Identification of Amino Acid Residues that Determine the Differential Ligand Specificities of Folate Receptors α and β[†]

Feng Shen, Xuan Zheng, Jianyong Wang, and Manohar Ratnam*

Department of Biochemistry & Molecular Biology, Medical College of Ohio, P.O. Box 10008, Toledo, Ohio 43699-0008

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ABSTRACT: The homologous folate receptor (FR) types α and β from both human and murine sources have opposite stereospecificities for reduced folate coenzymes and different affinities for a variety of (anti)folate compounds. The present study identifies the critical amino acid sequence divergence underlying functional differences between FR-α and FR-β. Chimeric constructs of the cDNAs encoding human FR-α and FR-β were expressed in human 293 fibroblasts. The resulting membrane associated proteins were characterized in terms of their ability to bind [^{3}H]folic acid and their relative affinities for the (6*S*) and (6*R*) diastereoisomers of N^5-methyltetrahydrofolate. Substitution of the amino-terminal portion (residues 1−92) in the mature FR-α polypeptide with the corresponding segment of FR-β resulted in folate binding characteristics similar to FR-β. Next, a series of chimeric constructs were generated, involving substitution of progressively shorter segments within residues 1−92 in FR-α with the corresponding peptides of FR-β. In this fashion, it was determined that the alanine residue at position 49 in FR-α was critical for its functional divergence from FR-β, since substitution at this position with Leu (the corresponding residue in FR-β) resulted in the folate binding characteristics of FR-β. Reciprocal substitution in FR-β with peptide 1−92 of FR-α resulted in poor expression of a [^{3}H]folic acid binding protein. By analysis of chimeric constructs, the poor [^{3}H]folic acid binding of the FR-$\alpha_{1-92}/\beta_{93-237}$ chimera could be attributed to interference of a short segment from FR-α in the vicinity of Ala 49 (peptide 39−59) with proper folding of the chimera. Conversion of the ligand binding properties of FR-β to those of FR-α required the reciprocal mutation of Leu 49 to Ala, but in addition, substitution of one or more residues downstream of amino acid 92 of FR-β with the corresponding residues in FR-α was essential. The homologous murine FR types α and β, which are functionally analogous to the human receptor isoforms, also contain a similar Ala vs Leu substitution. These results indicate that steric/hydrophobic effects of the side chains of Leu vs Ala at position 49 will critically modulate the affinities and stereospecificities of FR isoforms for folate compounds. Furthermore, additional amino acid sequence divergence at one or more positions downstream of residue 92 in FR-α is also an essential determinant of the unique functional characteristics of this receptor isoform.

Folate receptors belong to a class of proteins which bind folic acid with a high affinity (K_D < 1 nM) [reviewed in Antony (1992)]. The mature proteins are single polypeptides of 220−237 amino acid residues and are variably N-glycosylated. Three FR isoforms have been identified and their cDNAs have been characterized; the cDNA for FR-α was isolated by three groups from placenta, KB cells, and CaCo-2 cells (Elwood, 1989; Sadasivan & Rothenberg, 1989; Lacey et al., 1989); the cDNA for FR-β was isolated from placenta (Ratnam et al., 1989); the cDNA for FR-γ and a corresponding truncated form, FR-γ', were recently identified in malignant hematopoietic cells (Shen et al., 1994). The receptor isoforms are approximately 70 percent identical in amino acid sequence. FR-α and FR-β are attached to the cell surface by a glycosyl-phosphatidylinositol (GPI) anchor (Lacey et al., 1989; Luhrs & Slomiany, 1989; Verma et al., 1992; Yan & Ratnam, 1995), while FR-γ is constitutively secreted due to the lack of an efficient signal for GPI modification (Shen et al., 1995). The FR isoforms are expressed in a tissue-specific manner and are frequently upregulated in malignant tissues. In general, FR-α is specific for certain epithelial cells and is greatly elevated in several malignant tissues of epithelial origin, e.g., ovarian carcinoma and uterine carcinoma (Campbell et al., 1991; Coney et al., 1991; Weitman et al., 1992; Ross et al., 1994). Most normal tissues contain very low or moderate (spleen, thymus) levels of FR-β, which is also elevated in several malignancies of non-epithelial origin (Ross et al., 1994). FR-γ and FR-γ' are specific for hematopoietic tissues (Shen et al., 1994, 1995). FR-α and FR-β are functionally distinct. The receptor isoforms from both human (Wang et al., 1992) and murine (Brigle et al., 1991) sources have opposite stereospecificities for reduced folate coenzymes, with FR-α having a relatively high affinity for the physiologic (6*S*) diastereoisomer. The FR isoforms also have different affinities for antifolates (Wang et al., 1992; Brigle et al., 1994). There is abundant kinetic evidence for the cellular transport of folate and antifolate compounds by FR (Antony et al., 1985; Kamen & Capdevila, 1986; Kane et al., 1986; Kamen et al., 1988; Jansen et al., 1989a,b). The uptake occurs by an endocytic process (Kamen et al., 1988, 1989; Rothberg et al., 1990; Leamon & Low, 1991; Birn et al., 1993; Turek et al., 1993).

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* To whom correspondence should be addressed.

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Although in certain cultured cells FR is required for folate uptake (Matsue et al., 1992; Luhrs et al., 1992), physiologic reduced folate coenzyme uptake appears to be mediated by a universally present anion carrier which is an integral membrane protein termed the classical reduced folate carrier (RFC) [reviewed in Ratnam and Freisheim (1990)]. In contrast to FR, the RFC does not bind folic acid. Although the RFC has a relatively low affinity for reduced folate coenzymes (~1000-fold lower) compared to FR and it functions suboptimally in physiologic solutions, transport by the carrier occurs rapidly (within seconds) and with a high capacity that is sufficient to fulfill cellular folate requirements. The RFC also provides the primary transport route for the uptake of the classical antifolate, methotrexate, at pharmacologic concentrations of the drug. In cultured cells expressing both RFC and high levels of FR, however, it has been established that FR offers the preferred pathway for the transport of novel classes of antifolate drugs at low extracellular drug concentrations (Jansen et al., 1989a, 1990, 1991; Henderson & Strauss, 1990; Westerhof et al., 1991).

Because of the tissue specificity of FR and its elevation in malignant tissues, several experimental approaches for targeting FR on tumors are currently being tested. These methods include the use of novel antifolates (Jones et al., 1981; Jackman et al., 1990; Beardsley et al., 1989; Habeck et al., 1994), folic acid conjugated cytotoxics (Leamon & Low, 1992, 1994; Leamon et al., 1993), folate-coated liposomes (Lee & Low, 1994), and also mobilization of the host immune response (Bolhuis et al., 1992; Canevari et al., 1992; Ferrini et al., 1993). The above folate/novel antifolate-mediated approaches will greatly benefit, in terms of tissue selectivity, from the design of compounds that are selective for the one or the other tissue specific FR isoform and that will not bind to the RFC. While fundamental differences in ligand specificities exist between FR and the RFC, the differences between the FR isoforms may be more subtle.

A detailed understanding of the structural basis for the differential binding of folate compounds by FR-α and FR-β is thus important. At present, we have no knowledge of regions in the primary structure of FR that constitute the folate-binding site. The present study undertakes mutagenesis to identify amino acid residues that are directly responsible for the unique ligand binding characteristics of FR isoforms. The results are expected to complement pending X-ray crystallographic data and to provide additional information about FR structure and function. The approach we chose in these studies was to make a series of chimeric constructs of FR-α and FR-β in order to identify amino acid residues that are responsible for the functional differences between these two isoforms. Specifically, our interest was to localize amino acid sequence differences between FR-α and FR-β that could account for their differences in stereospecificity for reduced folate compounds. A major advantage of studying chimeric constructs of the structurally homologous proteins is that it overcomes, to a certain extent, ambiguities in the interpretation of mutagenesis experiments that result in inactive proteins. By this approach we are able to obtain functional proteins with properties similar to either FR-α or FR-β.

MATERIALS AND METHODS

Mutagenesis and Recombinant Plasmids. Chimeric constructs of FR-α and FR-β were made in one of the following three ways: (i) in some cases, common restriction sites were used to exchange the desired portions of FR-α and FR-β (Table 1); (ii) in cases where no suitable restriction site was found, such sites were created by polymerase chain reaction (PCR). Oligonucleotides were designed to contain appropriate restriction sites without changing the amino acids they encoded (Table 1). (iii) Point mutants were made by oligonucleotides containing both the restriction sites and the mutations (Table 1).

PCR was performed using Vent (New England Biolabs) DNA polymerase with FR-α or FR-β cDNA as template. Amplified PCR products were digested with the appropriate restriction enzymes and subcloned into the expression vector pCDNAI. The resulting plasmids were amplified in *Escherichia coli* MC1061/p3. The entire DNA sequence of every mutant cDNA construct was verified by dideoxy sequencing using Sequenase Version 2.0 (USB).

Cell Culture and Transfection. Human 293 fibroblasts were grown in Eagle's Minimal Essential medium (MEM, Irvine Scientific, Santa Ana, CA) supplemented with FBS (10%, v/v), penicillin (100 units/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM). Transfections were carried out using lipofectamine (Gibco BRL, Grand Island, NY) according to the manufacturer's protocol. Transfection efficiencies were normalized to a co-transfected β-galactosidase standard. The transfection efficiencies were generally uniform.

Preparation of Crude Plasma Membranes. Crude plasma membranes were prepared as described (Wang et al., 1992). Essentially cells from confluent cultures of transfected 293 cells were washed with acid buffer (10 mM sodium acetate, pH 3.5, 150 mM NaCl) at 4 °C to remove endogenously bound folates followed by washing with PBS (10 mM sodium phosphate, pH 7.5, 150 mM NaCl). The cells were scraped off the plates, allowed to swell in lysis buffer (1 mM $NaHCO_3$, 2 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM PMSF) for 30 min at 4 °C, and then homogenized by 50 strokes of a glass dounce homogenizer. The homogenate was centrifuged to sediment the nuclei and residual cells. The resulting supernatant was centrifuged for 45 min at 40000*g* to sediment the membranes. The membranes were then washed at 4 °C with acid buffer and PBS, respectively, by resuspension in the buffers followed by sedimentation. The resulting membrane preparation was resuspended in PBS.

[^{3}H]Folic Acid Binding Assay. The radioactive binding assay for FR in membrane preparations was performed by direct binding of [^{3}H]folic acid to the membranes as described (Shen et al., 1994). The specificity of binding of the radiolabel was determined by competition with unlabeled folic acid. Membranes from untransfected cells were used as a negative control. The [^{3}H]folic acid binding assay for FR on whole cells was performed as described (Shen et al., 1995).

Inhibition Studies. IC_{50} values for the inhibition of [^{3}H]folic acid binding to FR by folic acid and reduced folate coenzymes were determined as described (Wang et al., 1992). Membranes were assayed as described above in the presence of a range of concentrations (1−500 nM) of inhibitor and a fixed concentration (2 nM) of [^{3}H]folic acid. The resulting data were used to calculate IC_{50} values for the inhibitors using the computer program Inplot (GraphPad Software Inc., Version 4.03).

Table 1: Restriction Sites and Oligonucleotides (5′-3′) Used To Generate the Chimeric Constructs of FR-α and FR-β[a]

constructs	oligonucleotides	sequences of oligonucleotides	restriction sites
FR-$\alpha_{1-92}/\beta_{93-237}$	NA	NA	*Bam*HI
FR-$\beta_{1-92}/\alpha_{93-232}$	NA	NA	*Bam*HI
FR-$\alpha_{1-38}/\beta_{39-92}/\alpha_{93-232}$	NA	NA	*Bsm*I, *Bam*HI
FR-$\beta_{1-38}/\alpha_{39-92}/\beta_{93-237}$	NA	NA	*Bsm*I, *Bam*HI
FR-$\alpha_{1-38}/\beta_{39-237}$	NA	NA	*Bsm*I
FR-$\beta_{1-38}/\alpha_{39-232}$	NA	NA	*Bsm*I
FR-$\alpha_{1-59}/\beta_{60-92}/\alpha_{93-232}$	AF1 (*Mun*I)	TAGATTCAATTGGAACCACTCTGG	*Mun*I
FR-$\beta_{1-59}/\alpha_{60-92}/\beta_{93-237}$	AR1 (*Mun*I)	TGGTTCCAATTGAATCTATATAGG	
FR-$\alpha_{1-38}/\beta_{39-59}/\alpha_{60-232}$	BF1 (*Mun*I)	CAACTTCAATTGGGACCACTGC	
FR-$\beta_{1-38}/\alpha_{39-59}/\beta_{60-237}$	BR1 (*Mun*I)	TGGTCCCAATTGAAGTTGTACAGG	
FR-$\alpha_{1-38}/\beta_{39-53}/\alpha_{54-232}$	AF2 (*Csp*6I)	GATGTTTCGTACCTATATAG	*Csp*6I
	BR2 (*Csp*6I)	ATACAGGTACGAGGTGTCC	
FR-$\alpha_{1-53}/\beta_{54-59}/\alpha_{60-232}$	AYR	GTGGTTCCAATTGAATCTATATAG**GCG**GGAAAC	*Mun*I
	ARN	GTGGTTCCAATTGAA**GTT**ATATAGG	
FR-$\beta_{1-53}/\alpha_{54-59}/\beta_{60-237}$	B (RY/NR)	GTGGTCCCAATTGAAGCGGTACAGGTAGGAGGTGT	*Mun*I
FR-$\alpha_{1-38}/\beta_{39-46}/\alpha_{47-232}$	ANS-F (*Spe*I)	CCAGCACTAGTCAGGAAGC	*Spe*I
	BLA-R (*Spe*I)	CTTGTG**CGC**CTCCTGACTAGTGCTGGCCTG	
FR-α_{A49-L}	AAL-F (*Aat*II)	CAGGAA**CTC**CATAAGGACGTCTCCTA	*Aat*II
	AAL-R (*Aat*II)	ATGGAGACGTCCTTATG**GAG**TTCCTG	
FR-α_{V53-T}	AVT-F	CATTAAGGAT**ACG**TCCTACC	NA
	AVT-R	GGTAGGA**CGT**ATCCTTATG	
FR-$\alpha_{1-46}/\beta_{47-53}/\alpha_{54-232}$	AAL-F, AAL-R	see above	*Aat*II
	AVT-F, AVT-R	see above	NA
FR-β_{L49-A}	BLA-F (*Spe*I)	CAGGCCAGCACTAGTCAGGAG**GCG**CACAAG	*Spe*I
	BLA-R (*Spe*I)	CTTGTG**CGC**CTCCTGACTAGTGCTGGCCTG	
FR-$\beta_{1-38}/\alpha_{39-46}/\beta_{47-237}$	B(*Spe*I)F	CCAGCACTAGTCAGGAGCTGC	*Spe*I
	A(*Spe*I)R	GCTCCTGACTAGTGTTGGTAGAAC	
FR-$\beta_{1-46}/\alpha_{47-53}/\beta_{54-237}$	BLA-F, BLA-R	see above	*Spe*I
	BTV-R (*Aat*II)	CACAAGGAC**GTC**TCACGACTGTACAAC	*Aat*II
	BTV-F (*Aat*II)	GTTGTACAGTCGTGA**GAC**GTCCTTGTG	
FR-$\beta_{1-92}/\alpha_{93-232}$(L49-A)	BLA-F	see above	*Spe*I
	BLA-R		

[a] Underlined sequences indicate restriction sites. Highlighted residues indicate positions of point mutations. NA, not applicable because the restriction sites occur naturally.

Western Blot Analysis. Cells (1×10^6) were dissolved in 30 μL of PBS containing 0.5% Triton X-100 at 37 °C for 30 min. Insoluble material was removed by sedimentation at 10000*g* for 2 min. A portion of the sample (15 μL) was used for protein estimation by the Bio-Rad protein assay (Bio-Rad Laboratories). To the remaining sample were added 5 μL of 10% sodium dodecyl sulfate (SDS) and 15 μL of 2 × SDS sample buffer. The samples were electrophoresed on 12.5% SDS−polyacrylamide gels. Western blot analysis was carried out as described (Wang et al., 1992) using affinity-purified rabbit antibody against FR from human placenta (Ratnam et al., 1989) as the probe.

Treatment with Phosphatidylinositol-Specific Phospholipase C (PI-PLC). Cells expressing FR-β were treated with PI-PLC for 1.5 h at 37 °C as described previously (Yan & Ratnam, 1995).

RESULTS AND DISCUSSION

The rationale for the mutagenesis approach in the present study was based on the premise that because of the structural homology and the functional similarity between FR-α and FR-β, the majority of the divergent amino acid residues in the two proteins may be interchanged without significantly impacting function. Thus, specific functional differences between the FR isoforms may be accounted for by amino acid sequence divergence at one or a few positions. The experimental method adopted to test the above hypothesis was to construct and characterize a series of chimeric FRs by exchanging progressively shorter peptides to either bracket or pinpoint the critical divergent residues. While a normal level (comparable to wild-type FRs) of cell surface expression of [^{3}H]folic acid binding protein would be an indication of normal folding of a chimeric protein, it may be anticipated that in some chimeras, the nature of interactions of peptide sequences from the different proteins may adversely affect the efficiency of protein folding. However, such effects may also be due to divergence of one or a few amino acids, which may be identified. An important advantage of the above experimental approach to identifying the amino acids responsible for the functional differences between FR-α and FR-β is that it simultaneously excludes the possibility of involvement of the rest of the divergent amino acid residues. On the other hand, the method would detect possible synergism between amino acid residues in producing specific functional effects.

Substitution in FR-α with Segments of the FR-β Polypeptide. Chimeric constructs of FR-α and FR-β were made and transiently expressed in human 293 fibroblasts. Membrane preparations from the transfected cells were tested for their affinities for the diastereoisomers of 5-methyltetrahydrofolate. First the chimera FR-$\beta_{1-92}/\alpha_{93-232}$ was constructed by exploiting a common *Bam*HI site in the FR-α and FR-β cDNAs. FR-$\beta_{1-92}/\alpha_{93-232}$ showed ∼30-fold higher relative affinity for (6*R*)-5-methyltetrahydrofolate than for (6*S*)-5-methyltetrahydrofolate, similar to wild-type FR-β (Figure 1). The similarity of the FR-$\beta_{1-92}/\alpha_{93-232}$ chimera to FR-β suggests that amino acid(s) responsible for the stereospecificities of FR for reduced folate are within peptide 1−92. Initial attention was, therefore, focused on substituting shorter segments of FR-β within residues 1−92 of FR-α. Using

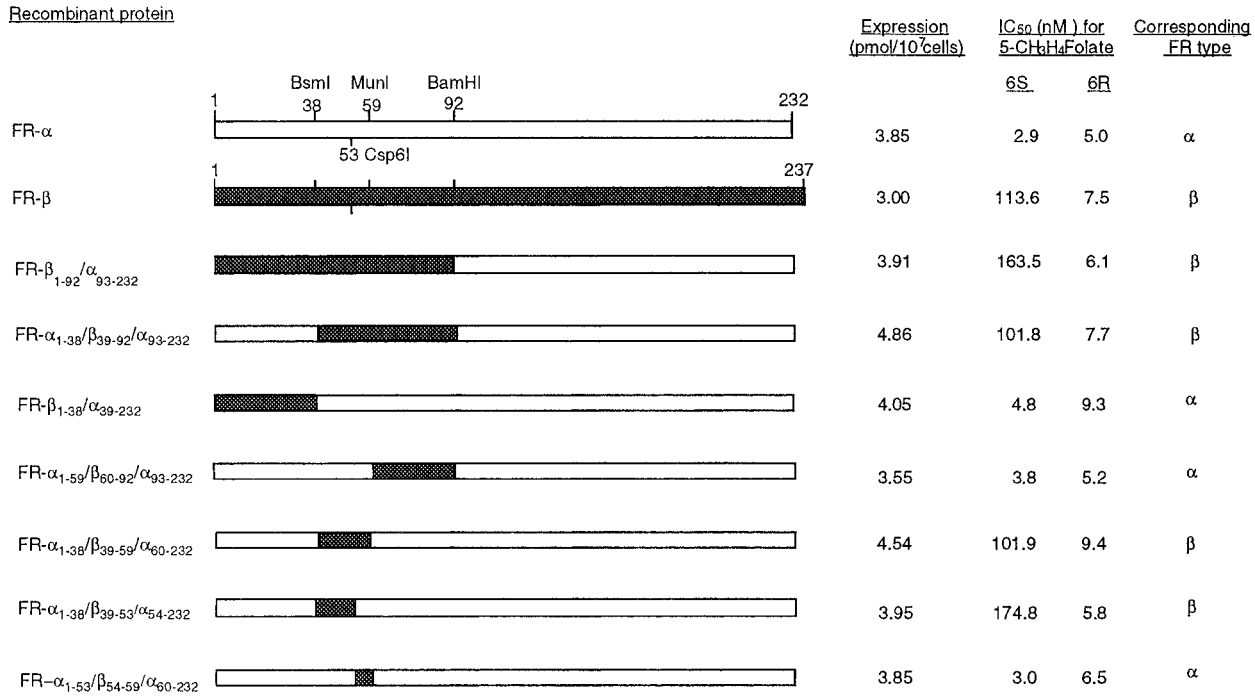

FIGURE 1: Properties of chimeric forms of FR-α and FR-*β*. Either naturally occurring restriction sites or restriction sites introduced by mutagenesis in the cDNAs for FR-α and FR-*β* were used as indicated in the schematic diagram. The horizontal bars represent either the partial or the complete polypeptide chains of FR-α (open bars) and FR-*β* (hatched bars). The numbers on the bars represent positions of amino acid residues. The columns on the right indicate the expression levels of the recombinant proteins in terms of the amount of [^{3}H]folic acid bound at the cell surface, their IC_{50} values for diastereoisomers of 5-methyltetrahydrofolate, and the wild-type FR isoform to which they correspond functionally. Transfection efficiencies were normalized to a co-transfected *β*-galactosidase standard.

Recombinant protein	Amino Acid sequence 39-53	Expression (pmol/10^7 cells)	IC_{50}(nM) 5-CH_3H_4Folate 6S	6R	Corresponding FR type
FR-α	A C C **S T N** T S Q E **A** H K D **V**	3.85	2.9	5.0	α
FR-β	A C C **T A S** T S Q E **L** H K D **T**	3.00	113.6	7.5	β
FR-$\alpha_{1-38}/\beta_{39-46}/\alpha_{47-232}$	A C C **T A S** T S Q E **A** H K D **V**	3.86	4.5	5.2	α
FR-$\alpha_{1-46}/\beta_{47-53}/\alpha_{54-232}$	A C C **S T N** T S Q E **L** H K D **T**	3.80	188.1	5.5	β
FR-α_{A49-L}	A C C **S T N** T S Q E **L** H K D **V**	3.76	179.0	6.8	β
FR-α_{V53-T}	A C C **S T N** T S Q E **A** H K D **T**	3.90	5.1	7.2	α

FIGURE 2: Properties of mutant and chimeric FR. The aligned amino acid sequences for a peptide (residues 39−53) are indicated. Highlighted residues indicate the positions at which the amino acid sequences of FR-α and FR-*β* are divergent. The columns on the right indicate the expression levels of the recombinant proteins in terms of the amount of [^{3}H]folic acid bound at the cell surface, their IC_{50} values for the diastereoisomers of 5-methyltetrahydrofolate, and the wild-type FR isoform to which they correspond functionally. Transfection efficiencies were normalized to a co-transfected *β*-galactosidase standard.

restriction sites in the two FR cDNAs that were either already available or created by PCR without changing the amino acid sequence of the proteins, progressively shorter segments within residues 1−92 in FR-α were substituted with the corresponding peptides of FR-*β* and the mutant proteins were tested for their stereospecificities for reduced folate (Figure 1). FR-$\alpha_{1-38}/\beta_{39-92}/\alpha_{93-232}$ showed properties similar to FR-*β*, while FR-$\beta_{1-38}/\alpha_{39-232}$ retained the properties of FR-α. Further substitution with the FR-*β* peptide 60−92 or 54−59 in FR-α did not significantly alter its properties while substitution with the FR-*β* peptides 39−59 or 39−53 resulted in chimeric FRs with the ligand specificities of FR-*β* (Figure 1). These results indicate that one or more residues within the amino acid sequence 39−53 is responsible for its functional distinction from FR-*β* (Figure 1). Among the 15 residues in this segment of FR-α, only five are unconserved (Figure 2).

Further mutations in FR-α involving three (FR-$\alpha_{1-38}/\beta_{39-46}/\alpha_{47-232}$) or two (FR-$\alpha_{1-46}/\beta_{47-53}/\alpha_{54-232}$) substitutions with the corresponding unconserved residues from the FR-*β* peptide 39−53 were made (Figure 2). The results showed that while amino acid divergence at positions 42, 43, and 44 did not account for the functional divergence of FR-α, substitution at the divergent positions 49 and 53 resulted in the ligand specificity of FR-*β* (Figure 2). Next, point mutations were made to obtain FR-α_{A49-L} and FR-α_{V53-T} (Figure 2). Substitution of Val 53 in FR-α with the corresponding Thr in FR-*β* did not significantly alter its properties (Figure 2). On the other hand, when Ala 49 was substituted with Leu, the corresponding amino acid in FR-*β*, the mutant protein showed relative affinities for the diastereoisomers of 5-methyltetrahydrofolate that were similar to those of FR-*β* (Figure 2), indicating that divergence at position 49 is critical for the different properties of FR-α and FR-*β*.

Substitution of FR-β with *Segments of the FR-α Polypeptide*. A simple test of whether the Ala vs Leu substitution alone will account for the functional differences between FR-α and FR-*β* is to test the effect of the reciprocal substitution of Leu 49 with Ala in FR-*β*. If the mutation

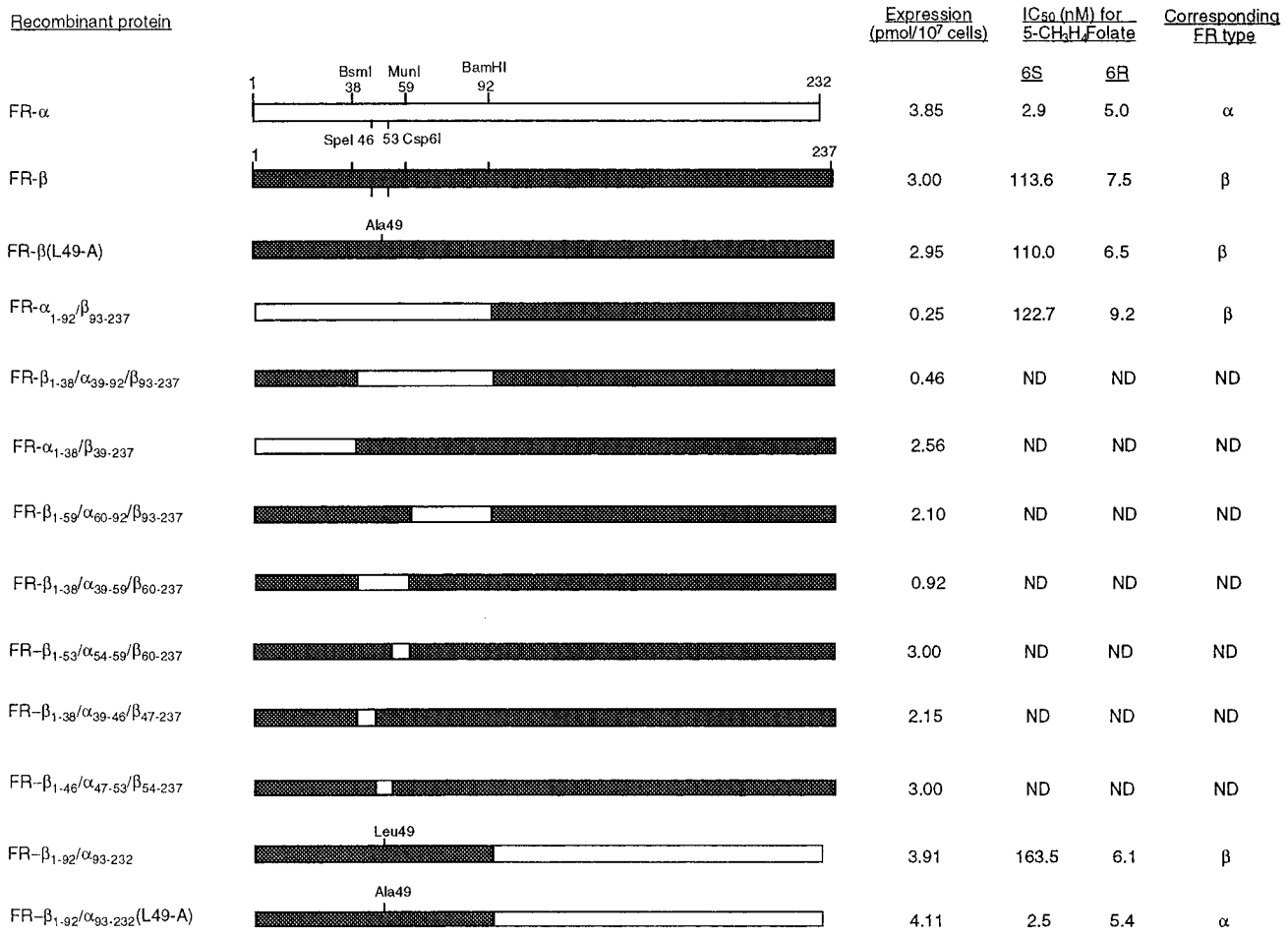

FIGURE 3: Properties of chimeric forms of FR-α and FR-β and chimeras or wild-type FR containing further point mutations. Either naturally occurring restriction sites or restriction sites introduced by mutagenesis in the cDNAs for FR-α and FR-β were used as indicated in the schematic diagram. The horizontal bars represent either the partial or the complete polypeptide chains of FR-α (open bars) and FR-β (hatched bars). The numbers on the bars represent positions of amino acid residues. The columns on the right indicate the expression levels of the recombinant proteins in terms of the amount of [^{3}H]folic acid bound at the cell surface, their IC_{50} values for the diastereoisomers of 5-methyltetrahydrofolate, and the wild-type FR isoform to which they correspond functionally. Transfection efficiencies were normalized to a co-transfected β-galactosidase standard. ND; not determined.

failed to confer the propeties of FR-α, it would mean that amino acid sequence divergence at one or more additional positions is also a critical determinant of the differential ligand binding by the FR isoforms. Therefore, the mutant FR-β_{L49-A} was constructed and its IC_{50} values for the 6*S* and 6*R* diastereoisomers of 5-methyltetrahydrofolate were measured. This mutant behaved like the wild-type FR-β (Figure 3), indicating that in addition to Ala 49, other amino acid(s) may be needed for the differential folate binding characteristics of FR-α.

When a systematic analysis of chimeric FR-β mutants that behave similarly to FR-α was attempted, an initial problem encountered was the very low cell surface expression of the FR-$\alpha_{1-92}/\beta_{93-237}$ chimera (<10% of wild-type FR-β; Figure 3). It was, therefore, necessary to first address the problem of low cell surface expression of [^{3}H]folic acid binding protein for the FR-$\alpha_{1-92}/\beta_{93-237}$ chimera. From Western blot analysis which is discussed below in detail, it is clear that under conditions of uniform transfection efficiencies, while the low cell surface expression of the mutant FR-$\alpha_{1-92}/\beta_{93-237}$ (Figure 3) is most likely due to either unproductive interactions or lack of the appropriate interactions between the amino-terminal portion (residues 1−92) of FR-α with a downstream portion (residues 93−237) of FR-β resultiing in a low efficiency of protein folding and targeting to the cell surface. In order to identify the shortest segment of FR-α that was responsible for the low expression of the chimera, progressively shorter fragments of peptide 1−92 of FR-α were placed in FR-β and the expression levels of the mutant proteins were measured (Figures 3 and 4). The mutants were made using the same strategy used above in constructing the FR-α mutants. The cell surface expression levels of FR-$\alpha_{1-92}/\beta_{93-237}$, FR-$\beta_{1-38}/\alpha_{39-92}/\beta_{93-237}$ and FR-$\beta_{1-38}/\alpha_{39-59}/\beta_{60-237}$ were approximately 1/10, 1/6, and 1/3, respectively, of that of wild-type FR-β, while FR-$\alpha_{1-38}/\beta_{39-237}$ and FR-$\beta_{1-59}/\alpha_{60-92}/\beta_{93-237}$ were expressed at levels comparable to that of wild-type FR-β (Figure 3). Western blot analysis of these mutants was consistent with this observation (Figure 4). On the Western blot in Figure 4, the mature cell surface [^{3}H]folic acid binding protein is represented by the diffuse band (arrow) having the lowest mobility indicated by its susceptibility to treatment of intact cells with PI-PLC, which specifically cleaves the GPI membrane anchor; the lower bands in Figure 4 may represent the immature protein as well as degradation products of misfolded protein in intracellular compartments. The multiple N-glycosylation sites in FR may also be expected to contribute to heterogeneity in electrophoretic mobility due to heterogeneity in glycosylation. The mature protein band is very strong in wild-type FR-β, FR-$\alpha_{1-38}/\beta_{39-237}$, and FR-

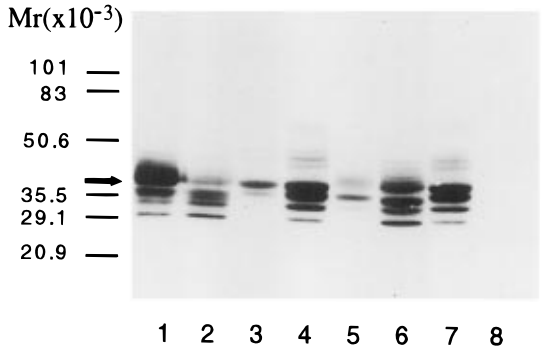

FIGURE 4: Western blot analysis of detergent extracts of human 293 fibroblasts transfected with FR-β and some chimeric constructs. The blot was probed with affinity purified rabbit polyclonal antibodies to FR-β as described under Materials and Methods. Lane 1, FR-β; lane 2, FR-β obtained from cells treated with PI-PLC; lane 3, FR-$\alpha_{1-92}/\beta_{93-237}$; lane 4, FR-$\alpha_{1-38}/\beta_{39-237}$; lane 5, FR-$\beta_{1-38}/\alpha_{39-92}/\beta_{93-237}$; lane 6, FR-$\beta_{1-38}/\alpha_{39-59}/\beta_{60-237}$; lane 7, FR-$\beta_{1-59}/\alpha_{60-92}/\beta_{93-237}$; lane 8, vector alone (negative control). The arrow indicates the position of the mature cell surface [^{3}H]folic acid binding protein. Transfection efficiencies were normalized to a co-transfected β-galactosidase standard.

$\beta_{1-59}/\alpha_{60-92}/\beta_{93-237}$ and is relatively faint in FR-$\alpha_{1-92}/\beta_{93-237}$, FR-$\beta_{1-38}/\alpha_{39-92}/\beta_{93-237}$, and FR-$\beta_{1-38}/\alpha_{39-59}/\beta_{60-237}$ (Figure 4). These results suggest that although peptide 39−59 of FR-α may contribute the most to the low expression of these mutants, probably due to the nature of its interactions with the carboxyl-terminal portion of FR-β, the effect is enhanced when a longer stretch of FR-α is present.

When shorter fragments within peptide 39−59 of FR-β were replaced with corresponding fragments from FR-α, the resulting mutant constructs (FR-$\beta_{1-38}/\alpha_{39-46}/\beta_{47-237}$, FR-$\beta_{1-46}/\alpha_{47-53}/\beta_{54-237}$, and FR-$\beta_{1-53}/\alpha_{54-59}/\beta_{60-237}$) did not show a significant difference in expression compared with wild-type FR-β (Figure 3). This result shows that peptide 39−59 derived from FR-α is nearly the shortest possible fragment that, when present, significantly disrupts the proper folding of the mutant FR-β constructs. The IC_{50} values for the (6*S*) and (6*R*) diastereoisomers of FR-$\alpha_{1-92}/\beta_{93-237}$ was determined despite its low expression levels and it was found that it still behaved like FR-β (Figure 3). These results suggest that in addition to the occurrence of Ala at position 49, the differential folate binding properties of FR-α may be caused by amino acid sequence divergence downstream of residue 92.

Simultaneous Mutation at Position 49 and a Downstream Portion of FR-β. The most direct way to test the above hypothesis is to convert Leu 49 to Ala in FR-$\beta_{1-92}/\alpha_{93-232}$, whose carboxyl-terminal region (residues 93−232) is derived from FR-α but which still behaves like FR-β. When the mutant construct FR-$\beta_{1-92}/\alpha_{93-232}$ (L49−A) was made and expressed in cells, the resulting protein showed a normal level of cell surface expression and furthermore, exhibited the reduced folate binding characteristics of FR-α (Figure 3). This result indicates that Ala at position 49 and amino acid residue(s) downstream of position 92 act synergistically in producing the differential ligand specificity of FR-α.

From the above study, it may be concluded that Ala 49 in FR-α and Leu 49 in FR-β are critical amino acid residues in determining their unique ligand binding characteristics. It may also be concluded that while the amino acid divergence at position 49 is essential for the differential ligand binding characteristics of FR-α vs FR-β, additional amino acid divergence in peptide 93−232 of FR-α is also essential for its isoform specific functional characteristics. Further mutagenesis studies should enable the pinpointing of such residue(s). Consistent with the results of this study, murine FR-α and FR-β which are functionally analogous to the human FR isoforms (Figure 5) also contain Ala (FR-α) or Leu (FR-β) at position 49 (Brigle et al., 1991, 1994).

Human FR-α	N	T	S	Q	E	**A**	H	K	D	V	S
Human FR-β	S	T	S	Q	E	**L**	H	K	D	T	S
Murine FR-α	N	T	S	Q	E	**A**	H	K	D	I	S
Murine FR-β	N	T	S	Q	E	**L**	H	K	A	D	S

FIGURE 5: Aligned amino acid sequences of human and murine FR-α and FR-β between residues 44 and 54. The amino acid at position 49 is highlighted.

The subtle nature of the Ala vs Leu substitution suggests that the effects of these amino acids are likely to be due to a localized perturbance of protein structure, such as local steric or hydrophobic effects possibly at or near the folate binding site of FR. Characterization of the exact nature of the interactions of critical amino acid residues identified by mutagenesis in this and future studies will be possible when complemented by pending X-ray crystallographic data. The present study demonstrates the feasibility of a systematic mutagenesis approach for the unambiguous identification of specific amino acid residues that play a critical functional role in the three-dimensional structure of the mature FR isoforms.

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