Folate Receptor Type γ Is Primarily a Secretory Protein Due to Lack of an Efficient Signal for Glycosylphosphatidylinositol Modification: Protein Characterization and Cell Type Specificity[†]

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ABSTRACT: A novel isoform of the human folate receptor (FR, type γ) was recently identified in hematopoietic tissues [Shen et al. (1994) *Biochemistry 33*, 1209−1215]. In that report, Cos-1 cells, transiently transfected with the cDNA for FR-γ, produced relatively poor expression of the receptor on the cell surface. In this study, several recombinant Chinese hamster ovary (CHO) cell lines were produced by stable transfection with the cDNA for FR-γ followed by amplification. Similar recombinant CHO cell lines were produced that expressed the glycosylphosphatidylinositol- (GPI-) anchored FR type β and a truncated form of FR type β (FR-$\beta\Delta$), in which the normal carboxyl-terminal signal for GPI anchor attachment was deleted. Both FR-γ- and FR-$\beta\Delta$-expressing CHO cells produced a [^{3}H]folic acid binding protein in the medium with a similar time course over a 24-h period; in contrast to intact FR-β, relatively insignificant amounts of either FR-γ or FR-$\beta\Delta$ were associated with the CHO cell surface and this was unaltered by the absence of serum in the medium. The FR-γ- and FR-$\beta\Delta$-producing CHO cells did not differ significantly in intracellular FR levels. Furthermore, the mRNA level for FR-γ did not exceed that for FR-$\beta\Delta$. When deglycosylated with hydrogen fluoride, both FR-γ and FR-$\beta\Delta$ showed similar apparent molecular weights on Western blots as predicted for the intact polypeptides. Chimeric constructs of FR-γ and FR-β cDNAs resulting in interchanging of the unconserved, carboxyl-terminal segments of the two proteins were expressed in human 293 fibroblasts; the results indicate that FR-γ is primarily a secretory protein due to a divergence of its carboxyl-terminal amino acid sequence from the other members of the FR gene family, resulting in an inefficient signal for GPI modification. The relative affinities of the secreted FR-γ for folic acid and the diastereoisomers of reduced folate compounds were in the order folic acid ($K_D = 0.42 \times 10^{-9}$ M) > (6*S*)/(6*R*)-N^5-methyltetrahydrofolate > (6*S*)/(6*R*)-N^5-formyltetrahydrofolate. Evidence is presented that expression of the secreted full-length FR-γ is restricted to certain hematopoietic cell types. FR-γ is, therefore, a potential serum marker for certain malignancies of hematopoietic tissues and also a candidate protein for the high-affinity serum folate binder that is elevated during folate deficiency.

The folate receptor (FR) is an N-glycosylated protein consisting of a single polypeptide of molecular weight ~28 000 Da (Antony, 1992). The receptor binds folate with a 1:1 stoichiometry (Antony, 1992). The initial report of a glycosylphosphatidylinositol (GPI) membrane anchor for FR (Lacey et al., 1989) was confirmed by others (Luhrs & Slomiany, 1989; Verma et al., 1992). It has been proposed that the soluble folate binding protein (sFBP) in the serum and in tissue culture medium is derived from its membrane-bound counterpart either by proteolysis or by the action of serum phospholipase (Luhrs & Slomiany, 1989; Verma et al., 1992; Antony et al., 1989; Elwood et al., 1991). Recent studies have shown that malignant tissues have elevated expression of FR suggesting its potential exploitation for the diagnosis or targeting of malignant tumors with antibodies or alternately for the transport of potent, novel, chemotherapeutic antifolate drugs (Campbell et al., 1991; Coney et al., 1991, Weitman et al., 1992, Ross et al., 1994).

Multiple isoforms of FR that are 70−80% identical have been identified in human and mouse tissues by molecular cloning (Ratnam et al., 1989; Lacey et al., 1989; Elwood, 1989; Brigle et al., 1992; Shen et al., 1994). The receptor isoforms in the same species differ in their affinities and stereospecificities for folate compounds and antifolates (Wang et al., 1992; Brigle et al., 1994). The human FR isoforms are tissue-specific. In general, FR-α is specific for primary cultures of epithelial cells and is elevated in explants of various carcinomas while FR-β is elevated in malignant tumors of nonepithelial origin (Ross et al., 1994). We recently identified a third human FR isoform (type γ) and a corresponding truncated form (FR-γ') (Shen et al., 1994). FR-γ and FR-γ' appear to be quite specific for hematopoietic tissues (Shen et al., 1994). The functional differences among the FR isoforms and their tissue specificities further render them attractive targets for cancer diagnosis and therapy.

We observed that when the cDNA for FR-γ was expressed transiently in Cos-1 cells, the increase in cell surface associated [^{3}H]folic acid binding protein was very low (<2%) compared to transfections with the cDNAs for other FR isoforms (Shen et al., 1994). In addition, significant amounts of FR-γ were not detected in the Cos-1 cell culture medium in the very short-term transfection experiments using FR-β-specific antibodies. Furthermore, the very high concentrations of folate in the Cos-1 cell culture medium interfered with direct quantitation of [^{3}H]folic acid binding protein in the medium. In this study, we further investigated those ob-

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servations by establishing stably transfected Chinese hamster ovary (CHO) cells with amplified expression of FR-γ.

The unconserved carboxyl-terminal segments of FR-α and FR-β are relatively hydrophobic and are characteristic of the proteolytically processed signal peptide for GPI attachment in the endoplasmic reticulum (Caras, 1991). Inspection of the amino acid sequence of the corresponding carboxyl-terminal segment of FR-γ revealed that it is shorter and less hydrophobic than those of FR-α and FR-β, although similar features occur in the GPI signal sequences of several proteins (Sikorav et al., 1987; Boothroyd, 1985; Seki et al., 1985; Millan, 1986). Herein, we test the hypothesis that FR-γ is poorly expressed on the cell surface because it lacks an efficient signal for GPI anchor attachment. Since the inability to process a GPI signal peptide could result in retention and degradation of the protein in intracellular membrane compartments (Field et al., 1994), we examined the fate of FR-γ in the recombinant CHO cells. For controls in these studies, we produced stable recombinant CHO cells with amplified expression of FR type β and also FR-β in which the carboxyl-terminal hydrophobic segment was deleted (FR-$\beta\Delta$) and, therefore, would be expected to be secreted in the medium. In addition, chimeric constructs of FR-β and FR-γ were produced and transiently expressed in human 293 fibroblasts in order to test the possible role of the carboxyl-terminal portion of FR-γ in determining its final destination. In these studies, cells were grown in low-folate (physiologic) medium in order to enable quantitative removal of folate from the medium prior to [^{3}H]folic acid binding assays. The folate binding properties of FR-γ were characterized. Finally, possible cell type specificity of the full-length FR-γ (243 residues) vs the truncated (104 residue) FR-γ' polypeptide was investigated.

MATERIALS AND METHODS

Cell Culture. All cell culture media were supplemented with 50 000 units of penicillin, 50 000 μg of streptomycin, and 146 mg of glutamine (Gibco-BRL, Grand Island, NY) per 500 mL of medium. Cells were purchased from American Type Culture Collection (Rockville, MD) unless otherwise indicated. Tissue culture media were purchased from Irvine Scientific (Santa Anna, CA) unless otherwise indicated. Human 293 fibroblasts were grown in Eagle's minimal essential medium (MEM) with 10% fetal bovine serum (FBS, Gibco-BRL). CHO cells deficient in dihydrofolate reductase (CHO-dhfr$^-$ cells) were grown in Dulbecco's modified Eagle's medium (DMEM) and 10% FBS, supplmented with 75 mg of L-proline and 5 mg each of adenosine, deoxyadenosine, and thymidine (Sigma Chemical Co., St. Louis, MO) per 500 mL of medium. CHO cell transfectants containing the neomycin resistance gene were grown in the above-described medium containing 1 mg/mL geneticin (active concentration: 0.7 mg/ml; Gibco-BRL). Transfectants containing both the neomycin resistance gene and the dihydrofolate reductase gene were grown in the above-described medium with various concentrations of methotrexate (MTX; Sigma Chemical Co.) instead of nucleosides. CHO cell transfectants were alternately grown in folate-free RPMI 1640 supplemented with all the appropriate components, as described above. HL-60 cells were grown in RPMI 1640 medium with 20% FBS. For growth in serum-free conditions, the parental and recombinant CHO cells were transferred to McCoy's 5A medium (Sigma) supplemented with human transferrin (4 μg/mL), bovine pancreas insulin (20 μg/mL), and epidermal growth factor (50 ng/mL).

Tissues. Spleen samples, leukocyte fractions of bone marrow aspirates, and normal and malignant human tissues were purchased from the Cooperative Human Tissue Network (CHTN), Columbus, OH.

Construction of Plasmids and Transfections. The cDNA for FR-γ (nucleotide 6−850) was placed between *Hin*dIII and *Xba*I sites in the polylinker region of the expression vector pcDNAIneo (Invitrogen) and the resulting plasmid (FR-γ-pcDNAIneo) was amplified in *Escherichia coli* MC1061/p3. Transfections into CHO cells were carried out using lipofectamine (Gibco-BRL) according to the manufacturer's protocol.

Production of Recombinant CHO Cells. CHO-dhfr$^-$ cells were transfected with FR-γ-pcDNAIneo. Transfectants expressing the neomycin resistance gene were selected by growing in 1 mg of geneticin/mL (active concentration 0.7 mg/mL) for 20 days. The cells were then transfected with a second plasmid (pSV2) containing the dhfr gene (pSV2-dhfr). This plasmid has a DNA sequence that is in part homologous to that of pCDNAIneo. The genes in the transfected cells were amplified by stepwise selection in methotrexate (10 nM−2 μM). Stable CHO cell transfectants were plated out at a dilution of 200 cells/15-cm plate and grown for 14 days. Isolated clones were picked using cloning rings and further subcloned by the same procedure.

Northern Blot Analysis. Total RNA (15 μg) from each sample was electrophoresed through a 1.0% agarose gel and blotted onto a nylon membrane essentially as described (Ross et al., 1994). An oligonucleotide, 58 bases long, with the sequence 5′-GCCAGCACCAGCCAGGAGCTGCACAAG-GACACCTCCCGCCTGTACAACTTTAACTGGG-3′ was labeled with ^{32}P, and hybridization of the Northern blot was carried out as described (Ross et al., 1994). This oligonucleotide spans a region perfectly conserved between FR-β and FR-γ and should therefore bind to the two transcripts with equal affinity.

The filter was erased by pouring boiling 0.1% SDS over it and allowing the solution to come to room temperature. Removal of the probe was verified by autoradiography. The filter was rehybridized with a probe for detecting β-actin mRNA. The template for the β-actin probe was prepared by amplifying a 660 base pair β-actin cDNA fragment by polymerase chain reaction (PCR). The PCR primers were purchased from Stratagene and have the following sequences:

sense
5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′

antisense
5′-CTAGAAGCATTGCGGTGGACGATGGAGGG-3′

The probe was labeled and the hybridization was carried out as described above.

Production of Chimeric cDNA Constructs and Transient Expression in 293 Cells. Chimeric constructs of FR-β and FR-γ were made by exchanging their unconserved carboxyl-terminal hydrophobic peptides. A common *Not*I site (at nucleotide 861 in FR-β and at nucleotide 625 in FR-γ) in the two cDNAs was used for this purpose. The cDNAs for FR-β and FR-γ and the chimeric FR-β/γ and FRγ/β cDNAs were placed in the polylinker region of the vector pcDNAI and used to transfect human 293 cells using lipofectamine (Gibco−BRL) according to the manufacturer's protocol. The cells were harvested 48 h after transfection. Untransfected

cells and cells transfected with the vector without a cDNA insert were used as negative controls in all experiments.

[^{3}H]Folic Acid Binding Assays. [^{3}H]Folic acid binding assays were performed on both cell-surface and soluble FR. In the binding assay of cell-surface FR, cells (3×10^6) in the logarithmic phase of growth or 48 h after transfection were washed in a 35-mm tissue culture dish at 4 °C, once with Hanks' balanced salt solution (HBSS; 1 mL), twice with acid saline (10 mM sodium acetate, pH 3.5, and 150 mM NaCl; 1 mL), and once again with HBSS (1 mL). The cells were then incubated with 2 pmol of [^{3}H]folic acid (Moravek) in 1 mL of HBSS at 4 °C for 1 h. After the cells were washed at 4 °C, once with 1 mL of HBSS, cell-surface bound [^{3}H]folic acid was recovered in 0.5 mL of acid saline and subjected to liquid scintillation counting. For the binding assay of soluble FR, cell culture medium (folate-free RPMI 1640) was collected and the pH was lowered to 3.5 by the addition of $^1/_{10}$ volume of 100 mM sodium acetate, pH 2.5, at 4 °C. Simultaneously Triton X-100 was added to a final concentration of 1%. Then an equal volume of an ice-cold suspension of Norit A charcoal (80 mg/mL) in 10 mM sodium acetate, pH 3.0, and 1% Triton X-100 was added to remove endogenous folic acid. The pH of the medium was adjusted back to 7.5 using a pretitered amount of NaOH. Aliquots of the treated medium were taken for the assay in a final volume of 0.5 mL. The samples were incubated with 2 pmol of [^{3}H]folic acid at 37 °C for 2 h, and [^{3}H]folic acid binding protein was assayed by a charcoal binding assay as described (Wang et al., 1992). Intracellular folic acid binding protein was estimated by the same procedure used to estimate the protein in the medium except that whole cells were first dissolved in 10 mM sodium phosphate, pH 7.5/150 mM NaCl/1% Triton X-100 before acidification with sodium acetate.

Phospholipase C Cleavage. Cells (3×10^6) in the logarithmic phase of growth were washed at 4 °C, once with 1 mL of HBSS, twice with 1 mL of acid saline, and once again with 1 mL of HBSS. The cells were treated with phospholipase C (1.5 units) (Boehringer Mannheim, Indianapolis, IN) in 1 mL of 25 mM Tris-HCl, pH 7.5, 250 mM sucrose, 10 mM glucose, and 1% bovine serum albumin at 37 °C for 2 h. Following this, [^{3}H]folic acid binding assay was carried out as described above.

Antiserum and Affinity-Purified Antibodies. A rabbit antiserum to purified FR from placenta was obtained as described (Ratnam et al., 1989). Purified placental FR was immobilized on AH-Sepharose (Pharmacia) and the fraction of the IgG in the rabbit antiserum that specifically bound to the immobilized FR was purified as described (Ratnam et al., 1989).

Western Blot Analysis. Western blot analysis of protein samples from cell culture medium was carried out using affinity-purified rabbit antibody to purified human placental FR as the primary antibody and alkaline phosphatase conjugated goat anti-rabbit antibody (Promega) as described previously (Wang et al., 1992).

Deglycosylation. Proteins from culture medium (200 μL) were lyophilized and deglycosylated by treatment with anhydrous HF as described (Sojar & Bahl, 1987).

K_i Determinations. K_i values for the inhibition of [^{3}H]-folic acid binding to FR-γ by folic acid and reduced folate coenzymes were determined as described (Wang et al., 1992).

Identification of Transcripts for FR-γ and FR-γ'. Total RNA was prepared and reverse-transcribed, and a 735 or 733 base pair fragment of the cDNA for either FR-γ or FR-γ', respectively, was amplified by polymerase chain reaction (PCR) using a ^{32}P-labeled "upstream" oligonucleotide primer and an unlabeled "downstream" primer as described previously (Shen et al., 1994). FR-γ' contains a 2-base deletion resulting in an in-frame stop codon within this cDNA fragment. The 2-base deletion in FR-γ' fortuitously generates a *Dde*I restriction site that is absent in FR-γ. A second *Dde*I restriction site occurs downstream of this site in both FR-γ and FR-γ' within the PCR-amplified cDNA fragment. Digestion of the PCR product with *Dde*I should give rise to ^{32}P-labeled end fragments of 484 and 316 base pairs, respectively, for FR-γ and FR-γ'. The PCR product was subjected to *Dde*I digestion. The ^{32}P-labeled cDNA fragments were then analyzed by polyacrylamide gel electrophoresis and autoradiography as described (Shen et al., 1994) with the inclusion of ^{32}P-end-labeled molecular size standards (1-kb ladder; Gibco−BRL). The oligonucleotide sequences used for PCR amplification were (5′ to 3′) GGACATGGC-CTGGCAGATGATGC (^{32}P-labeled upstream primer) and CAGGAATCAATAATCCCACGAGACGG (unlabeled downstream primer).

RESULTS AND DISCUSSION

In contrast to the highly expressed GPI-anchored FR isoforms, α and β, the cell surface expression of FR-γ in transiently transfected Cos-1 cells (Shen et al., 1994) is poor and could be explained by any one or a combination of the following broad reasons: (i) poor expression *per se* of FR-γ in Cos-1 cells due to several possible factors, including rapid protein degradation; (ii) trapping of the expressed protein in intracellular compartments as observed for some proteins with defective signal peptides for GPI anchor attachment; (iii) secretion of the expressed protein into the medium; (iv) rapid clearance from the cell surface due to proteolysis or serum phospholipase action on a putative GPI-anchored receptor; and (v) production of a cell surface associated FR with a poor capacity for binding [^{3}H]folic acid, leading to an apparent low expression.

Fate of FR-γ Expressed in Recombinant CHO Cells. In order to address the above possibilities, we first established stably transfected CHO cells that gave amplified expression of FR-γ, the GPI-anchored FR-β, and FR-$\beta\Delta$ in which the carboxyl-terminal 19 hydrophobic amino acids of FR-β were deleted. The deletion mutant of FR-β lacks a hydrophobic carboxyl-terminal signal peptide for GPI anchor attachment and would therefore be expected to be secreted, since the constitutive secretory pathway is believed to operate by default in the absence of protein targeting signals. Table 1 summarizes data on the binding of [^{3}H]folic acid on the cell surface and in the culture media of five CHO cell clones transfected with FR-γ together with similar recombinant cells expressing FR-β and FR-$\beta\Delta$. As expected, FR-β was primarily cell surface anchored while FR-$\beta\Delta$ was secreted into the medium. In all of the CHO cell clones expressing FR-γ, the [^{3}H]folate binding was detected primarily in the cell culture media, similar to FR-$\beta\Delta$. The time course of the appearance of [^{3}H]folic acid binding protein in the media showed a similar pattern for confluent cultures of FR-$\beta\Delta$ and FR-γ expressing CHO cells over a 24-h period, in contrast to FR-β (Figure 1), indicating similar rates of synthesis and secretion of FR-$\beta\Delta$ and FR-γ.

The levels of FR-$\beta\Delta$ and FR-γ in intermediate intracellular compartments were also similar (Table 1), indicating that in

Table 1: Distribution of [^{3}H]Folic Acid Binding Protein Produced by Recombinant CHO Cells[a]

CHO cell clone	[^{3}H]folic acid bound (pmol/10^7 cells)		
	cell surface	24-h culture medium	intracellular
CHO-β	12.40	3.5	ND
CHO-$\beta\Delta$	0	31.4	22.0
CHO-γ 1	0.14	25.7	ND
CHO-γ 2	0.05	21.5	ND
CHO-γ 3	0.13	27.1	ND
CHO-γ 4	0.05	28.0	ND
CHO-γ 5	0.06	30.5	15.2
CHO-dhfr$^-$	0	0	0

[a] Standard error <10%. The assays were repeated at least four times, as described under Materials and Methods. CHO-β, CHO-$\beta\Delta$, and CHO-γ denote recombinant CHO cells expressing FR-β, FR-$\beta\Delta$ (with truncated carboxyl terminus), and FR-γ, respectively. The numbers next to CHO-γ denote individual clones. ND indicates not determined. Although the actual assays were carried out on 3×10^6 cells as described under Materials and Methods, the values were normalized to 10^7 cells in this table so as not to exceed two decimal places. Confluent cell cultures were used in this experiment.

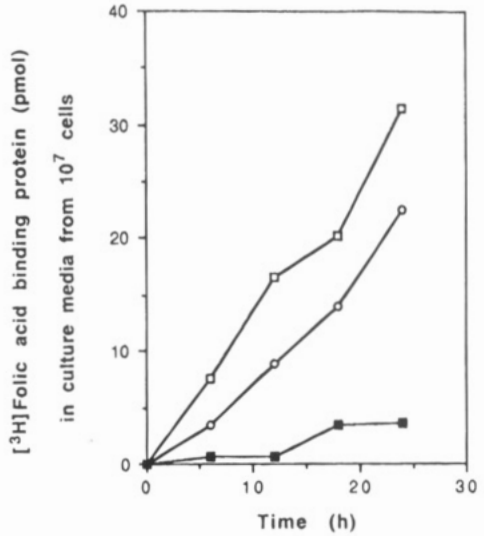

FIGURE 1: Time course of appearance of [^{3}H]folic acid binding protein in the media of confluent cultures of recombinant CHO cells expressing FR-β (■), FR-$\beta\Delta$ (□), and FR-γ (CHO-γ, clone 4) (○). Parental CHO-dhfr$^-$ cells were used as the negative control. The culture medium used was folate-free RPMI 1640 containing 10% FBS. At the indicated times, aliquots of the media were treated to remove endogenous folate and assayed for folate binding protein as described under Materials and Methods. The assays were repeated three times and gave a standard error <10%. The results were normalized according to cell number in confluent cultures.

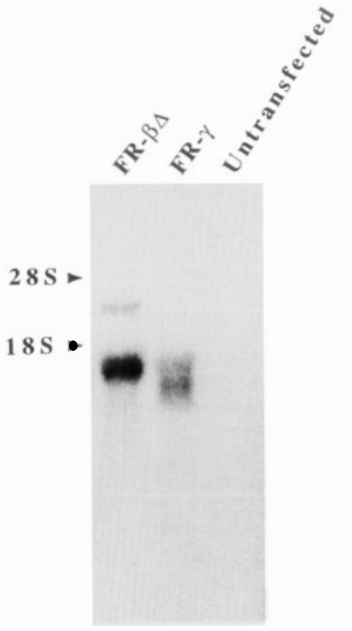

FIGURE 2: Northern blot analysis of total RNA from untransfected CHO cells and the cells transfected with the cDNA for FR-$\beta\Delta$ or FR-γ (clone 5). The filter was probed with an oligonucleotide spanning a region identical between FR-$\beta\Delta$ and FR-γ as described in Materials and Methods. The position of the 18S and 28S ribosomal RNA bands are indicated. The integrity of the RNA samples was confirmed by visualization of the rRNA bands by ethidium bromide staining and also by probing the blot for the mRNA for β-actin as described under Materials and Methods.

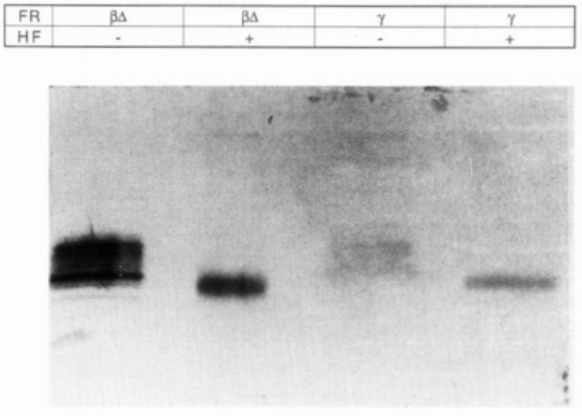

FIGURE 3: Western blot analysis of cell culture media from recombinant CHO cells expressing FR-$\beta\Delta$ or FR-γ before and after deglycosylation with HF. Culture media (200 μL) were dialyzed against 2.5 mM sodium phosphate, pH 7.5, and freeze-dried. HF treatment and Western blot analysis was carried out as described under Materials and Methods.

contrast to certain proteins with defective GPI signals (Field et al., 1994), the divergent carboxyl-terminal region of FR-γ did not result in intracellular retention of the protein. Futhermore, estimation of relative mRNA levels by Northern blot analysis (Figure 2) did not show higher transcript levels for FR-γ compared to FR-$\beta\Delta$, suggesting that FR-γ is not synthesized at significantly higher levels than FR-$\beta\Delta$. These results indicate that it is unlikely that the FR-γ polypeptide is produced and degraded intracellularly at an extraordinarily high rate compared to other FR isoforms.

The predicted polypeptide molecular weights of FR-γ and FR-$\beta\Delta$ are 25 154 and 24 962 Da, respectively. Both the polypeptides possess predicted N-linked glycosylation sites that give rise to diffuse or multiple bands on Western blots (Figure 3). Upon deglycosylation with hydrogen fluoride, both proteins recovered from the recombinant CHO cell culture media gave bands of similar apparent molecular weights (M_r ~25 000) on Western blots (Figure 3). This result indicates that the occurrence of FR-γ in the cell culture media is not the result of quantitative and selective proteolysis of a large peptide fragment from a putative GPI-anchored FR-γ. From the discussion below, if such a proteolytic cleavage site exists, it must occur 19−32 amino acids upstream of the carboxyl terminus, resulting in a polypeptide that would migrate appreciably faster than FR-$\beta\Delta$ by Western blot analysis.

When the recombinant CHO cells expressing FR-β, FR-$\beta\Delta$, or FR-γ were grown briefly in serum-free medium as described under Materials and Methods, the results in Table 1 were essentially unaltered (results not shown). This observation rules out the possibility that serum phospholipase may quantitatively release a putative GPI-anchored FR-γ into the medium.

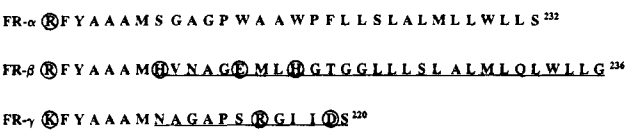

FIGURE 4: Aligned amino acid sequences of carboxyl-terminal segments of FR-α, FR-β, and FR-γ. Charged residues are circled. The numbers represent amino acid residue numbers in the mature polypeptides. The underlined peptides in FR-β and FR-γ were interchanged in the construction of FR-β/FR-γ chimeras.

Table 2: Transient Expression of FR-β, FR-γ, and the Receptor Chimeras in 293 Human Fibroblasts[a]

transfected cDNA	[^{3}H]folic acid bound to cell surface (pmol/10^7 cells)	
	−PI-PLC	+PI-PLC
FR-β	4.10	0.45
FR-γ	0.03	0.02
FR-β/γ	0.05	0.03
FR-γ/β	15.80	1.00

[a] Standard error <10%. The assays were repeated at least four times, as described under Materials and Methods. FR-β/γ and FR-γ/β denote chimeric constructs with the carboxyl terminus of FR-γ and FR-β, respectively. −PI−PLC and +PI-PLC indicate cells that were untreated and treated with PI-PLC, respectively, as described under Materials and Methods.

Structural Basis for the Secretion of FR-γ. In order to understand the physical basis for the secretory nature of FR-γ vs the GPI-anchored FR-α and FR-β, we inspected the carboxyl-terminal amino acid sequences of the three proteins which should contain the signal sequence for GPI anchor attachment (Figure 4). The signal for GPI anchor attachment is typically characterized by an uncharged carboxyl terminal region of 11−20 amino acids, the site of cleavage/GPI attachment and a spacer of 8−12 amino acids that separates the modification site from the relatively hydrophobic sequence (Coyne et al., 1993; Caras, 1991). The carboxyl-terminal peptides of the three FR isoforms are unconserved in contrast to their upstream amino acid sequence. Both FR-α and FR-β contain the structural elements commonly observed for a GPI signal peptide (Figure 4). In FR-γ, on the other hand, the 18 carboxyl-terminal amino acids are mostly uncharged but are interrupted by charged residues at positions 2 and 6 from the carboxyl terminus resulting in the longest contiguous stretch of 12 uncharged amino acids (Figure 4). Such a weakly hydrophobic sequence with charged residues near the carboxyl terminus may still serve as a GPI modification signal as exemplified by *Drosophila* acetylcholinesterase (Sikorav et al., 1987), group 1 MITat 1.1BC (Boothroyd, 1985), rat and human Thy-1 (Seki et al., 1985), and placental alkaline phosphatase (Millan, 1986). If this were true for FR-γ, the GPI attachment site would extend into a highly conserved region of FR-γ.

In light of the above reasoning, chimeric constructs of FR-γ and FR-β were produced by exchanging the unconserved portions of their carboxyl-terminal regions (beginning with histidine in FR-β and asparagine in FR-γ; Figure 4). As seen in Table 2, FR-β and FR-γ with the FR-β carboxyl terminus were both highly expressed on the cell surface and were GPI-anchored. In contrast, FR-γ and the second chimera were expressed very poorly on the cell membrane (Table 2); these two proteins were quantitatively recovered in the cell culture media and bound [^{3}H]folic acid (49.4 and 6.2 pmol, respectively, per 10^7 cells). This result clearly indicates that the secretory nature of FR-γ is due to the divergence of its unconserved carboxyl-terminal peptide, which cannot serve as an efficient signal for GPI modification. Unlike other proteins with defective signals for GPI attachment, that are retained and degraded in intracellular compartments (Field et al., 1994), FR-γ is efficiently secreted, perhaps because of the relative hydrophilicity conferred by the two charged residues (arginine and aspartic acid; Figure 4) in its carboxyl-terminal segment.

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

compound	K_i ($\times 10^{-9}$ M)
folic acid	0.42
(6*S*)-N^5-methyltetrahydrofolate	2.20
(6*R*)-N^5-methyltetrahydrofolate	2.40
(6*S*)-N^5-formyltetrahydrofolate	20.60
(6*R*)-N^5-formyltetrahydrofolate	15.50

[a] Standard error <10%. The K_i values were determined as described previously (Wang et al., 1992).

Affinities of Folic Acid and Reduced Folate Compounds for FR-γ. A major functional difference between FR-α and FR-β is their different affinities for folate compounds and their opposite stereospecificities (6*S* and 6*R* diastereoisomers for FR-α and FR-β, respectively) for reduced folate coenzymes (Wang et al., 1992). Because of the relatively low affinity of FR-β for the physiologic circulating 6*S* diastereoisomer of N^5-methyltetrahydrofolate (Wang et al., 1992), a role for this receptor isoform that is expressed in tissues of nonepithelial origin (Ross et al., 1994) in physiologic folate transport, is suspect. It was therefore of interest to examine the folate-binding characteristics of the secreted FR-γ. Table 3 summarizes the affinities of folic acid and the 6*S* and 6*R* diastereoisomers of N^5-methyltetrahydrofolate and N^5-formyltetrahydrofolate for FR-γ in terms of their K_i values for the competitive inhibition of binding of [^{3}H]folic acid. In contrast to other FR isoforms, FR-γ does not display a significant stereospecificity for reduced folate coenzymes, indicating differences in its mode of binding folate compounds. It does, however, display a high affinity for folic acid ($K_i = 0.42 \times 10^{-9}$ M) and for the circulating (6*S*)-N^5-methyltetrahydrofolate ($K_i = 2.2 \times 10^{-9}$ M).

Cell Type Specificity of FR-γ and FR-γ′. The predicted amino acid sequence of FR-γ′ differs from that of FR-γ due to a deletion of 139 amino acid residues resulting in a 104-residue amino-terminal polypeptide (Shen et al., 1994). Previously, the mRNA for either FR-γ or FR-γ′ (not distinguished) was detected in normal spleen, thymus, and bone marrow and in the bone marrow of patients with chronic myelogenous leukemia (CML), acute myelogenous leukemia (AML), and acute lymphocytic leukemia (ALL); a variety of nonhematopoietic tissues did not express the transcripts except some carcinoma explants (Shen et al., 1994).

In this study, we developed a method for distinguishing between transcripts for FR-γ and FR-γ′ by exploiting a fortuitous restriction site for *Dde*I that is introduced due to the occurrence of a 2 base pair deletion that results in a premature stop codon in the cDNA for FR-γ′, as described under Materials and Methods. When total RNA from various tissues was reverse-transcribed and amplified by PCR using a ^{32}P-labeled upstream oligonucleotide, both FR-γ and FR-γ′ transcripts gave an expected cDNA fragment of 735/733 base pairs (Figure 5). When digested with *Dde*I, the PCR product gave an expected ^{32}P-labeled end fragment of 484 base pairs for FR-γ and a radioactive 316 base pair fragment for FR-γ′ (Figure 5). Normal spleen, HL60 promyelocytic leukemia cells, and bone marrow samples of myeloid (CML and AML) leukemias predominantly expressed FR-γ′ (Figure 5 and results not shown). Among three samples of bone

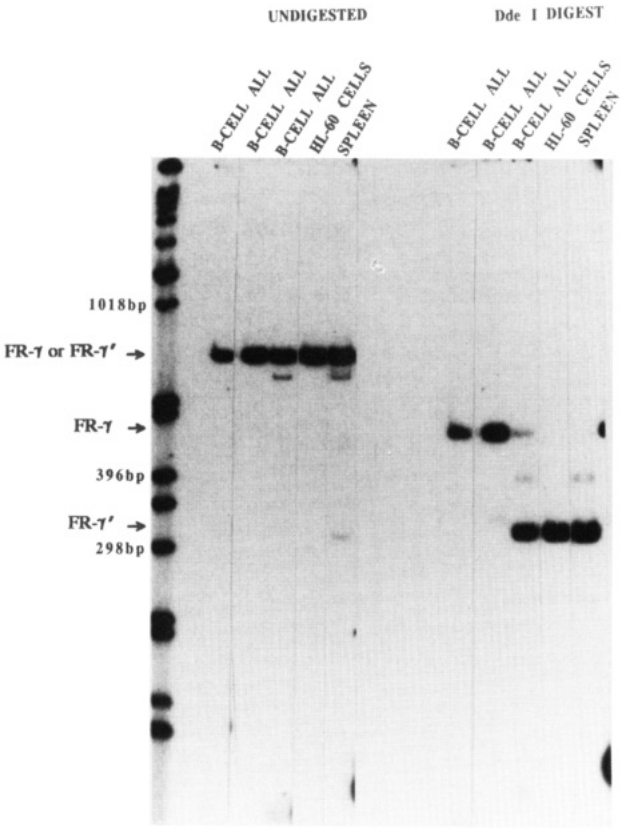

FIGURE 5: Autoradiograph showing cell type specificity of mRNA for FR-γ and FR-γ'. Total RNA from the sources indicated was reverse-transcribed and amplified using a radiolabeled PCR primer and electrophoresed on a 6% polyacrylamide gel as described under Materials and Methods. The full-length PCR product representing FR-γ or FR-γ' will be 735 or 733 base pairs, respectively (the small size difference is undetectable). When the PCR product is digested with the restriction enzyme *Dde*I, the resulting labeled fragment sizes will be 484 and 316 base pairs for FR-γ and FR-γ', respectively. The extreme left lane contains a radiolabeled 1-kb ladder of molecular size standards (Gibco-BRL), with some representative fragment sizes indicated.

marrow from B-cell ALL that were tested, two produced FR-γ and the third ALL sample showed a relatively greater proportion of FR-γ' (Figure 5). Regardless of whether the latter observation reflects different proportions of leukemic vs normal cells in the ALL bone marrow samples or variations in leukemic subtypes, the results clearly indicate that the expression of the secreted full-length FR-γ is restricted to certain hematopoietic cell types.

FR-γ in Physiologic and Clinical Perspective. A soluble high-affinity folate-binding protein (sFBP) has been identified in the culture media of cells (e.g., KB cells) expressing high levels of the membrane-anchored FR and in certain biological fluids, i.e., milk (Svendsen et al., 1982), umbilical cord serum (Kamen & Caston, 1975), and the serum of folate-deficient individuals (Waxman & Schreiber, 1973). An obvious function of sFBP *in vivo* is to ensure the stability and bioavailability of (6*S*)-N^5-methyltetrahydrofolate (Tani & Iwai, 1984). It has been shown in cell cultures that the predominantly membrane-anchored FR is the precursor of a relatively small proportion of sFBP, which is produced either by proteolysis (Antony et al., 1989; Elwood et al., 1991; Verma et al., 1992) or due to the action of serum phospholipase (Luhrs & Slomiany, 1989; Verma et al., 1992). The milk sFBP corresponds to FR-α in the receptor-rich mammary epithelium. However, the origin of sFBP *in vivo* in serum is unknown. FR-γ, which is quantitatively secreted and has a high affinity for the circulating form of folate, is a good candidate for the sFBP that is observed at very low levels in normal serum and elevated in folate deficient states [reviewed in Ratnam and Freisheim (1990)]. The observed cell type specificity of expression of the full-length FR-γ in hematopoietic tissues, coupled with its secretory nature, may render this FR isoform a useful serum marker for certain malignant subtypes of lymphoid or other cells. Further detailed studies of specificities of expression of FR-γ, in respect to various differentiation stages in hematopoiesis, are required in order to identify those malignancies.

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