Preferred Sites of Glycosylphosphatidylinositol Modification in Folate Receptors and Constraints in the Primary Structure of the Hydrophobic Portion of the Signal†

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ABSTRACT: The divergent carboxyl-terminal signal peptides for glycosylphosphatidylinositol (GPI) membrane anchor attachment in folate receptor (FR) types α and β were characterized. All of the candidate amino acid residues for GPI modification were identified and tested by substituting individually and in combination with amino acids that cannot be modified by GPI. Thus the GPI modification in FR-α was decreased to 22% by mutation of Ser234 to Thr but unaltered by changing the other candidate, Gly235, to Met. However, the double mutant FR-$\alpha_{Ser234-Thr,Gly235-Met}$ showed half of the GPI modification seen in FR-$\alpha_{Ser234-Thr}$. This result suggests that Ser234 is the preferred GPI modification site, while Gly235 is a minor, alternate GPI modification site. Similarly, in FR-β, mutation of Asn230 to Gln decreased GPI modification to 32%, while mutation of the other candidate site, Gly237, to Met had no effect. However, mutation at both sites further reduced the GPI modification by a half. A five amino acid carboxyl-terminal deletion (FR-βΔ5) caused no decrease in the extent of GPI modification. However, the same deletion in FR $\beta_{Asn230-Gln}$ decreased the residual GPI modification by 66%. These results suggest that Asn230 is the preferred GPI modification site in FR-β, while Gly235 offers a minor alternate modification site; consistent with this conclusion is the fact that modification at the downstream site is hindered by its proximity to the carboxyl terminus in FR-βΔ5. Further, the suggestion that the hydrophobic portion of the GPI signal is a random sequence of neutral amino acids with overall moderate hydrophobicity was tested. Mutation of Ala246, which is near the carboxyl terminus of FR-βΔ5, to Asp or Pro reduced GPI modification of FR-βΔ5 to 0% and 12%, respectively. This is the first demonstration of the influence of a neutral amino acid (proline) substitution in the hydrophobic region of the GPI signal even though the occurrence of proline is not uncommon in GPI signal peptides. The results suggest that (i) although candidate sites for GPI modification may occur adjacently or in close proximity on the linear sequence of the GPI signal, a single residue, Ser234 in FR-α and Asn230 in FR-β, is the preferred site of proteolysis/GPI attachment; however, (ii) the stringency of selection of the GPI modification site is limited because alternate sites may also be modified albeit to a limited extent; and (iii) the efficiency of recognition of the hydrophobic portion of the GPI signal may be constrained to a limited extent by its amino acid sequence. The uniqueness and efficiency of a GPI signal peptide may thus be determined by a combination of limited constraints involving various parts of the peptide.

Attachment of the glycosylphosphatidylinositol (GPI) membrane anchor to the carboxyl terminus of polypeptides occurs by a transamidation reaction within the endoplasmic reticulum following proteolytic cleavage of a relatively hydrophobic carboxyl-terminal peptide (Bangs et al., 1985, 1986; Ferguson et al., 1986). The signal for GPI modification has been characterized in a few model proteins and is composed of (i) a moderately hydrophobic carboxy-terminal region of 10−20 amino acids, (ii) the site of proteolysis/GPI modification, and (iii) a spacer of 8−12 amino acids that separates the modification site from the hydrophobic sequence (Ferguson et al., 1988; Cross, 1990; Moran et al., 1991). The hydrophobic region is believed to be characterized by a minimal length and a moderate overall hydrophobicity and not by the actual sequence of amino acids (Caras et al., 1991). Only certain amino acids can be modified efficiently, while others can be modified either partially or not at all (Moran et al., 1991). Detailed studies have also been reported on the effects of various amino acid substitutions, in the second and third positions after the modified residue, on the efficiency of GPI modification (Kodukula et al., 1993; Nuoffer et al., 1993). In general, only certain amino acid residues are tolerated at these positions. It has also been demonstrated that attachment of a hydrophilic polypeptide to the carboxyl terminus of the GPI signal does not prevent recognition of the signal (Caras, 1991).

The somewhat nonrestrictive nature of the present concept of the GPI signal peptide leads to the question of what features determine the specific recognition of only certain polypeptide protein domains as GPI signals. A clear understanding of structural requirements of the signal for GPI modification will also enable a better understanding of topographical and mechanistic aspects of the various components of the GPI modification machinery in the endoplasmic reticulum. Although the signal sequences for a diverse variety of GPI anchored proteins are published, a comprehensive analysis of the data is complicated by the fact that their relative efficiencies as GPI signals are not known; presumably, the same cellular machinery will modify vari-

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Table 1: Synthetic Oligonucleotides Used To Generate the Various Mutants

mutants	oligonucleotides used
FR-α_{G235-M}	CTATGCTGCAGCCATGAGTATGGCTGGGGC
	GGCAGGGATTTCTAGATATCAGGGCAG
FR-α_{S234-T}	CTATGCTGCAGCCATGACTGGGGCTGG
	GGCAGGGATTTCTAGATATCAGGGCAG
FR-$\alpha_{G235-M,S234-T}$	GGCAGGGATTTCTAGATATCAGGGCAG
	CTATGCTGCAGCCATGACTATGGCTGGGCC
FR-β_{G237-M}	CACAGCCAGCACCAGCCAGGAGCTG
	GATGCTTCATATGACTGGGG
	CCCCAGTCATATGAAGCATC
	GGGTTAGGTGACTTCTAGAAGCATGC
FR-β_{N230-Q}	CAGCCATGCATGTGCAAGCTGGTGAG
	GGGTTAGGTGACTTCTAGAAGCATGC
FR-$\beta_{G237-M,N230-Q}$	CACAGCCAGCACCAGCCAGGAGCTG
	GATGCTTCATATGACTGGGG
	CCCCAGTCATATGAAGCATC
	GATTTAGGTGACACTATAG
FR-$\beta\Delta5$	CGACTTAAGCTTGGCCTGGATCTATTGCCTAC
	TCAGCCAAGTCTAGACTATTGCAGCATCAG
FR-$\beta\Delta5_{N230-Q}$	CAGCCATGCATGTGCAAGCTGGTGAG
	TCAGCCAAGTCTAGACTATTGCAGCATCAG
FR-$\beta\Delta5_{A246-D}$	CAGTCTGGACCTGATGCTG
FR-$\beta\Delta5_{A246-P}$	CGACTTAAGCTTGGCCTGGATCTATTGCCTAC
	TCAGCCAAGTCTAGACTATTGCAGCATCAGGGGCAGACTGAG

ously expressed proteins with a wide range of efficiencies, adequate for their biological function. Mutational analysis of model proteins, therefore, assumes importance in studies of the GPI signal.

For the present study, we chose the two major human folate receptor (FR) isoforms, type α (Lacey et al., 1989) and type β (Ratnam et al., 1989). The receptors are ~28 000 Da polypeptides that are N-glycosylated and bind [^{3}H]folic acid with a high affinity (K_d < 1 nM) and with a 1:1 stoichiometry (Antony, 1992). The two proteins have an amino acid sequence identity of about 70% but show poor homology in the carboxyl terminal regions (Ratnam et al., 1989; Lacey et al., 1989). FR-α and FR-β are differentially tissue specific. In general, FR-α is expressed in epithelial cells and is elevated in malignancies of epithelial origin, whereas FR-β is elevated in malignancies of nonepithelial origin (Ross et al., 1994). The proteins are attached to the plasma membrane by GPI membrane anchors (Lacey et al., 1989; Luhrs & Slomiany, 1989; Verma et al., 1992). A third FR isoform, type γ, that is expressed in hematopoietic tissues is constitutively secreted because of a divergence of its carboxyl-terminal sequence (Shen et al., 1994, 1995).

In this report, amino acid residues in the carboxyl-terminal regions of FR-α and FR-β were substituted individually and in combination by site-directed mutagenesis. The degree of GPI modification in each mutant protein, relative to the corresponding wild-type protein, was determined by measuring the phosphatidylinositol-specific phospholipase C (PI-PLC), sensitive [^{3}H]folic acid binding protein expressed on the cell surface. Protein expression and PI-PLC cleavage were also monitored by Western blotting. Herein, we discuss information on structural constraints within the GPI signal sequence obtained by characterizing the mutant proteins in relation to their capacity for GPI modification.

MATERIALS AND METHODS

Materials. Oligonucleotides were made by Genosys Biotechnologies, Inc. Lipofectamine and opti-MEM I reduced serum medium were purchased from Gibco BRL. MEM medium (powder) and fetal bovine serum (FBS) were purchased from Irvine Scientific. Phosphatidylinositol-specific phospholipase C (PI-PLC) was from Boehringer Mannheim Biochemicals. [^{3}H]Folic acid (specific radioactivity, 27 Ci/mmol) was from Moravek Biochemicals, Brea, CA. Alkaline phosphatase conjugated goat anti-rabbit IgG was purchased from Promega. Vent and Taq DNA polymerase were purchased from New England Biolabs and Perkin Elmer Cetus, respectively.

Antibodies. Rabbit antisera to purified FR from human placenta were obtained as described (Ratnam et al., 1989). The antibodies specific for FR were affinity purified using immobilized FR as described (Ratnam et al., 1989).

Mutagenesis and Recombinant Plasmids. All mutants were constructed using the polymerase chain reaction (PCR). The reactions were performed using Vent or Taq DNA polymerase with the cDNA for FR-α or FR-β as templates. The PCRs were primed in two alternate ways. In some cases, a mutagenic oligonucleotide encompassing an appropriate restriction site was used as the downstream primer and the upstream primer was an oligonuclotide encompassing another restriction site. In cases where no suitable restriction site was found downstream from the mutation locus, two complementary oligonucleotides containing the mutation were used in combination with upstream and downstream primers containing appropriate restriction sites. The PCR products were purified by agarose gel electrophoresis and digested at either end with the appropriate restriction enzymes. FR-β or FR-α cDNAs inserted in the polylinker of the plasmid pcDNA1Neo (Invitrogen) were digested with the same restriction enzymes, and the PCR products were appropriately subcloned into them. Plasmid DNA was amplified in *Escherichia coli* MC1061 and purified using the Qiagen plasmid kit (Qiagen). For each mutant cDNA, the entire DNA sequence was verified by dideoxy sequencing using Sequenase Version 2.0 (USB). The synthetic oligonucleotides used in generating the various mutants are listed in Table 1.

Cell Culture and Transfection. Human 293 fibroblasts were maintained in MEM medium supplemented with FBS (10% v/v), penicillin (100 units/mL), streptomycin (100 μg/

mL) and L-glutamine (2 mM). Transfections using cDNAs cloned into the polylinker region of plasmid pcDNA1Neo were carried out in six-well tissue culture plates (Corning) using lipofectamine (Gibco BRL), according to the manufacturer's protocol. In negative controls, cells were transfected with the plasmid carrying no insert. The cells were analyzed 48 h after transfection.

Folate Binding Assay. Transfected cells in each tissue culture well were washed with 1 mL of 10 mM sodium phosphate buffer, pH 7.5/150 mM NaCl (PBS), then with 1 mL of ice-cold acid buffer (10 mM sodium acetate, pH 3.5/150 mM NaCl), and again with PBS. PBS (1 mL) containing a mixture of 5 pmol of [^{3}H]folic acid and 25 pmol of unlabeled folic acid was added at 4 °C and incubated for 30 min. The cells were then washed twice with 1 mL of ice-cold PBS. Acid buffer (1 mL) was used to extract cell surface bound [^{3}H]folic acid and the radioactivity measured by liquid scintillation counting. The specificity of [^{3}H]folic acid binding was established by measuring the binding of the radiolabel after prior incubation with 100 nM unlabeled folic acid. Another negative control included cells that were either untranfected or transfected with vector alone.

Treatment of Cells with PI-PLC. Cells in six-well tissue culture plates were washed twice with 1 mL of PBS and once with PI-PLC buffer (50 mM Tris-HCl, pH 7.5/10 mM EDTA). Subsequently, duplicate wells were treated for 1 h at 37 °C with either 1.0 mL of PI-PLC buffer containing 0.3 unit of PI-PLC or with the buffer alone.

Immunoblotting. Cells (1×10^6) were scraped from the tissue culture wells and dissolved in 30 μL of PBS containing 0.5% Triton X-100. 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 (20 μL per well) were electrophoresed on 12.5% SDS–polyacryalmide gels and transferred to nitrocellulose paper. The blots were probed with affinity-purified anti-FR antibodies as described (Ratnam et al., 1989).

RESULTS AND DISCUSSION

Control of Transfection Efficiencies. In the foregoing experiments, several precautions were taken to ensure that the relative expression of each wild-type and mutant FR on the surface of transfected cells was due to structural differences between the wild-type and mutant proteins and not due to possible differences in transfection efficiencies of the corresponding cDNAs. *A priori*, since all of the cDNAs were inserted in the same expression vector and since the mutant cDNAs contain only minor nucleotide substitutions or deletions, the cDNAs would not be expected to display inherent differences in transfection efficiencies or in protein expression levels. However, variations in transfection efficiencies between different purification batches of the same plasmid or between similar transfections conducted in separate experiments are known to occur.

When the expression of cell-surface-associated [^{3}H]folic acid binding was studied as a function of the amount of plasmid DNA used for transfection, a pattern similar to that depicted in Figure 1 was obtained for FR-α, FR-β, and the corresponding FR mutants that were capable of binding to

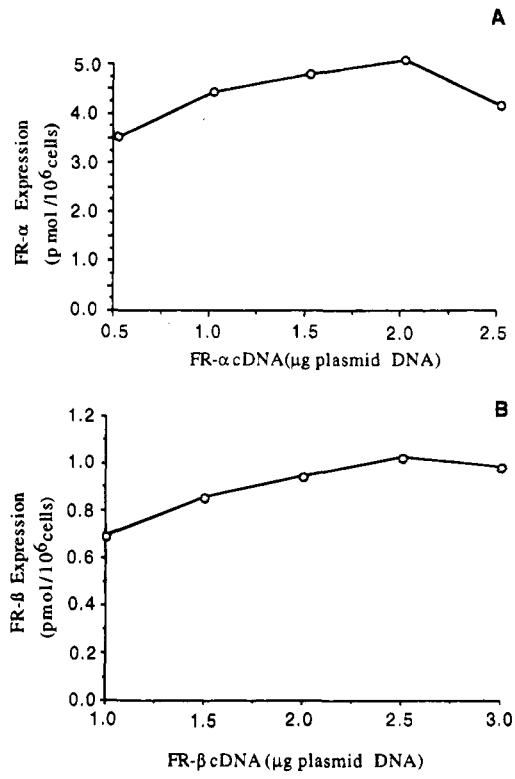

FIGURE 1: Relationship between FR expression and the amount of plasmid DNA used for transfection. Different amounts of plasmid DNA containing the cDNA for FR-α (panel A) or FR-β (panel B) were used to transfect human 293 fibroblasts as described under Materials and Methods. [^{3}H]Folic acid binding protein on the surface of transfected cells was assayed as described under Materials and Methods. For the mutant constructs of FR-α and FR-β, the plateau in transfection efficiency was reached at cDNA concentrations similar to those of the corresponding wild-type protein. Standard error < 10%.

[^{3}H]folic acid (see below). In Figure 1, similar FR expression levels were obtained between 1 and 2 μg of DNA for FR-α and between 1.5 and 3 μg of DNA for FR-β. Therefore, by using 1.5 or 2.0 μg of DNA for all of the transfection experiments described below, it was ensured that small differences in transfection efficiencies between different plasmids in the same experiment did not result in significant differences in protein expression levels.

In Figure 2, an amount of plasmid containing the β-galactosidase gene (internal control) within its linear range of transfection efficiency was used to cotransfect cells simultaneously with the wild-type and mutant FRs described below; constancy in the expression of β-galactosidase activity by the transfected samples was taken as futher proof of uniform transfection efficiencies. It should be noted that in Figure 2, while the β-galactosidase control plasmid was used at a concentration that was in the linear range of transfection efficiency, the FR cDNAs were in the plateau range. Thus, small variations in β-galactosidase activity, if any, would reflect much smaller differences in the expression of wild-type and mutant FRs.

As further precautions, all of the plasmids used in the transfection experiments were purified in an identical fashion. FR cDNAs being compared were transfected simultaneously in each experiment. All of the transfections and subsequent quantitations of cell-surface FR expression and Western blot

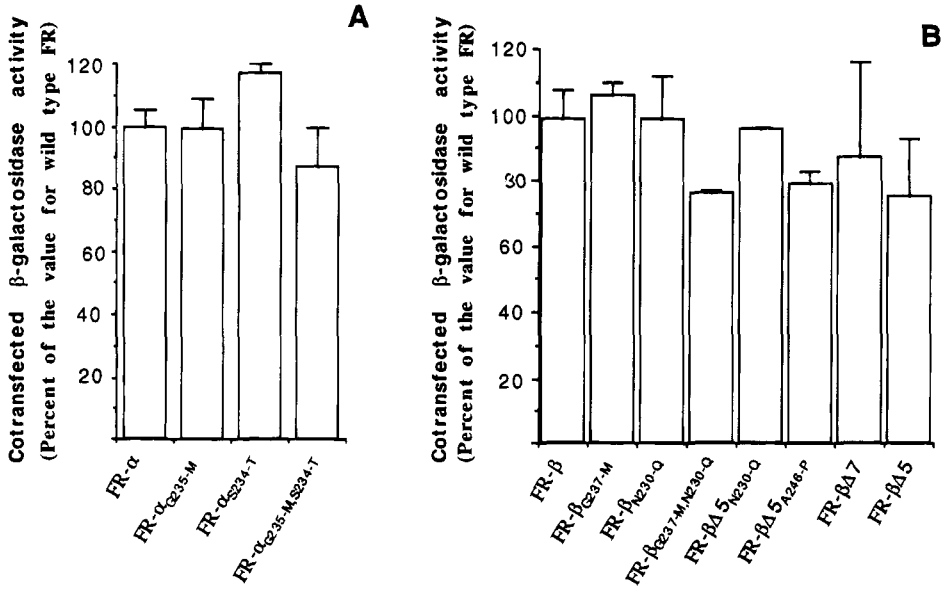

FIGURE 2: Transfection efficiencies of wild-type and mutant FR cDNA constructs. Human 293 fibroblasts were cotransfected with the cDNA for β-galactosidase and with individual wild-type and mutant forms of FR-α cDNA (panel A) or with wild-type and mutant forms of FR-β cDNA (panel B) as described under Materials and Methods. Cell lysates were then prepared and assayed for β-galactosidase activity as described under Materials and Methods.

analyses were repeated at least four times, and concordant results were obtained.

Primary Sites of GPI Modification in FR-α and FR-β. It has been demonstrated that certain amino acid residues (Gly, Ala, Asp, Ser, Asn) are efficiently modified by GPI and that a few residues (Cys, Val) are modified with a relatively low efficiency. Other amino acid residues cannot serve as the site of GPI modification. It has also been demonstrated (Kodukula et al., 1993; Moran et al., 1991; Nuoffer et al., 1993) that certain uncharged residues are tolerated at positions +1 and +2 after the GPI modification site (Gly, Ala, Arg, Asp, Cys, Asn, Gln, Met, Ser, and Thr at +1; Ala, Gly, Ser, and Thr at +2), while several other residues are not. When this information was combined with previous mass spectrometric data (Zhang et al., 1994) that bracketed the GPI modification sites in FR-α and FR-β to within 10 amino acids (Ser234 to Pro243 and His228 to Gly237, respectively), it was possible to designate candidate GPI modification sites. These sites are Ser234 and Gly235 in FR-α and Asn230 and Gly237 in FR-β. The rationale for determining candidate GPI modification sites in FR-α and FR-β is detailed in Table 2. In order to pinpoint the exact GPI modification sites, the candidate GPI modification sites in FR-α and FR-β were substituted individually with amino acids that could not themselves be modified and, furthermore, that could not interfere with modification of neighboring residues (Kodukula et al., 1993; Moran et al., 1991; Nuoffer et al., 1993). In all of these mutants, the amino acid substitutions were chosen in order to be as conservative as possible. In making these mutants care was also taken to substitute with amino acids that could not be modified even with a very low efficiency. Furthermore, it was ensured that the substituting amino acid chosen was not capable of positional effects on GPI modification at neighboring sites. Accordingly, the mutants, FR-$\alpha_{Ser234-Thr}$, FR-$\alpha_{Gly235-Met}$, FR-$\beta_{Asn230-Gln}$, and FR-$\beta_{Gly237-Met}$ were constructed.

As seen in Figure 3, the cell-surface expression of FR-α was unaltered by conversion of Gly235 to Met but decreased to 22% of wild type when Ser234 was changed to Thr. The cell-surface FR in both cases was sensitive to PI-PLC treatment. Western blot analysis of total cell membranes prior to and after treatment with PI-PLC (Figure 3) is consistent with the cell-surface [^{3}H]folic acid binding data. The PI-PLC-sensitive cell-surface FR-α is represented by a diffuse band at M_r ~40 000 Da on the Western blot (Figure 3) which also shows PI-PLC-insensitive bands that presumably arise from immature protein inside intracellular compartments. (Such additional bands may actually be consid-

Table 2: Rationale for Selecting Candidate Sites for GPI Modification of FR-α and FR-β within a 10 Amino Acid Window[a]

FR isoform	residues	candidate for GPI modification	reasons[b]
FR-α	Ser234	yes	no obstruction
	Gly235	yes	no obstruction
	Ala236	no	Pro at ω + 2
	Gly237	no	Pro at ω + 1, Trp at ω + 2
	Pro238	no	unmodifiable
	Trp239	no	unmodifiable
	Ala240	no	Trp at ω + 2
	Ala241	no	Pro at ω + 2
	Trp242	no	unmodifiable
	Pro243	no	unmodifiable
FR-β	His228	no	unmodifiable
	Val229	no	unmodifiable
	Asn230	yes	no obstruction
	Gly231	no	Glu at ω + 1
	Glu232	no	unmodifiable
	Met233	no	unmodifiable
	Leu234	no	unmodifiable
	His235	no	unmodifiable
	Gly236	yes	no obstruction
	Thr237	no	unmodifiable

[a] From mass spectrometric analysis, the peptide sequences upstream of Ser234 in FR-α and His228 in FR-β were identified in the purified proteins (Zhang et al., 1994). The mass spectrometric analysis also confirmed the absence of residues downstream of Pro243 in FR-α and Thr237 in FR-β in the mature proteins. Furthermore, it has been established that the GPI-modified residue should be at least 18 amino acids from the free carboxyl terminus (Caras et al., 1991). [b] Based on reports of Moran et al. (1991), Kodukula et al. (1993), and Nuoffer et al. (1993).

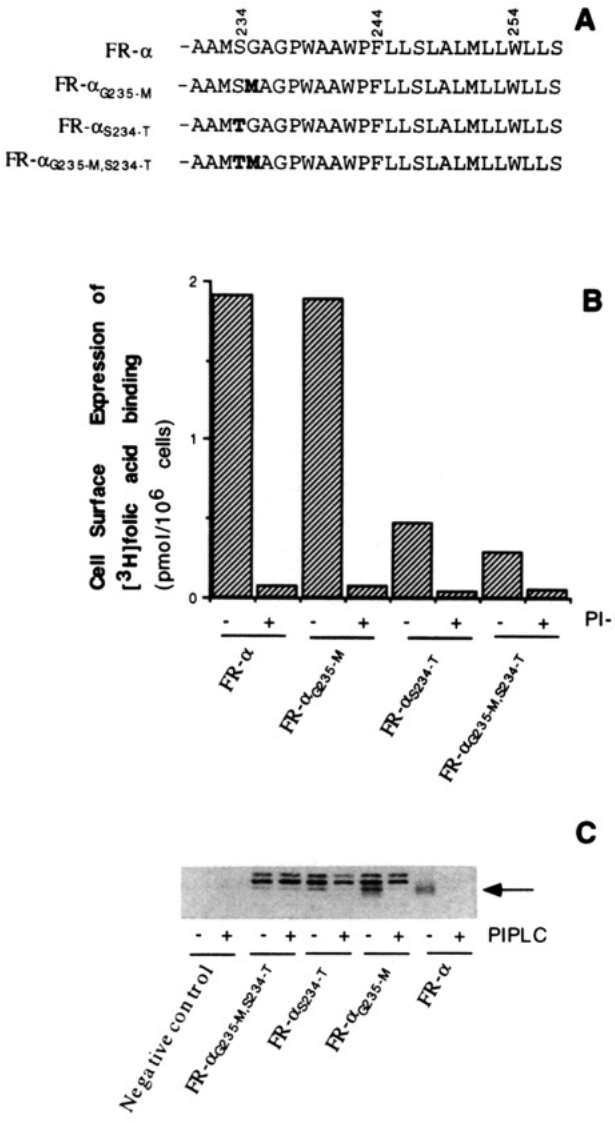

FIGURE 3: Mutation of candidate GPI modification sites in FR-α. (A) Two candidate sites in FR-α—Ser234 and Gly235—were mutated respectively to Thr and Met either individually or together. Amino acids indicated by bold letters represent mutations. The numbers indicate positions of amino acid residues. (B) FR-α and mutant FR-α cDNA-plasmid constructs were transfected in human 293 fibroblasts as described under Materials and Methods. The transfected cells were treated with or without PI-PLC followed by [^{3}H]folic acid binding assay as described under Materials and Methods. Each experiment was repeated four times in duplicate, and similar results were obtained. Standard error <10%. (C) After the binding assay, cells were scraped and dissolved in 10 mM sodium phosphate buffer, pH 7.5/150 mM NaCl containing 1% Triton X-100. After the detergent-insoluble residue was sedimented, the cell lysates were subjected to Western blot analysis, using antibodies to placental FR as described under Materials and Methods. The arrow indicates the position of PI-PLC-sensitive bands. The negative control represents cells transfected with vector alone.

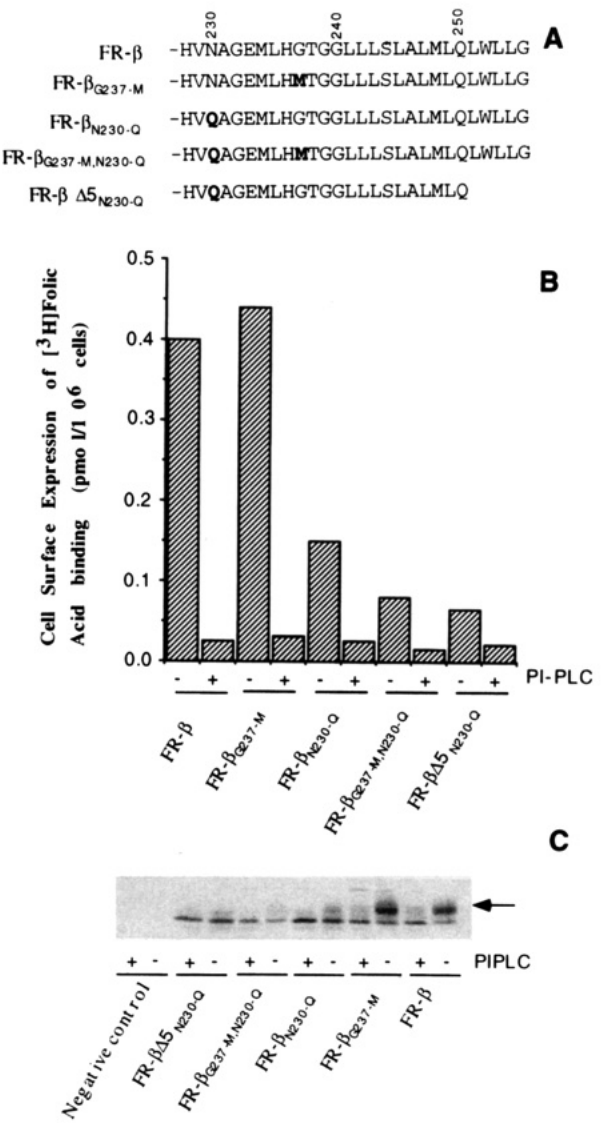

FIGURE 4: Mutation of candidate GPI modification sites in FR-β. (A) Two candidate sites in FR-β—Gly237 and Asn235—were mutated respectively to Met and Gln either individually or together. Asn230 was mutated to Gln in FR-βΔ5. Amino acids indicated by bold letters represent mutations. The numbers indicate positions of amino acid residues. (B) FR-β and mutant FR-β cDNA-plasmid constructs were transfected in human 293 fibroblasts as described under Materials and Methods. Treatment of the transfected cells with or without PI-PLC was followed by the [^{3}H]folic acid binding assay as described under Materials and Methods. Each experiment was repeated four times in duplicate, and similar results were obtained. Standard error <10%. (C) After the binding assay, cells were scraped and dissolved in 10 mM sodium phosphate buffer, pH 7.5/150 mM NaCl containing 1% Triton X-100. After the detergent-insoluble residue was sedimented, the cell lysates were subjected to Western blot analysis, using antibodies to placental FR as described under Materials and Methods. The arrow indicates the position of the PI-PLC-sensitive bands. The negative control represents cells transfected with vector alone.

ered as indicators of protein expression for the mutant constructs). Ser234 is thus most likely the primary site of GPI modification in FR-α. A similar analysis of wild-type and mutant forms of FR-β (Figure 4) showed that while substitution of Gly237 with Met had no effect on its expression and PI-PLC sensitivity, mutation of Asn230 to Gln decreased FR-β expression to 23% of wild type. Thus, Asn230 appears to be the primary GPI modification site in FR-β.

Secondary or Alternate Sites of GPI Modification in FR-α and FR-β. The data in Figures 3 and 4 indicated that individual mutations at Gly235 in FR-α and Gly237 in FR-β to Met did not affect the GPI modification efficiencies of FR-α and FR-β, respectively. Thus, Ser234 in FR-α and Asn230 in FR-β are the preferred sites for GPI modification to the exclusion of the other candidates, Gly235 and Gly237, and possibly additional sites in their proximity that are intrinsically capable of GPI modification with lower ef-

ficiencies. However, in both FR-$\alpha_{Ser234-Thr}$ and FR-$\beta_{Asn230-Gln}$, there is residual GPI modification of 22% and 23%, respectively, although Thr and Gln residues should not be modified by GPI even with a low efficiency (Moran et al., 1991; Kodukula et al., 1993; Nuoffer et al., 1993).

In order to identify possible secondary sites of GPI modification in FR-α and FR-β, the double mutants FR-$\alpha_{Ser234-Thr,Gly235-Met}$ and FR-$\beta_{Asn230-Gln,Gly237-Met}$ were generated. The corresponding second mutation further decreased GPI modification in FR-$\alpha_{Ser234-Gly}$ and in FR-$\beta_{Asn230-Gln}$ by 40% and 50%, respectively (Figures 3 and 4). Furthermore, a carboxyl-terminal deletion of five amino acids did not decrease GPI modification of wild-type FR-β (discussed below) but caused a further 66% reduction of the residual GPI modification in FR-$\beta_{Asn230-Gln}$ (Figure 3). Thus when Ser235 in FR-α and Asn230 in FR-β are unavailable, residual modification occurs at other sites, which include but may not be limited to Gly235 in FR-α and Gly237 in FR-β. Consistent with this hypothesis, deleting the five carboxyl-terminal residues in FR-$\beta_{Asn230-Gln}$ further decreased GPI modification, presumably by affecting minor downstream modification site(s) that was(were) brought too close to the free carboxyl terminus in the deleted construct.

Therefore, while a single amino acid residue is the preferred site for GPI modification, vis-à-vis its exact location in the GPI signal sequence, the GPI signal affords a limited amount of flexibility in providing secondary modification sites. The lack of recognition of Gly235 in FR-α as a significant site for GPI attachment is particularly intriguing. Even when the adjacent modification site (Ser234) is abolished, Gly235 accounts for only some of the residual modification. Glycine and serine residues, however, are both intrinsically capable of GPI modification (Kodukula et al., 1993). Gly235 in FR-α also occurs within the established distance limits in relation to the downstream hydrophobic sequence (Coyne et al., 1993). Since amino acid residues upstream of the GPI modification site are unimportant for the modification, it may be concluded that the choice of Ser234 as the modification site in FR-α is dictated by its exact location in relation to downstream elements in the GPI signal.

Structural Constraints in the Hydrophobic Portion of the GPI Signal Peptide. Previous reports on the distance requirements between the hydrophobic signal sequence and the modification site have shown that they should be separated by a spacer of at least eight amino acids. However, the upper limit for this distance is not so well defined, with the GPI modification efficiency tapering off above a spacer length of 11 amino acids (Coyne et al., 1993). Since many GPI-anchored proteins have rather long hydrophobic stretches in their GPI signal peptides, it is conceivable that overlapping or distinct hydrophobic peptides within this region may serve the same role in signaling GPI modification. Therefore, in order to study the structural requirements of the hydrophobic signal sequence in FR by mutagenesis, it was first necessary to reduce this sequence to a minimal length that was still functional. By systematic deletion of amino acids from the carboxyl terminus, it was established that up to five amino acids could be deleted from the carboxyl terminus of FR-β without decreasing GPI modification efficiency (Figure 5).

It is generally believed that the hydrophobic signal sequence for GPI-anchor attachment is independent of the exact amino acid sequence but is, rather, dependent upon

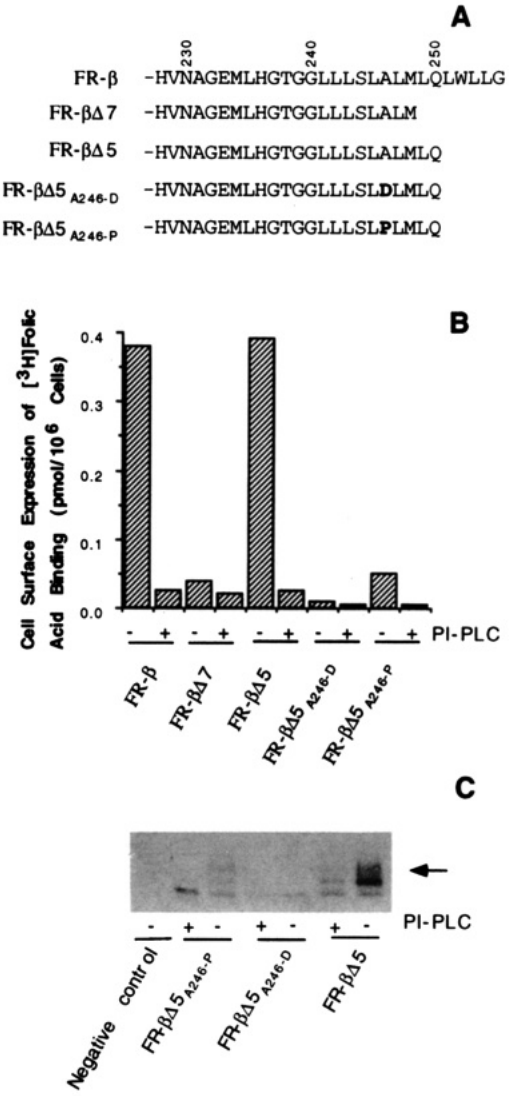

FIGURE 5: Mutation of the hydrophobic region in the GPI signal peptide of FR-β. (A) Seven and five amino acids were deleted at the carboxyl terminus of FR-β. Ala246 was mutated to Asp or Pro in FR-$\beta\Delta5$. Amino acids indicated by bold letters represent mutations. The numbers indicate positions of amino acid residues. (B) FR-β and mutant FR-β cDNA-plasmid constructs were transfected in human 293 fibroblasts as described under Materials and Methods. Treatment of transfected cells with or without PI-PLC was followed by the [^{3}H]folic acid binding assay as described under Materials and Methods. Each experiment was repeated four times in duplicate, and similar results were obtained. Standard error <10%. (C) After the binding assay, cells were scraped and dissolved in 10 mM sodium phosphate buffer, pH 7.5/150 mM NaCl containing 1% Triton X-100. After the detergent-insoluble residue was sedimented, the cell lysates were subjected to Western blot analysis using antibodies to placental FR as described under Materials and Methods. The arrow indicates the position of PI-PLC-sensitive bands. The negative control represents cells transfected with vector alone.

the physical properties of the peptide (i.e., its hydrophobicity) (Caras, 1991). The degree of hydrophobicity of this sequence may vary. Thus, within a stretch of 10−20, uncharged amino acid residues, up to 30% of the residues may be polar (Ferguson, 1988; Udenfriend et al., 1991). However, this signal sequence is very sensitive to single substitutions with charged residues (Lowe, 1992). Even when charged residues do exist close to the carboxyl terminus prior to GPI modification in a few proteins, a relatively hydrophobic sequence of at least about 10 contiguous

uncharged residues is seen at an appropriate distance from the GPI modification site (Ferguson, 1988). Consistent with these observations, substitution of Ala246 in FR-$\beta\Delta5$ with Asp abolished GPI modification (Figure 5), indicating that this position is an essential part of the hydrophobic region. In order to determine whether it is the introduction of charge *per se* at this position that affected its function as a GPI signal, an unconserved but neutral substitution to proline was made at this position. Proline was chosen both because of the nonconservative nature of the substitution and because it would be predicted to disrupt an α-helical element of seven residues at the carboxyl terminus of FR-$\beta\Delta5$. The proline substitution dramatically decreased GPI modification of FR-$\beta\Delta5$ by 84% (Figure 5). This is the first demonstration of the adverse effect of a single neutral amino acid substitution within the hydrophobic GPI signal sequence. However, it should be noted that the occurrence of proline is not uncommon in the hydrophobic portion of GPI signal peptides. This observation contradicts the view that the hydrophobic portion of the GPI signal peptide is a random sequence of amino acids of moderate to high overall hydrophobicity. On the other hand, in addition to overall hydrophobicity, specific structural (or polarity) constraints within small segments may dictate whether a hydrophobic peptide will serve to signal GPI modification efficiently. Thus, for example, although proline occurs commonly in the hydrophobic GPI signal sequences of various proteins, this residue may not be well tolerated in a putative subdomain within the hydrophobic sequence. Further analysis by systematic substitution at Ala246 and neighboring positions in FR-$\beta\Delta5$ with various amino acids should help pinpoint the exact structural requirements in this region of the GPI signal.

The results of this study suggest that the function of a GPI signal peptide may be governed by complex structural requirements involving both the hydrophobic sequence and the modification site. Although individual elements of the GPI signal may have a limited flexibility, their combination could determine the overall stringency of the GPI signal structure.

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