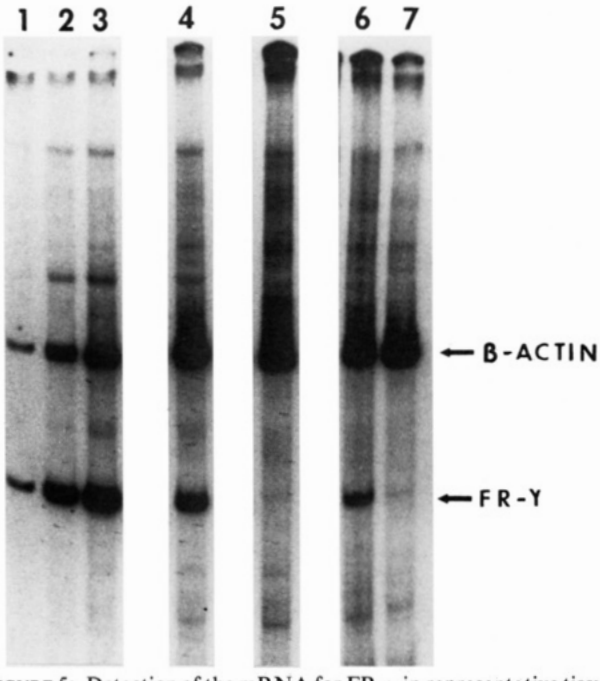

FIGURE 5: Detection of the mRNA for FR-γ in representative tissue samples by (nonquantitative) RNA–PCR analysis. Total RNA prepared for various tissues was analyzed using ^{32}P-labeled primers specific for FR-γ and β-actin as described under Materials and Methods. Arrows indicate the positions of specific PCR products obtained for FR-γ (361 base pairs) or β-actin (661 base pairs). The samples shown in this figure are the following; lanes 1, 2, and 3 (0.005, 0.05, and 0.5 μg, respectively, of total RNA), CML spleen; lane 4, normal spleen (0.5 μg of total RNA); lane 5, meningioma (0.5 μg of total RNA); lane 6, ovary carcinoma (0.5 μg of total RNA); lane 7, KB cells (0.5 μg of total RNA). Because of the abundance of mRNA for β-actin, the primers for β-actin were introduced after the first five PCR cycles (as described under Materials and Methods).

binding proteins (sFBPs) isolated from leukemic spleen (Sadasivan et al., 1986, 1987) are in all likelihood derived from FR-β or FR-γ since (a) FRs have been clearly shown to be the precursors of sFBPs in KB cells and placenta (Luhrs & Soliamy, 1989; Verma et al., 1992; Antony et al., 1989; Elwood et al., 1991) and (b) the soluble and membrane-associated folate binding proteins in leukemic spleen were immunologically cross-reactive (Sadasivan et al., 1986, 1987). Serum sFBP is elevated in cancer and several pathologic states (Rothenberg & da Costa, 1971; Corrocher et al., 1979; Rochman et al., 1985). Molecular characterization of these serum FBPs and identification of their isoform types may enable their exploitation as serum markers. Further investigations should also explore the value of FR-β and FR-γ as cell-surface markers for certain hematopoietic cell types. The structural, functional, and regulatory differences among the FR isoforms may enable their exploitation for differential transport of novel types of anti-folates and for immunotargeting of certain malignant tissues. The physiological significance of alternative splicing of FR-γ is at present unclear, although the resulting truncated polypeptide may be expected to be secreted because it lacks a membrane-anchoring domain.

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