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Complete Mapping of Divergent Amino Acids Responsible for Differential Ligand Binding of Folate Receptors α and β*

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Karen M. Maziarz, Hugo L. Monaco‡, Feng Shen, and Manohar Ratnam§

From the Department of Biochemistry and Molecular Biology, Medical College of Ohio, Toledo, Ohio 43614-5804 and the ‡Department of Genetics, University of Pavia, Via Abbiategrasso 207, 27100 Pavia, Italy

The folate receptor (FR) type α may be distinguished from FR-β by its higher affinity for the circulating folate coenzyme, (6*S*)-5-methyltetrahydrofolate (5-CH_3H_4folate), and its opposite stereospecificity for reduced folate coenzymes. Previous studies showed that a single leucine to alanine substitution at position 49 of the mature protein sequence is responsible for the functional divergence of FR-β (Shen, F., Zheng, X., Wang, H., and Ratnam, M. (1997) *Biochemistry* 36, 6157–6163); however, the results also indicated that the minimum requirement for conversion of FR-β to the functional equivalent of FR-α should include amino acid substitution(s) downstream of residue 92 in addition to mutation of L49A. To pinpoint those residues, chimeric FR-β_{L49A}/FR-α constructs including progressively shorter segments of FR-α downstream of position 92 as well as selected point mutants were studied. Simultaneous substitution of Leu-49, Phe-104, and Gly-166 in FR-β with the corresponding FR-α residues Ala, Val, and Glu, respectively, reconstituted the ligand binding characteristics of FR-α. The results also exclude a role for other residues in FR-α in determining its functional divergence. A homology model of FR-α based on the three-dimensional structure of the chicken riboflavin-binding protein is used to show the position of residues 49, 104, and 166 in relation to the hydrophobic cleft corresponding to the riboflavin-binding pocket.

The cell surface receptor for folic acid (folate receptor, FR)[1] has the ability to mediate physiologic folate uptake and to transport novel antifolate drugs and folate conjugates (1–20). The receptor is a current major focus as a tumor target for multiple experimental approaches in cancer therapy. Several studies have shown that FR, when expressed at high levels, could offer the preferred uptake route of novel classes of antifolate drugs that target glycineamide ribonucleotide formyltransferase and thymidylate synthase (3–6). Taking advantage of the non-destructive nature of FR-mediated internalization of folate-coupled macromolecules (7, 8), cytotoxins such as momordin, pseudomonas exotoxin, and maytansinoids were shown to produce selective killing in FR-rich cells (9–12). Furthermore, the toxicity of such conjugates was dependent upon receptor density on the cell surface (10). Folate-conjugated radiopharmaceuticals also appear to offer a means of tumor imaging/radiation therapy (13–16). Folate-coated liposomes were shown to selectively target FR-rich tumor cells (17) and selective killing of the malignant cells was obtained by encapsulating doxorubicin in the liposomes (18). By a similar strategy, it was possible to deliver antisense oligonucleotides against the epidermal growth factor receptor to FR-rich tumor cells (19). Furthermore, selective folate-mediated targeting of an adenoviral vector to FR-rich tumor cells has been achieved in the presence of an antibody to ablate the endogenous viral tropism (20).

The mechanism of folate uptake via FR has been shown to occur by an endocytic process (7–8, 21–25). The three known isoforms of FR, types α (26, 27), β (28), and γ/γ′ (29), bind folic acid with a high affinity ($K_d < 1$ nM) and a stoichiometry of 1:1 (30). The mature proteins are 68–79% identical in amino acid sequence, ranging in length from 220 to 236 amino acids. The proteins have either two (FR-β, FR-γ) or three (FR-α) sites of *N*-glycosylation which cumulatively contribute to their proper protein folding and intracellular trafficking but are not required for folate binding (31). FR-α and FR-β are attached to the cell surface by a glycosylphosphatidylinositol membrane anchor (26, 32–34), whereas, FR-γ and a truncated form of the protein, FR-γ′ are constitutively secreted (35). The sites of glycosylphosphatidylinositol modification in FRs-α and -β are Ser^{209} and Asn^{211}, respectively (34).

FR isoforms are expressed in a tissue specific manner and are selectively overexpressed in certain malignant tissues. FR-α is specific for particular epithelial cells and is vastly up-regulated in ovarian and uterine carcinomas (36–39). FR-β is not expressed at significant levels in most normal tissues with the known exceptions of placenta, spleen, thymus, and neutrophils (39, 40). Overexpression of FR-β was observed in some malignancies of non-epithelial origin including myeloid leukemia (29, 40). FR-γ and FR-γ′ are specific for hematopoietic tissues, particularly lymphoid cells (35).

FR-α and FR-β from both human (41) and murine (42) sources have opposite stereospecificities for the reduced coenzyme forms of folate, 5-methyltetrahydrofolate (5-CH_3H_4folate) (Fig. 1) and 5-formyltetrahydrofolate (5-$CHOH_4$folate). In contrast to FR-α, FR-β preferentially binds to the unphysiologic (6*R*)-diastereoisomers of these compounds relative to their physiologic (6*S*)-diastereoisomers. FR-β also exhibits a significantly lower affinity relative to FR-α for the (6*S*)-diastereoisomer of 5-CH_3H_4folate, the major circulating form of the vitamin. In addition, FR-β has a lower affinity for a variety of antifolate compounds compared with FR-α (41).

The tissue specificity of FR isoforms and their elevation in malignant tissues may be an important factor in selective targeting of malignant cells via FR-mediated uptake of novel antifolates and folate conjugates. It is therefore of significance to understand the structural basis for the functional difference between the membrane anchored FR isoforms. We have previ-

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§ To whom correspondence should be addressed.

[1] The abbreviations used are: FR, folate receptor; PBS, phosphate-buffered saline; H_4folate, tetrahydrofolate; cRBP, chicken riboflavin-binding protein.

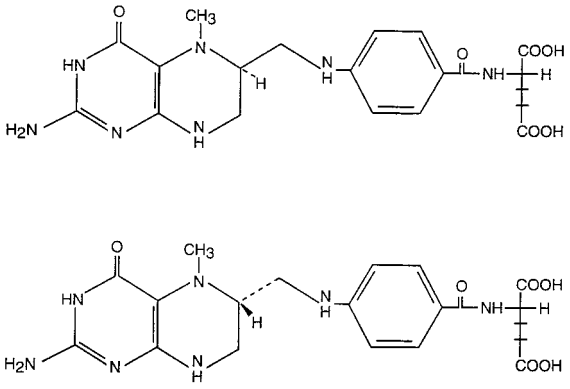

FIG. 1. **Structures of the (6*S*)- (*top*) and (6*R*)- (*bottom*) diastereoisomers of 5-methyltetrahydrofolate (N^5-methyl-5,6,7,8-tetrahydropteroyl glutamic acid).**

ously demonstrated a relatively unambiguous approach to identify peptide segments or specific amino acid residues that could account for the functional differences between FR-α and FR-β (43). The approach takes advantage of the fact that FR-α and FR-β are structurally homologous, and therefore chimeric constructs of the two proteins may be expected to result in functional proteins that could be characterized; by systematic construction of a series of such chimeras in which progressively shorter peptides of one protein are substituted in the other protein, residues that are significant for a specific functional difference may be mapped while simultaneously excluding a role for other residues. In this manner we have previously demonstrated that the functional divergence of FR-β from FR-α is due to Leu^{49} in FR-β (43); substitution of Ala^{49} in FR-α with Leu conferred the ligand binding characteristics of FR-β. However, conversion of FR-β to the functional equivalent of FR-α could only be accomplished when, in addition to the reciprocal mutation of L49A, the sequence downstream of residue 92 in FR-α was substituted in FR-β. Thus, the functional divergence of FR-α from FR-β could possibly be accounted for by one or more residues downstream of position 92 in addition to Ala^{49}. The purpose of this study is to identify those residues in FR-α.

MATERIALS AND METHODS

Mutagenesis and Recombinant Plasmids—Chimeric constructs of FR-α and FR-β were made by utilizing natural restriction sites or by creating restriction sites by the polymerase chain reaction using Vent DNA polymerase (New England Biolabs) and the cDNAs for FR-α or FR-β as template. Oligonucleotides (Life Technologies, Inc.) were designed in two different ways in order to construct the appropriate recombinant proteins. In cases where no suitable restriction site was found, complementary primers containing an appropriate restriction site or point mutation were used in conjunction with upstream and downstream primers containing restriction sites. Alternatively, mutagenic oligonucleotides were used as end primers to amplify the desired fragment. All of the synthetic oligonucleotides (Table I) were designed to contain restriction sites or point mutations without undesirable alterations of amino acid sequence. The polymerase chain reaction products were first digested at both ends with the appropriate restriction enzymes and subcloned into the plasmid pcDNA1 (Invitrogen) containing the FR-β_{L49A} mutant cDNA. The recombinant plasmid was amplified in *Escherichia coli* MC1061/p3 and purified using the Qiagen plasmid kit (Qiagen). The entire cDNA sequence was verified by dideoxy sequencing using AmpliCycle Sequencing Kit (Perkin-Elmer).

Cell Culture and Transfection—Human 293 fibroblasts were maintained in Eagle's minimal essential media (Irvine Scientific) supplemented with fetal bovine serum (10% v/v), penicillin (100 units/ml), and streptomycin (100 μg/ml). The cells were transfected using either LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's protocol or by the use of calcium phosphate (44). Transfection efficiencies were normalized to a co-transfected β-galactosidase standard and found to be relatively uniform.

Preparation of Crude Plasma Membranes—Crude plasma membranes were prepared essentially as described (41). Confluent cultures of transfected 293 cells were washed at 4 °C initially with PBS (10 mM sodium phosphate, pH 7.5, 150 mM NaCl) followed by acid wash (10 mM sodium acetate, pH 3.5, 150 mM NaCl) to remove endogenously bound folate from cell surface receptors and a subsequent PBS wash. The cells were scraped off the plates and suspended in lysis buffer (1 mM $NaHCO_3$, 2 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, pH 7.7), incubated at 4 °C for 30 min and then frozen at −70 °C. The cells were then thawed and homogenized by 60 strokes in a glass Dounce homogenizer. The homogenate was centrifuged at 1000 × *g* for 5 min to sediment nuclei and cell debris. The supernatant was then centrifuged at 4000 × *g* for 45 min to sediment the membranes. The membranes were washed in cold (4 °C) acid buffer by resuspending in the buffer using a 1-ml syringe followed by sedimentation. After an additional wash in PBS the membrane preparation was resuspended in PBS containing 1 mM phenylmethylsulfonyl fluoride.

[^{3}H]Folic Acid Binding Assay—[^{3}H]Folic acid (Morevek, specific radioactivity 37 Ci/mmol, 2.5 pmol/assay tube) was mixed with a membrane suspension in 0.5 ml of PBS and incubated for 60 min at room temperature on a rotary shaker. The membranes were sedimented at 12,000 × *g* for 30 min at 4 °C and washed once with PBS. The membranes were then dissolved in scintillation fluid (Eccoscint H, Fisher Scientific) and the associated radioactivity counted in a Beckman LS3801 liquid scintillation counter. Membranes from untransfected cells as well as membranes preincubated with excess unlabeled folic acid were used as negative control.

The [^{3}H]folic acid binding assay for FR on whole cells in 6-well tissue culture dishes was performed as described previously (34). The cells were washed sequentially at 4 °C with PBS, acid buffer, and again with PBS. The cells were then incubated in a 1-ml solution of PBS containing 3 pmol of [^{3}H]folic acid and 9 pmol of unlabeled folic acid and incubated for 30 min at 4 °C. The cells were then washed two times with PBS. Ice-cold acid buffer was used to remove bound folate and the amount of [^{3}H]folic acid measured by liquid scintillation counting. Untransfected cells as well as membranes preincubated with excess unlabeled folic acid were used as negative controls.

Inhibition Studies—The relative affinities of (6*S*)- or (6*R*)-diastereoisomers of 5-CH_3H_4folate for wild-type or mutant FRs were determined by measuring the IC_{50} values for the compounds for inhibition of [^{3}H]folic acid binding using a range of reduced folate concentrations (1–500 nM). A fixed concentration (2 nM) of [^{3}H]folic acid was used in these experiments. The assays were carried out as described above for the binding of [^{3}H]folic acid to FR-rich membranes either in the absence of inhibitor or with simultaneous addition of inhibitor and [^{3}H]folic acid. IC_{50} values were calculated using the Inplot computer program (GraphPad Software Inc., Version 4.03).

Structural Modeling—A sequence alignment between cRBP and FR-α was performed using the program ClustalW (45). A homology model of the conserved ligand-binding domain (peptide 12–188) was generated with the modeling package Quanta (Molecular Simulations) using the cRBP crystal structure (46) as template. Nonconserved residue replacements were carried out using the mutated function within Quanta. The three insertions all occur in the surface loop region, and their conformations were modeled using a main chain data base and side chain rotamer search procedure in program O (47). The model was used for the rotation and translation search in molecular replacement program Amore (48) against the x-ray diffraction data of FR-α.[2] The resulting model was further refined by rigid-body refinement and energy-minimization calculations in X-PLOR (49). Finally, the stereochemical quality of the model was confirmed using Procheck (50). The surface accessibility of residues was calculated in Quanta using the Lee and Richards algorithm (51) with the probe radius set as 1.4 Å.

RESULTS AND DISCUSSION

To determine the role of individual amino acids of FR-α responsible for its functional variance from FR-β, the general strategy adopted in this study was to examine the effect of substituting peptides or amino acids of FR-α at corresponding locations in FR-β. The basic premise of this approach is that one or a combination of a few amino acids in FR-α are solely responsible for its functional divergence and that they will similarly influence the function of FR-β when substituted in it. For the results of such studies to be most meaningful, it is also

[2] F. Zhang, M. Ratnam, J. Miller, C. Shih, and R. Schevitz, unpublished results.

TABLE I
5′-3′ Oligonucleotides and restriction sites used to generate the chimeric constructs of FR-α and FR-β

Constructs	Oligonucleotides	Sequence of oligonucleotides	Restriction sites
FR-$\beta_{1-139}/\alpha_{140-232}$ (L49A)	A139F (*Bsp*EI)	GAACTGGACTTCCGGATTTAACAAG	*Bsp*EI
	A139R (*Bsp* EI)	CTTGTTAAATCCGGAAGTCCAGTTC	
	B139F (*Bsp* EI)	GACTGGACCTCCGGAGTTAACAAG	
	B139R (*Bsp* EI)	CTTGTTAACTCCGGAGGTCCAGTC	
FR-$\beta_{1-92}/\alpha_{93-139}/\beta_{140-236}$ (L49A)	A139F, A139R	See above	*Bam*HI[a], *Bsp* EI
	B139F, B139R	See above	
FR-$\beta_{1-92}/\alpha_{93-125}/\beta_{126-236}$ (L49A)	A125F (*Eco*72I)	CCTCCTACACGTGCAAGAGC	*Bam*HI[a], *Eco*72I[b]
	A125R (*Eco*72I)	GCTCTTGCACGTGTAGGAGG	
FR-$\beta_{1-92}/\alpha_{93-115}/\beta_{116-236}$ (L49A)	A115F (*Eco*47III)	GACTGTGAGCGCTGGTGGGAAG	*Bam*HI[a], *Eco*47III[b]
	A115R (*Eco*47III)	TTCCCACCAGCGCTCACAGTC	
FR-$\beta_{1-125}/\alpha_{126-232}$ (L49A)	A125F, A125R	See above	*Eco*72I[b]
FR-$\beta_{1-115}/\alpha_{116-232}$ (L49A)	A115F, A115R	See above	*Eco*47III[b]
FR-$\beta_{1-92}/\alpha_{93-115(D96N)}/\beta_{116-236}$ (L49A)	A96F (*Bam*HI)	TGGGGCCCTGGATCCAGCAGGTG**AAT**CAG	*Bam*HI[a]
FR-$\beta_{1-92}/\alpha_{93-115(N106D}/\beta_{116-236}$(L49A)	ADN-F	GGTACTG**GAC**GTGCCCCTGTG	NA[c]
	ADN-R	ACAGGGGCAC**GTC**CAGTACC	
FR-$\beta_{1-92}/\alpha_{93-115(E115Q)/\beta116-236}$(L49A)	AEQR (*Eco*47III)	TCCTCCCACCAGCGCTGACAGTC	*Eco*47III[b]
FR-$\beta_{1-92}/\alpha_{93-115(V104F}/\beta_{116-236}$(L49A)	AVF-F	CAAAGAGCGG**TTC**CTGAACGTG	NA
	AVF-R	CACGTTCAG**GAA**CCGCTCTTTG	
FR-$\beta_{L49A,F104V}$	BFV-F	CAAAGAACGC**GTA**CTGGATGTG	NA
	BFV-R	CACATCCAG**TAC**GCGTTCTTTG	
FR-$\beta_{1-139}/\alpha_{140-155}/\beta_{156-236}$ (L49A,F104V)	A155F (*Eco*RI)	CAACCTTTCGAATTCTACTTCC	*Bsp*EI, *Eco*RI
	A155R (*Eco*RI)	GAAGTAGAATTCGAAAGGTTGG	
	B155F (*Eco*RI)	GCACCTTTGAATTCTACTTCCC	
	B155R (*Eco*RI)	GGGAAGTAGAATTCAAAGGTGC	
FR-$\beta_{1-155}/\alpha_{156-232}$ (L49A,F104V)	A155F, A155R	See above	*Eco*RI
	B155F, B155R	See above	
FR-$\beta_{L49A,F104V,L167I}$	B167F	CTTTGTGAAGGC**ATC**TGGAGTCAC	NA
	B167R	GTGACTCCA**GAT**GCCTTCACAAAG	
FR-$\beta_{L49A,F104V,S191P}$	B191F	ATGTGGTTTGAT**CCA**GCCCAGGGC	NA
	B191R	CCCTGGGC**TGG**ATCAAACCACATC	
FR-$\beta_{L49A,F104V,G166E}$	B166F	CCCTTTGTGAA**GAA**CTCTGGAGTC	NA
	B166R	GACTCCAGAG**TTC**TTCACAAAGGG	

Upstream and downstream primers used to create the protein constructs originated within the flanking regions of the expression vector pCDNA1. Underlined sequences indicate restriction sites. Mutated bases are in bold face.
[a] Denotes natural restriction site found in FR-α and FR-β.
[b] Denotes naturally occurring restriction site found only in FR-β.
[c] NA, not applicable because no restriction site within the protein was used.

important that the mutations not interfere with protein folding and cell surface expression of a functional receptor. Indeed it may be seen in the following sections that all of the FR-β mutants were functional and showed expression levels comparable to that of the wild-type protein. The focus of the functional analysis of the mutant proteins was to test for increase in affinity for (6*S*)-5-CH_3H_4folate and absence of stereospecificity for (6*R*)-5-CH_3H_4folate, both distinguishing characteristics of FR-α. It was previously demonstrated (43) that substitution of Ala[49] in FR-α with Leu, the corresponding residue in FR-β, conferred FR-β-like characteristics. Conversion of FR-β to the functional equivalent of FR-α by substituting the sequence downstream of position 92 of FR-α was only possible if Leu[49] was simultaneously mutated to Ala. Therefore in the following studies, all of the sequence substitutions and mutations were made downstream of position 92 in FR-β_{L49A}.

Functional Mapping of FR-α Peptides Downstream of Position 92—cDNA constructs were made to encode chimeric proteins in which portions of the carboxyl terminus downstream of amino acid 92 in the FR-β_{L49A} mutant were replaced by the corresponding peptides from FR-α. The initial chimeric proteins generated were FR-$\beta_{1-139}/\alpha_{140-232}$(L49A) and FR-$\beta_{1-92}/\alpha_{93-139}/\beta_{140-236}$(L49A) (Fig. 2). The mutants were transiently expressed in human 293 fibroblasts. Membrane preparations from the transfected cells were tested for their relative affinities for the (6*S*)- and (6*R*)-diastereoisomers of 5-CH_3H_4folate (Fig. 2). Both chimeric proteins showed approximately 10-fold greater affinity for (6*S*)-5-CH_3H_4folate than either the wild-type FR-β or FR-β_{L49A}; however, the values were approximately 4-fold lower than that of wild-type FR-α. These results appeared to suggest that amino acid residues from both segments of FR-α, *i.e.* peptides 93–139 and 140–232, would be required to completely confer FR-α-like ligand binding characteristics to FR-β_{L49A}.

Role of Divergent Amino Acids in Peptide 93–139 of FR-α—To further map the functionally relevant amino acid(s) within peptide 93–139, additional chimeric constructs in which shorter segments of FR-α were incorporated into the FR-β_{L49A} mutant were designed (Fig. 2) to produce: FR-$\beta_{1-92}/\alpha_{93-125}/\beta_{126-236}$(L49A) and FR-$\beta_{1-92}/\alpha_{93-115}/\beta_{116-236}$(L49A). These two constructs showed functional characteristics similar to FR-$\beta_{1-92}/\alpha_{93-139}/\beta_{140-236}$(L49A). Furthermore, chimeras in which peptides 126–232 and 116–232 of FR-α were substituted in FR-β_{L49A} (Fig. 2) behaved similar to FR-$\beta_{1-139}/\alpha_{140-232}$(L49A). It is therefore reasonable to conclude that the peptide sequence 116–139 in FR-α has little significance in the differential ligand specificities of the FR isoforms and that peptide 93–115 must contain functionally significant residues.

Among the 23 residues in peptide 93–115, only four, at positions 96, 104, 106, and 115, are unconserved in FR-α. As noted previously, the two known murine FR isoforms type 1 and type 2 correspond to the human FR-α and FR-β, respectively, in terms of their ligand binding characteristics. Alignment of the amino acid sequences of peptides 93–115 of FRs from both human and murine (52) sources (Fig. 3) indicates that amino acid residues at positions 96 and 106 are conserved in the murine FR isoforms. Furthermore, the amino acid at position 115 is the same (glutamic acid) in both human FR-β and murine FR-1. Therefore if one assumes the presence of analogous structure-function relationships among human and murine FRs, it would appear that the likely candidates contributing to functional differences between FR-α and FR-β do not

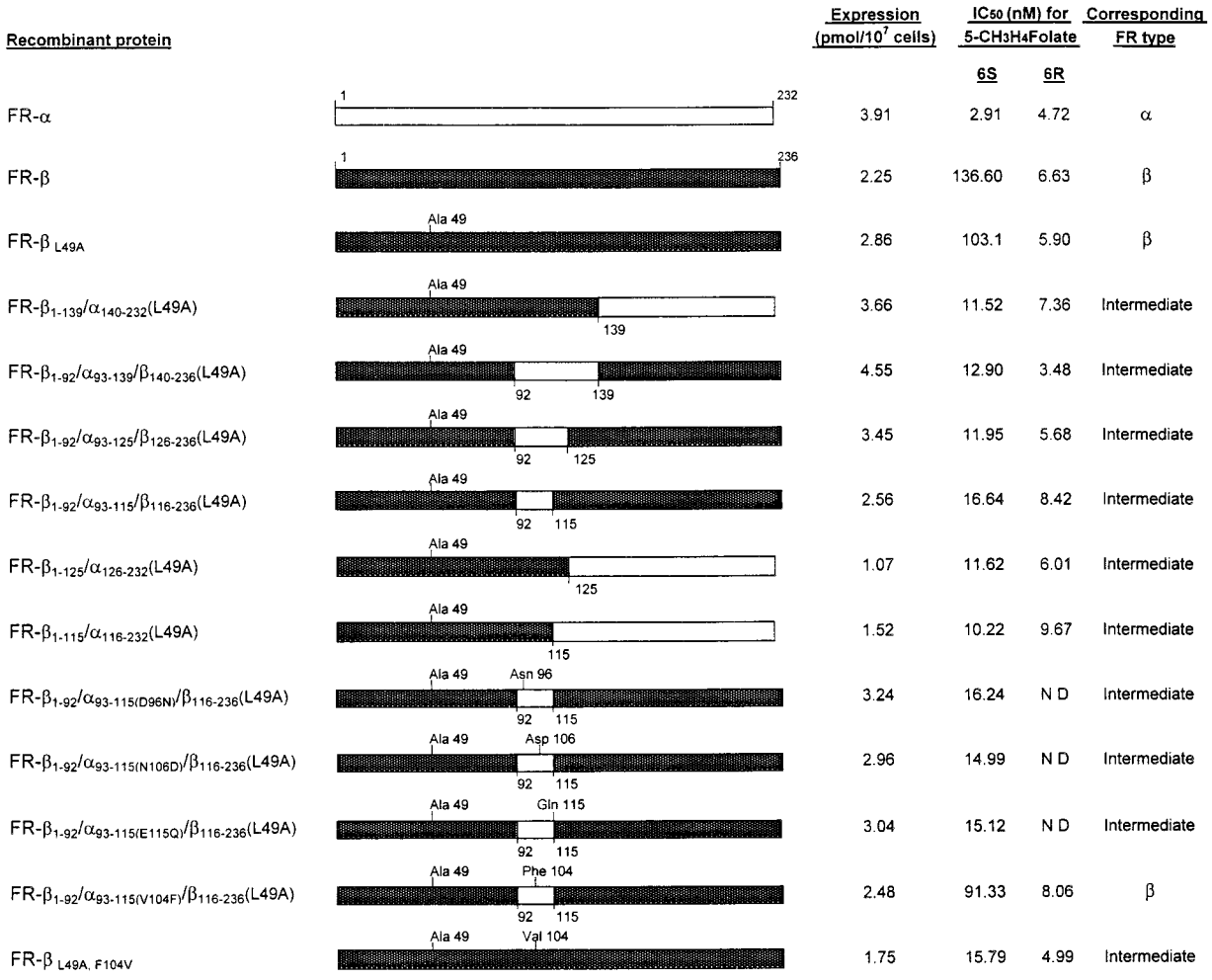

FIG. 2. **Characterization of chimeric and mutant forms of FR for mapping functional residues in peptide 93–139.** The *horizontal bars* represent the complete or partial polypeptide sequence of FR-α (*open bars*) or FR-β (*hatched bars*). The *numbers* on the diagram indicate the positions of amino acid residues in the protein after processing of the leader peptide. The *columns* at 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, the IC_{50} values for the diastereoisomers of 5-CH_3H_4folate, and the wild-type FR isoform to which they correspond functionally. The term *intermediate* refers to relative affinities for (6*S*)-5-CH_3H_4folate that are in between those of FR-α and FR-β. Transfection efficiencies were normalized to a co-transfected β-galactosidase standard. *ND* indicates not determined. From multiple determinations of IC_{50} values, percent error was <25.

	93	96	104	106	115
Human FR-α	Q Q V	**D** Q S W R K E R	**V**	**L**	**N** V P L C K E D C	**E**
Human FR-β	Q Q V	**N** Q S W R K E R	**F**	**L**	**D** V P L C K E D C	**Q**
Murine FR-1	Q Q V	**D** Q S W R K E R	**I**	**L**	**D** V P L C K E D C	**Q**
Murine FR-2	Q Q V	**D** Q S W R K E R	**F**	**L**	**D** V P L C K E D C	**H**

FIG. 3. **Alignment of amino acid sequences of human (26) and murine (52) FRs between amino acids 93 and 115.** The *numbers* indicate positions of amino acids. Residues that are different between FR-α and FR-β are in *bold face.*

occur at positions 96, 106, and 115. Indeed, when these amino acids in FR-$\beta_{1-92}/\alpha_{93-115}/\beta_{116-236}$(L49A) were individually changed back to the corresponding residues of FR-β, it was apparent that the amino acids at positions 96, 106, and 115 were inconsequential to the functional differences between FR-α and FR-β (Fig. 2); mutation of the amino acid at position 104, however, reverted the characteristics of the chimera to those of FR-β_{L49A}. Furthermore, the double mutant, FR-$\beta_{L49A,F104V}$, showed approximately 10-fold higher affinity for (6*S*)-5-CH_3H_4folate compared with FR-β (Fig. 2). These results clearly indicate that valine at position 104 in FR-α contributes partially to its distinct ligand binding properties.

Indentification of a Functionally Relevant Residue Downstream of Position 139 in FR-α—From the foregoing results it appears that to achieve complete conversion of FR-β to the functional equivalent of FR-α, it is necessary to substitute one or more residues downstream of position 139 in addition to creating L49A and F104V mutations in FR-β. To identify those residue(s), chimeras were first constructed in which FR-α peptide sequences 140–155 or 156–232 were substituted in the double mutant, FR-$\beta_{L49A,F104V}$ (Fig. 4). FR-$\beta_{1-139}/\alpha_{140-155}/\beta_{156-236}$(L49A,F104V) showed properties similar to those of FR-$\beta_{L49A,F104V}$ (Fig. 4). FR-$\beta_{1-155}/\alpha_{156-232}$(L49A,F104V), on the other hand, exhibited ligand binding specificity similar to FR-α (Fig. 4) thus mapping the relevant amino acid(s) to the sequence downstream of position 155. Since the carboxyl-terminal peptide downstream of the glycosylphosphatidylinositol modification site (Ser^{209}) in FR-α is absent in the mature protein (34), further mapping was restricted to peptide 156–208. Within this peptide only seven residues are unconserved between FR-α and FR-β. Alignment of the amino acid sequence of human and murine (52) FR isoforms (Fig. 5) shows that only three amino acids (positions 166, 167, and 191) are conserved between human FR-α and murine FR-1 and between human FR-β and murine FR-2. It was therefore undertaken to test the significance of those residues in contributing to functional differences between FR-α and FR-β. Mutation of Leu^{167} or Ser^{191} in FR-$\beta_{L49A,F104V}$ to Ile and Pro, respectively, the correspond-

Recombinant protein	Expression (pmol/10^7 cells)	IC_{50} (nM) for 5-CH_3H_4Folate 6S	6R	Corresponding FR type
FR-α	3.91	2.91	4.72	α
FR-β	2.25	136.60	6.63	β
FR-$\beta_{1-139}/\alpha_{140-155}/\beta_{156-236}$ (L49A, F104V)	1.37	8.10	3.48	Intermediate
FR-$\beta_{1-155}/\alpha_{156-232}$ (L49A, F104V)	1.41	3.58	8.57	α
FR-β L49A, F104V, L167I	3.23	10.17	7.07	Intermediate
FR-β L49A, F104V, S191P	1.61	10.07	5.68	Intermediate
FR-β L49A, F104V, G166E	2.43	3.84	5.02	α

FIG. 4. **Characterization of chimeric and mutant forms of FR for mapping functional residues downstream of position 139.** The *horizontal bars* represent the complete or partial polypeptide sequence of FR-α (*open bars*) or FR-β (*hatched bars*). The *numbers* on the diagram indicate the positions of amino acid residues in the protein after processing of the leader peptide. The *columns* at 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, the IC_{50} values for the diastereoisomers of 5-CH_3H_4folate, and the wild-type FR isoform to which they correspond functionally. The term *intermediate* refers to relative affinities for (6*S*)-5-CH_3H_4folate that are in between those of FR-α and FR-β. Transfection efficiencies were normalized to a co-transfected β-galactosidase standard. From multiple determinations of IC_{50} values, percent error was <25.

	156	161 162	165 166 167	169	191	208
Human FR-α	Y ... P	**T V** L C	**N E I** W	**T** H S Y ... W F D	**P**	A ... M
Human FR-β	Y ... P	**A A** L C	**E G L** W	**S** H S Y ... W F D	**S**	A ... M
Murine FR-1	Y ... S	**A A** L C	**E E I** W	**S** H S Y ... W F D	**P**	A ... M
Murine FR-2	Y ... P	**A S** L C	**E G L** W	**S** H S Y ... W F D	**S**	A ... M

FIG. 5. **Alignment of amino acid sequences of human (26) and murine (52) FRs between amino acids 155 and 208.** The *numbers* indicate positions of amino acids. Residues that are different between FR-α and FR-β are in *bold face*. The *dotted lines* indicate amino acid sequences that are identical between human FR-α and FR-β.

ing residues in FR-α, did not significantly alter the properties of the protein (Fig. 4). However, when Gly^{166} was substituted with Glu, the corresponding residue in FR-α, the ligand binding characteristics of FR-α were fully reconstituted in FR-$\beta_{L49A,F104V}$. Taken together, the results of this study indicate that alanine at position 49, valine at position 104, and glutamic acid at position 166 act synergistically to produce the distinguishing ligand binding characteristics of FR-α compared with FR-β.

A Structural Model of FR—FR shows considerable (~30%) amino acid sequence identity with the 219-residue chicken riboflavin-binding protein (cRBP) (53). The amino acid sequence alignment of the two proteins has been reported (46). The conserved amino acids include the 16 cysteine residues in FR and 6 tryptophan residues. The nine pairs of cysteine residues in cRBP form disulfide bridges to maintain its structural fold (54). In the three-dimensional structure of cRBP, the conserved tryptophan residues at positions 61, 91, 117, 131, 135, and 168 and tyrosine 82 stack into a hydrophobic pocket for binding the aromatic ring of the ligand (46). The sequence similarity between FR and cRBP implies structural similarity between these two protein families. We have built a homology model of the folate-binding domain of FR-α (Fig. 6) based on the crystal structure of cRBP (46). In the model, residues 104 and 166 are 13.9 Å apart and are respectively located inside and at the entrance of a cleft that corresponds to the ligand-binding pocket in cRBP. Residue 49 is peripheral to this cleft, 24.3 Å away from residue 104. The solvent accessible surface area of

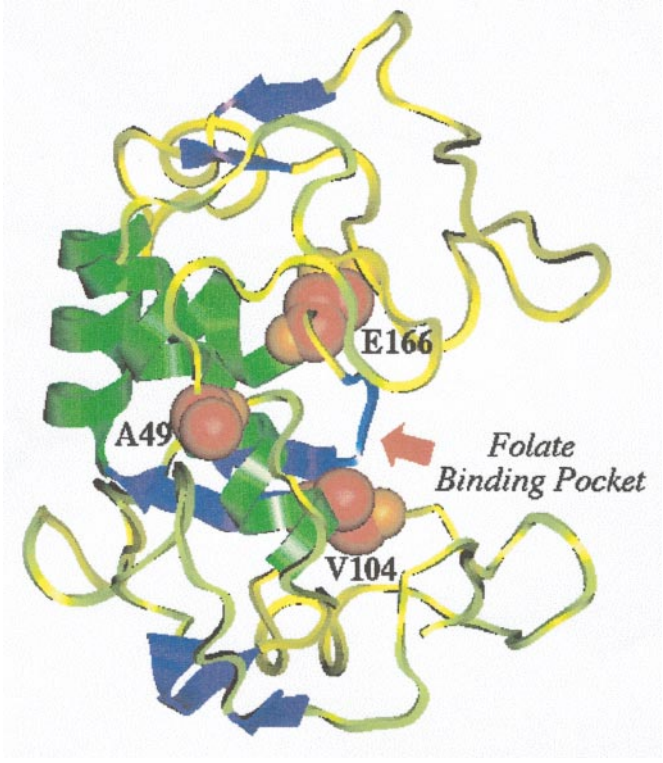

FIG. 6. **Homology model of the folate-binding domain of FR-α based on the crystal structure of the chicken riboflavin-binding protein.** The protein backbone is colored by the secondary structure: α helices in *green*, β sheets in *blue*, loops in *yellow*. Ala^{49}, Val^{104}, and Glu^{166} are highlighted with space-filling models and colored in *red*. The folate binding pocket is indicated by the *arrow*.

residues 49, 104, and 166 are 8.61, 27.5, and 91.7 $Å^2$, respectively, and their side chain fractional accessibilities are 0.11, 0.20, and 0.61, respectively. The model indicates that while conserved tryptophan and tyrosine residues may contribute to the affinity of ligand binding, residues 49, 104, and 166 may be

expected to play either a direct or an indirect role in causing the differential affinities and stereospecificities of FR-α and FR-β for the coenzyme forms of folate and for antifolate drugs.

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REFERENCES

1. Jansen, G., Westerhof, G. R., Kathmann, I., Rademaker, B. C., Rijksen, G. & Schornagel, J. H. (1989) *Cancer Res.* **49,** 2455–2459
2. Henderson, G. B. & Strauss, B. P. (1990) *Biochem. Pharmacol.* **39,** 2019–2025
3. Jansen, G., Schornagel, J. H., Westerhof, G. R., Rijksen, G., Newell, D. R. & Jackman, A. L. (1990) *Cancer Res.* **50,** 7544–7548
4. Jansen, G., Westerhof G. R., Kathmann, I., Rijksen, G. & Schornagel, J. H. (1991) *Cancer Chemother. Pharmacol.* **28,** 115–117
5. Westerhof, G. R., Jansen, G., van Emmerik, N., Kathmann, I., Rijksen, G., Jackman, A. L. & Schornagel, J. H. (1991) *Cancer Res.* **51,** 5507–5513
6. Westerhof, G. R., Schornagel, J. H., Kathmann, I., Jackman, A. L., Rosowsky, A., Forsch, R. A., Hynes, J. B., Boyle, F. T., Peters, G. J., Pinedo, H. M. & Jansen, G. (1995) *Mol. Pharmacol.* **48,** 459–471
7. Leamon, C. P. & Low, P. S. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 5572–5576
8. Turek, J. J., Leamon, C. P. & Low, P. S. (1993) *J. Cell Sci.* **106,** 423–430
9. Leamon, C. P. & Low, P. S. (1992) *J. Biol. Chem.* **267,** 24966–24971
10. Leamon, C. P. & Low, P. S. (1994) *J. Drug Targeting* **2,** 101–112
11. Leamon, C. P., Pastan, I. & Low, P. S. (1993) *J. Biol. Chem.* **268,** 24847–24854
12. Ladino, C. A., Chari, R. V., Bourret, L. A., Kedersha, N. L. & Goldmacher, V. S. (1997) *Int. J. Cancer* **73,** 859–864
13. Mathias, C. J., Wang, S., Lee, R. J., Waters, D. J., Low, P. S. & Green, M. A. (1996) *J. Nucl. Med.* **37,** 1003–1008
14. Mathias, C. J., Wang, S., Waters, D. J., Turek, J. J., Low, P. S. & Green, M. A. (1998) *J. Nucl. Med.* **39,** 1579–1585
15. Mathias, C. J. & Green, M. A., (1998) *Nucl. Med. Biol.* **25,** 585–587
16. Wang, S., Luo, J., Lantrip, D. A., Waters, D. J., Mathias, C. J., Green, M. A., Fuchs P. C. & Low, P. S. (1997) *Bioconj. Chem.* **8,** 673–679
17. Lee, R. J. & Low, P. S. (1994) *J. Biol. Chem.* **269,** 3198–3204
18. Lee, R. J. & Low, P. S. (1995) *Biochim. Biophys. Acta* **1233,** 134–144
19. Wang, S., Lee, R. J., Cauchon, G., Gorenstein, D. G. & Low, P. S. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 3318–3322
20. Douglas, J. T., Rogers, B. E., Rosenfeld, M. E., Michael, S. I., Feng, M. & Curiel, D. T. (1996) *Nat. Biotechnol.* **14,** 1574–1578
21. Kamen, B. A., Wang, M. T., Streckfuss, A. J., Peryea, X. & Anderson, R. G. W. (1988) *J. Biol. Chem.* **263,** 13602–13609
22. Leamon, C. P. & Low, P. S. (1993) *Biochem. J.* **291,** 855–860
23. Birn, H., Selhub, J. & Chrstensen, E. I. (1993) *Am. J. Physiol.* **264,** 302–310
24. Smart, E. J., Mineo, C. & Anderson, R. G. W. (1996) *J. Cell Biol.* **134,** 1169–1177
25. Wu, M., Fan, J., Gunning, W. & Ratnam, M. (1997) *J. Membr. Biol.* **159,** 137–147
26. Lacey, S. W., Sanders, J. M., Rotherburg, K. G., Anderson, R. G. W. & Kamen B. A. (1989) *J. Clin. Invest.* **84,** 715–720
27. Elwood P. C. (1989) *J. Biol. Chem.* **264,** 14893–14901
28. Ratnam, M., Marquardt, H., Duhring, J. L. & Freisheim, J. H. (1989) *Biochemistry* **28,** 8249–8254
29. Shen, F., Ross, J. F., Wang, X. & Ratnam, M. (1994) *Biochemistry* **33,** 1209–1215
30. Antony, A. C. (1992) *Blood* **79,** 2807–2820
31. Shen, F., Wang, H., Zheng, X. & Ratnam, M. (1997) *Biochem. J.* **327,** 759–764
32. Luhrs, C. A. & Slomiany, B. L. (1989) *J. Biol. Chem.* **264,** 21446–21449
33. Verma, K. S., Gullapalli, S. & Antony, A. C. (1992) *J. Biol. Chem.* **267,** 4119–4127
34. Yan, W. & Ratnam, M. (1995) *Biochemistry* **34,** 14594–14600
35. Shen, F., Wu, M., Ross, J. F., Miller, D. & Ratnam, M. (1995) *Biochemistry* **24,** 5660–5665
36. Campbell, I. G., Jones, T. A., Foulkes, W. D. & Trowsdale, J. (1991) *Cancer Res.* **51,** 5329–5338
37. Coney, L. R., Tomasetti, A., Carayannopoulos, L., Frasca, V., Kamen, B. A., Colnaghi, M. I. & Zurawski, V. R., Jr. (1991) *Cancer Res.* **51,** 6125–6132
38. Weitman, S. D., Lark, R. H., Corey, L. R., Fort, D. W., Frasca, V., Zurawski, V. R., Jr. & Kamen, B. A. (1992) *Cancer Res.* **52,** 3396–3401
39. Ross, J. F., Chauduri, P. K. & Ratnam, M. (1994) *Cancer* **73,** 2432–2443
40. Ross, J. F., Wang, H., Behm, F. G., Matthew, P., Wu, M., Booth, R. & Ratnam, M. (1999) *Cancer* **85,** 348–357
41. Wang, X., Shen, F., Freisheim, J. H., Gentry, L. E. & Ratnam, M. (1992) *Biochem. Pharmacol.* **44,** 1898–1901
42. Brigle, K. E., Spinella, M. J., Westin, E. H. & Goldman, I. D. (1994) *Biochem. Pharmacol.* **47,** 337–345
43. Shen, F., Zheng, X., Wang, J. & Ratnam, M. (1997) *Biochemistry* **36,** 6157–6163
44. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
45. Higgins, D. G., Thompson, J. D. & Gibson, T. J. (1996) *Methods Enzymol.* **266,** 383–402
46. Monaco, H. L. (1997) *EMBO J.* **16,** 1475–1483
47. Jones, T. A., Zou, J. Y., Cowan, S. W. & Kjeldgarrd, M. (1991) *Acta Crystallogr. Sect. A* **47,** 110–119
48. Navaza, J. (1994) *Acta Crystallogr. Sect. A* **50,** 157–163
49. Bruger, A. T. (1992) *X-PLOR, Version* 3.1, *A System for X-ray Crystallography and NMR,* Yale University Press, New Haven, CT
50. Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thronton, J. M. (1993) *J. Appl. Crystallogr.* **26,** 283–291
51. Lee, B. & Richards, F. M. (1971) *J. Mol. Biol.* **55,** 379–400
52. Brigle, K. E., Westin, E. H., Houghton, M. T. & Goldman, I. D. (1991) *J. Biol. Chem.* **266,** 17243–17249
53. Norioka, N., Okada, T., Hamazume, Y., Mega, T. & Ikenama, T. (1985) *J. Biochem.* (*Tokyo*) **97,** 19–28
54. Hamazume, Y., Mega, T. & Ikenaka, T. (1987) *J. Biochem.* (*Tokyo*) **101,** 217–223

Complete Mapping of Divergent Amino Acids Responsible for Differential Ligand Binding of Folate Receptors α and β

Karen M. Maziarz, Hugo L. Monaco, Feng Shen and Manohar Ratnam

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