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Molecular Requirements for Attachment of the Glycosylphosphatidylinositol Anchor to the Human Alpha Folate Receptor

Antonella Tomassetti,* Federica Bottero, Mimma Mazzi, Silvia Miotti, Maria I. Colnaghi, and Silvana Canevari

Experimental Oncology E, Istituto Nazionale per lo Studio e la Cura dei Tumori, 20133 Milano, Italy

Abstract The α isoform of the folate receptor (FR) is a 38-KDa glycosylphosphatidylinositol (GPI) protein which mediates the internalization of folates. The FR amino acid sequence has features typical of GPI-linked proteins, including the presence of a hydrophobic carboxyl-terminus, a hinge region, and a stretch of small and uncharged amino acids. Substitution of predicted cleavage/attachment Ser_{234} with arginine or threonine, or replacement of Gly_{235} with proline by site-directed mutagenesis had no effect on GPI processing. In fact, CHO cells transfected with each of the three cDNA variants or with FR wild-type showed comparable amounts of phosphatidylinositol-specific phospholipase C-resistant FR in double-determinant radioimmunoassay. Western blot analysis of total cell lysates from all transfectants consistently revealed the 38-KDa FR band. Deletion of residues 233–237 in the amino-terminal portion of the FR cDNA constructs derived by a polymerase chain reaction strategy abrogated GPI processing, with only a small proportion of the FR remaining in the cytoplasm in four of the five clones tested. This finding suggests that FR residues 233–237 are essential in properly juxtaposing the FR hydrophobic domain. Together, these data support the hypothesis that the postulated Ser_{234} is not the only potential cleavage/attachment site of the α isoform of FR. J. Cell. Biochem. 72:111–118, 1999.

Key words: alpha-folate receptor; glycosylphosphatidylinositol anchor; cleavage/attachment site; site-directed mutagenesis; membrane receptor

A minor proportion (6–10%) of cell surface proteins is inserted into the membrane through a tail of phosphoinositol, carbohydrates and ethanolamine called the glycosylphosphatidylinositol (GPI) anchor, which is attached to the last amino acid of the COOH-terminus of the protein [Udenfriend et al., 1995b]. More than 150 GPI proteins are known, including membrane regulatory proteins, adhesion molecules, enzymes, receptors, and other cell surface species of as yet unknown function. Processing to a GPI anchor takes place in the endoplasmic reticulum (ER), where the nascent protein is first glycosylated and then annexed to the presynthesized GPI tail. A nascent peptide destined to become GPI-anchored must have a hydrophobic NH_2-terminus to direct it to the ER and a hydrophobic COOH-terminus which is cleaved in the ER by a transpeptidase and replaced with the GPI-tail [Amthauer et al., 1993; Field et al., 1994].

Despite extensive efforts to determine the nature of the cleavage/attachment site, also called the ω site, [Udenfriend et al., 1995b; Moran et al., 1991b], many of the molecular details remain unclear. Analysis of cDNA-deduced sequences and transfection of mutated or engineered cDNAs into intact cells, as well as analysis using in vitro translation systems, have all suggested likely cleavage/attachment sites of the GPI moiety. The COOH-terminal signal peptide typically comprises a terminal hydrophobic tail of 10–15 amino acids, a proline-rich hinge region, and a cleavage/attachment site of three small uncharged amino acids. Studies on placental alkaline phosphatase have contributed to defining rules that predict the amino acid residues of the nascent protein likely to accept the GPI moiety. Only six amino acids (serine, glycine, alanine, aspartic acid, asparagine, and cysteine) appear to be allowable at

*Correspondence to: Antonella Tomassetti, Experimental Oncology E, Istituto Nazionale per lo Studio e la Cura dei Tumori, Via Venezian 1, 20133 Milano, Italy.

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the ω site and three (alanine, glycine, and serine) at the ω + 2, whereas all amino acids except proline and tryptophan potentially occupy the ω + 1 site [Udenfriend et al., 1995a]. The decay accelerating factor appears to have two possible cleavage/attachment sites and in one of the two ω + 2 sites, threonine is also allowable [Moran et al., 1991b].

In the last decade, our studies have focused on the cellular and biochemical characterization of the α isoform of the folate receptor (FR), a GPI protein of 38 KDa which mediates the internalization of folates [Miotti et al., 1997]. Three different FR isoforms have been discovered so far: α and β which are both GPI-linked membrane proteins, and γ, for which there is no evidence of processing to GPI [Shen et al., 1995; Ratnam et al., 1989; Elwood, 1989]. Like most GPI-anchored proteins, the α isoform of the FR is susceptible to phosphatidylinositol-specific phospholipase C (PI-PLC) [Tomassetti et al., 1993], a bacterial enzyme that cleaves PI between glycerol and phosphate, removing the linked protein from membrane-associated 1,2-diacylglycerol. The FR contains a hydrophobic COOH-terminus of 15 amino acid residues rich in leucine, a hinge region at residues 238–243 and a stretch of eight small and uncharged amino acids. Based on rules postulated by Gerber et al. [1992] and Kodukula et al. [1993], Ser_{234} is the predicted cleavage/attachment site. In the present study, we find that single amino acid substitution at the FR ω and ω + 1 putative sites Ser_{234} and Gly_{235}, respectively, with residues not likely to result in a GPI-anchored protein had no apparent effect on the efficiency of GPI attachment. By contrast, deletion of the stretch of residues 233–237, which includes Ser_{234}, the putative cleavage/attachment site, abrogated GPI processing.

MATERIALS AND METHODS

Cell Lines and Reagents

IGROV1 cells (Dr. J. Benard, Institut G. Roussy, Villejuif, France) and CHO (ATCC) cells were maintained in RPMI 1640 (Gibco BRL, Paisley, UK) supplemented with 5% FCS, 2 mM L-glutamine and 100 U/ml gentalyn. Geneticin G418 sulfate and glutamine were purchased from Gibco Europe (Paisley, Scotland). Dialyzed (cut-off, 3500 Da) FCS (batch 669141) was from Hyclone (Holland). Recombinant PI-PLC (EC 3.1.4.10) was purchased from Oxford GlycoSystems (England) octyl-β-glucoside was from Boehringer-Mannheim, (Germany); and restriction enzymes from New England Biolabs (Beverly, MA).

Mutagenesis and Recombinant Plasmids

Ser_{234} and Gly_{235} substitution mutants of FR were prepared by PCR amplification from the FR cDNA cloned into the *Eco*RI sites of pBluescript II KS +/- (Stratagene, La Jolla, CA) and named BS#31 [Bottero et al., 1993]. Table 1 lists the sense primers used. The anti-sense primer used was the M13 forward 20-mer primer (Boehringer-Mannheim). AmpliTaq DNA polymerase (Perkin Elmer, Roche, Branchburg, NJ) was used for PCR amplification consisting of a denaturation cycle at 95°C for 5 min and 30 cycles as follows: 1 min at 95°C, 1 min at 55°C, 2 min at 72°C. The PCR products were digested with *PstI*, gel purified, subcloned into BS#31 previously digested with the same enzyme and amplified in *Escherichia coli* XL-1 Blue (Stratagene). Each *HindIII/XbaI* fragment from the BS constructs containing the mutated FR cDNAs was subcloned into pcDNAI/neo vector and amplified in *Escherichia coli* MC1061/P3 (Invitrogen, San Diego, CA). The internal deletion construct was also generated by PCR using the procedure described above and the sense primer reported in Table 1. The sequence of each construct, including specific mutation, deletion and subcloning site, was verified by dideoxy sequencing using Sequenase Version 2.0 (USB, Cleveland, OH).

DNA Transfection

CHO cells were seeded 5,000 cells/well in a 96-well plate (Costar, Cambridge, MA) and

TABLE I. Sequences of the Oligonucleotides Used to Obtain cDNA Variants of FR

Mutation/deletion	Sequences[a]
$Ser_{234} \rightarrow Arg_{234}$	AGG TCC TAT GCT GCA GCC ATG AG**A** GGG GCT GGG CCC
$Ser_{234} \rightarrow Thr_{234}$	TTC TAT GCT GCA GCC ATG A**C**T GGG GCT GGG CCC TGG
$Gly_{235} \rightarrow Pro_{235}$	TTC TAT GCT GCA GCC ATG AGT **CC**G GCT GGG CCC TGG
$Del_{233–237}$	AAA ATT GCT GCA GCG ‖ CCC TGG GAC GCC TGG CCT

[a]The sense strand sequence of the oligonucleotides used as primers in PCR are shown in 5′ to 3′ orientation. Mutated sequences are in boldface. Double slash indicates the position of the deleted sequences. *Pst* I site is underlined.

grown for 1 day. After washing once with serum-free medium, cells were transfected with 10 µg of purified plasmid using Lipofectin (Gibco, BRL, Gaithersburg, MA) according to the manufacturer's protocol. Mock-transfected cells were obtained by transfection with the plasmid carrying no insert. After 24 h, the medium was replaced with fresh medium containing 10% FCS. Geneticin G418 sulfate (Gibco) at 800 µg/ml was added to the culture 48 h later. Mutant FR clones were selected by FACS analysis (see below) and only those with high cell surface expression of FR were characterized further. Genomic DNA from either mutated and deleted clones was extracted, amplified by PCR (sense primer, from nucleotide 1600 to 1621 of the CMV promoter inserted into pcDNAIneo; anti-sense primer, from nucleotide 753 to 774 of the FR cDNA), and the gel-purified products sequenced.

Flow Cytometric Immunofluorescence Analysis

Purified murine anti-FR (MAbs) MOv 18 and Mov19 [Miotti et al., 1987] were used to evaluate cell surface expression of wild-type FR and mutants as described [Sen et al., 1996]. Briefly, 100 µl anti-FR MAbs was diluted to 10 µg/ml in PBS with 0.3% BSA and added to the cells. After incubation at 4°C and subsequent washing with PBS, cells were further incubated for 1 h with 100 µl fluorescein-conjugated goat anti-mouse IgG diluted 1:80 in 3% BSA in PBS and analyzed in a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

PI-PLC Treatment and Cell Lysis

Confluent cells were rinsed twice with PBS and 5×10^6 cells/ml were incubated with 0.3 U/ml of PI-PLC at 37°C for 1 h. After centrifugation at 1,000*g* for 10 min and two washes with PBS, cells (5×10^6 cells/ml) were lysed with lysis buffer [Tris-HCl pH 7.4, 0.15 M NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1.1% octyl β-glucoside] for 1 h at 0°C. Detergent lysates were clarified by centrifugation at 15,000*g* for 15 min at 4°C. The protein concentration was determined by BCA protein assay (Pierce, Rockford, IL). For the preparation of total lysate, trypsinized cells were directly treated with lysis buffer under the same conditions described above.

DDIRMA

PI-PLC soluble protein, further clarified by centrifugation at 15,000*g* for 15 min at 4°C, and the lysate after PI-PLC treatment were analyzed for immunoreactive units by DDIRMA using immobilized MOv18 and ^{125}I-MOv19 as described [Mantovani et al., 1994; Tomassetti et al., 1993]. Immunoreactive units are defined as the amount of immunoreactivity in 1 ml of standard solution of IGROV 1 supernatant [Bottero et al., 1993].

Western Blot Analysis

Total lysates were separated on 12% SDS-PAGE slab gels and blotted to nitrocellulose membranes (Hybond C-Super, Amersham). Blots were saturated with Blotto (5% non-fat dried-milk in PBS) containing 0.2% Tween 20. After incubation with the primary antibody (in Blotto plus Tween 20) at the dilution recommended by the manufacturer, the nitrocellulose sheets were incubated with relevant biotinylated secondary antibodies (Amersham) diluted in the same solution, followed by horseradish peroxidase (HRP)-streptavidin, and were developed with enhanced chemiluminescence reagent (ECL) (Amersham). Biotinylated protein molecular weight markers for ECL (range 14.3 to 97.4 KDa) were from Amersham. A semi-quantitative evaluation was performed using Eagle Sight™ 3.2 software.

RESULTS

GPI Processing of Mutated FR

Figure 1 shows the amino acid substitutions introduced into the FR sequence by site-directed mutagenesis. Both arginine and threonine at position 234 seemed to abrogate GPI processing since arginine is a large basic amino acid not allowable at the ω site, while threonine, a polar amino acid, was experimentally found not allowable at the ω site of placental alkaline phosphatase [Udenfriend et al., 1995a]. At the predicted ω + 1 site, a proline substituted for the native Gly_{235} expected give rise to a protein resistant to modification by the transamidase [Kodukula et al., 1993].

CHO cells were stably transfected with the three cDNA variants and FR_{wt} under the control of the CMV promoter, selected in G418-containing medium, and analyzed for maximal FR cell surface expression by FACS analysis with anti-FR MAbs. To test for cell surface

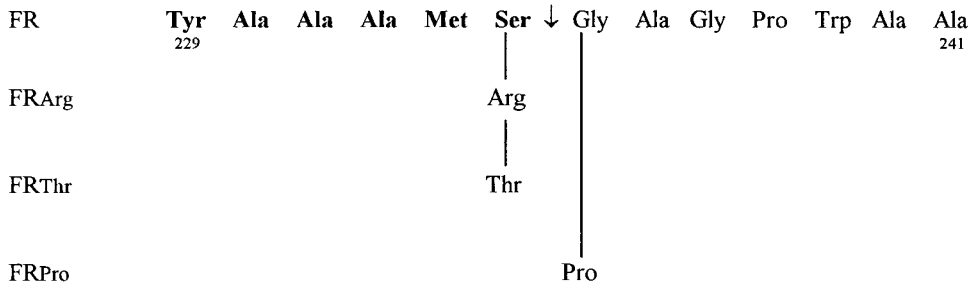

Fig. 1. Schematic diagram showing amino acid substitutions of the FR. Residues on the NH_2-terminal side of the ω site (arrow) are in boldface. Mutated amino acids at the ω and ω + 1 site are below the native sequence.

TABLE II. Sensitivity of Mutated FR Variants to PI-PLC

Protein variants	PI-PLC sensitive	PI-PLC resistant[a]	% of total expression resistant to PI-PLC
	imm. units/mg[b]		
FRwt	2.51	0.60	19
FRArg	2.57	0.46	15
FRThr	8.86	1.90	18
FRPro	14.86	2	12

[a]Cells were lysed with buffer containing 1.1% β-octylglucoside after digestion with PI-PLC.
[b]Immunoreactive units defined as described in Materials and Methods.

expression of the mature GPI-anchored protein, each transfected cell line was digested with PI-PLC and then analyzed by FACS in parallel with the undigested cell lines. The mature GPI-anchored receptor on the surface of the FR_{Arg} and FR_{Thr} transfectants showed the same sensitivity to the action of the enzyme as Fr_{wt} transfectants. Only on FR_{Pro} transfectants was some residual reactivity (<10%) upon PI-PLC digestion detected with the anti-FR MAbs (data not shown).

To estimate the amount of processed versus unprocessed protein, each transfectant was digested with PI-PLC, lysed, and FR was quantitated by DDIRMA (Table 2). All mutated FRs were GPI-anchored proteins as demonstrated by sensitivity to PI-PLC. Although the amount of protein resistant to PI-PLC might reflect both residual membrane FR and FR retained in the cytoplasm, the similarity between the FR mutants and the wild-type in percent of PI-PLC resistant protein (19% in wild-type and from 12 to 18% in mutants) argue against any difference in ER processing. DDIRMA analysis of spent medium of each transfected cell line revealed no detectable amounts of spontaneously released receptor (data not shown).

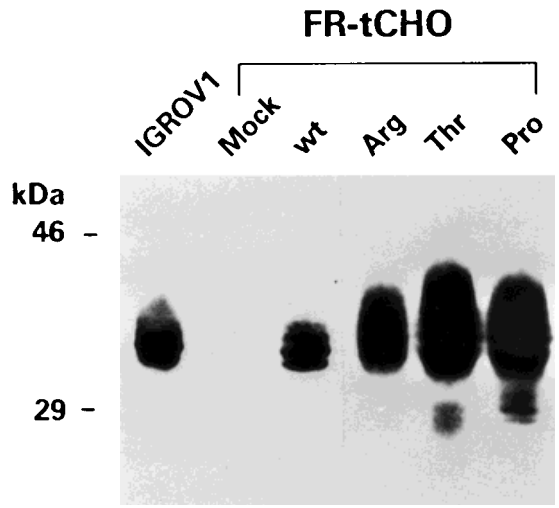

Fig. 2. Western blot analysis of lysates from mutated FR transfectants (FR-tCHO). Transfected CHO cells were lysed, and equal amounts of proteins (10 (g) were electrophoresed on a 12% SDS-polyacrylamide gel and transferred onto nitrocellulose. The filter was incubated with ant-FR MAbs. The cDNAs used for transfection are indicated above each lane. IGROV1 and Mock-tCHO lysates were also loaded as positive and negative control, respectively.

Western blot analysis of the total cell lysates with anti-FR Mabs (Fig. 2) showed a major immunoreactive band at about 38 KDa, corresponding to the processed protein, for all transfectants and the reference cell line IGROV1. FR_{Thr} and FR_{Pro} transfectants exhibited three and two bands, respectively, in the range of 30 and 33 KDa, representing about 15 and 20%, respectively, of the total detected FR. Minor bands at lower molecular weight, which might represent a precursor form of the receptor, were detected in the lysates of all transfectants except tested FR_{Arg}. IGROV1 and FR_{wt}t-CHO cell lysates showed an additional band at about 33 KDa (about 5%) in a longer exposure of the blot (data not shown). Thus, in FR_{Thr} and FR_{Pro} transfectants, a lower percentage of the mutated

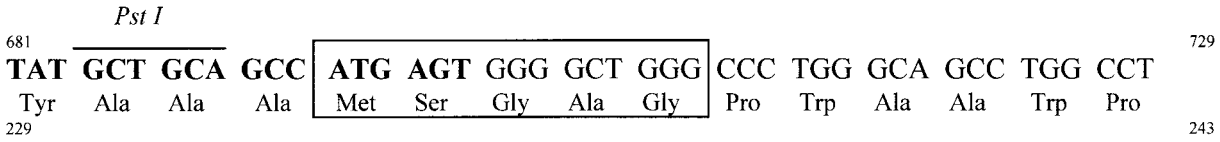

Fig. 3. Schematic diagram showing deletion at the FR COOH-terminus. Box delineates deleted nucleotide sequence and amino acids. Residues NH_2-terminal of the ω site are in boldface. See Materials and Methods for cloning strategy. *PstI* site used to construct the deleted cDNA is underlined.

TABLE III. Sensitivity of FR_Δ Variants to PI-PLC

Δ clones	PI-PLC sensitive	PI-PLC resistant[a]	% of total expression resistant to PI-PLC
	imm. units/mg[b]		
C2	0.036	0.202	85
D5	0.006	0.154	96
E5	0.011	0.164	94
F3	0.050	0.336	87
H12	0.027	0.142	84

[a]Cells were lysed with buffer containing 1.1% β-octylglucoside after digestion with PI-PLC.
[b]Immunoreactive units defined as described in Materials and Methods.

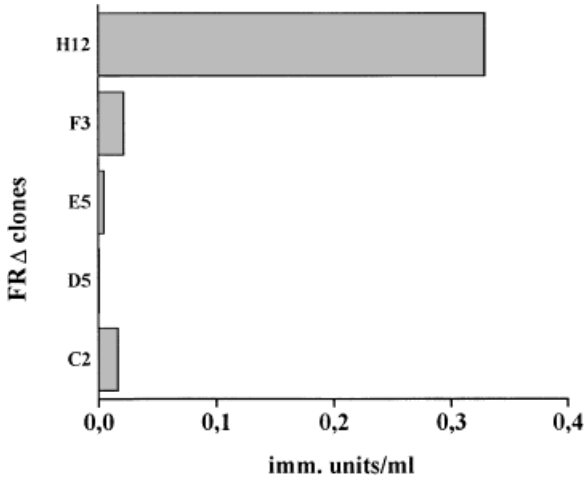

Fig. 4. Secretion of FR_Δ into tissue culture medium of transfected CHO cells. Five selected clones from FR_Δ transfectants were seeded into a 96-well plate at 50,000 cells/ml. Spent medium was collected after 5 days and tested by DDIRMA.

protein might undergo maturation. Despite these differences in processing efficiency, it appears that the three amino acid substitutions at the ω or ω + 1 site do not affect the ability of the transamidase to process native FR to the GPI-linked protein. Analysis of folic acid binding and 5-methyltetrahydrofolic acid uptake also showed no evidence of compromised function in mutated FRs compared to the FR_{wt} (data not shown).

GPI-Processing in a FR Deletion Mutant

To determine whether the putative cleavage/attachment site contained a unique site in the uncharged hinge region, we modified FR cDNA such that residues 233–237 were deleted (Fig. 3). CHO cells were stably transfected with the deletion construct (FR_Δ) and 15 clones resistant to G418 selection were tested by FACS analysis with anti-FR MAbs; none of the stable clones showed detectable membrane expression of FR (data not shown). However, more sensitive analysis by DDIRMA on PI-PLC-released proteins and cell lysates revealed FR expression in five of the 15 stable clones (Table 3). In comparison with the FR_{wt} transfectants (see Table 2), the total amount of cellular FR_Δ in the deleted clones was from 8 to 20 times lower. Clones C2, F3, and H12 expressed 13–16% of FR_Δ on the membrane which was released by PI-PLC treatment, while the majority is retained in the cytoplasm. Clones D5 and E5 showed an even smaller PI-PLC-sensitive fraction (4–6%) and expressed less total FR_Δ.

Because nascent GPI protein might undergo partial processing without the attachment of the GPI moiety and thus be secreted [Cross, 1990; Caras et al., 1989], the spent medium of FR_Δ-expressing clones was assayed by DDIRMA (Fig. 4). While clones D5 and E5 secreted no detectable FR_Δ, trace amounts of the soluble protein were detected in spent medium from clones C2 and F3, and very high levels were detected from clone H12. IGROV1 cells shed two- to three-fold higher amounts of soluble FR (see Materials and Methods for evaluation of immunoreactive units), while CHO cells transfected with FR_{wt} shed no detectable FR. Note that the soluble FR detected in IGROV1 cell spent medium represents the shed membrane form, not the secreted form [Antony, 1996; Tomassetti et al., 1993].

Western blot analysis of the lysates of clones F3 and H12 with anti-FR MAbs (Fig. 5A) re-

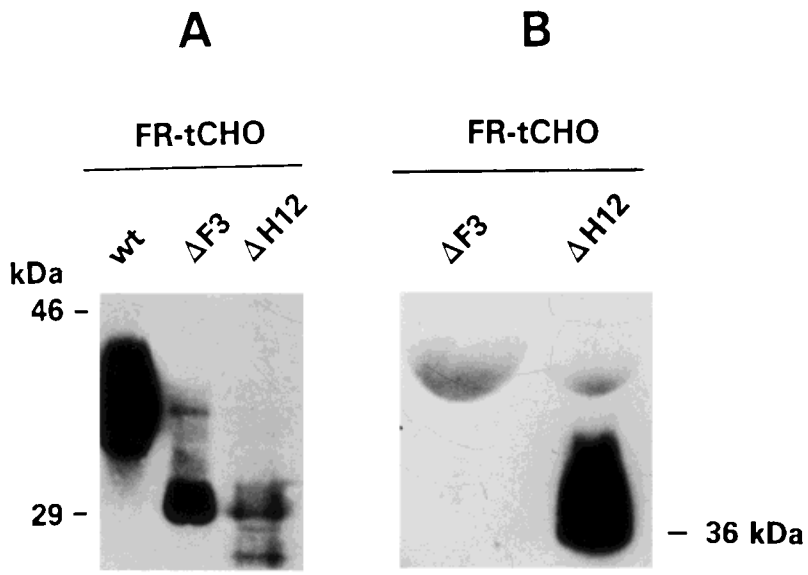

Fig. 5. Biochemical characterization of $FR_{\triangle}$ retained in the cytoplasm or secreted into tissue culture medium. Total lysates (**A**) and spent media (**B**) from clones F3 and H12 were analyzed by Western blot with anti-FR MAbs. While the same amount was loaded for the lysates (50 µg), the volume of clone F3 spent medium was five-fold that of clone H12 (100 and 20 µl, respectively). The nonspecific band at >46 KDa is due to overloading of serum proteins.

vealed a major band at about 30 KDa and a faint band at 38 KDa, which might represent the partially glycosylated protein and the mature protein lacking the GPI tail for the clone F3, while the H12 lysate showed several bands between 26 and 31 KDa, which might reflect different degrees of glycosylation, but no mature protein. In the spent medium of clone H12, a major band of about 36 KDa was detected (Fig. 5B), consistent with the molecular weight of the soluble form of FR [Tomassetti et al., 1993]. No specific immunoreactivity was detected in the spent medium from clone F3.

DISCUSSION

The FR amino acid sequence at the COOH-terminus is consistent with the general rules for correct GPI processing. However, unlike other GPI proteins [see Udenfriend et al., 1995a for review], the FR sequence contains a longer stretch of small uncharged amino acids from Ala_{230} to Gly_{237} located upstream of the hinge region and the hydrophobic COOH-terminal tail. Our present data suggest that Ser_{234} is not the only potential FR cleavage/attachment site. Moreover, our data are consistent with the hypothesis that FR residues Ser_{234}-Gly_{237} are essential to bring the hydrophobic domain into juxtaposition.

According to the postulated general rules [Kodukula et al., 1993; Gerber et al., 1992], only small uncharged amino acids (serine, glycine, alanine, cysteine, aspartic acid, and asparagine) can occupy the ω site, although some activity has also been observed with valine and leucine [Moran et al., 1991b]. Moreover proline was found to completely inhibit the processing to a GPI-linked protein when located at either the ω + 1 or ω + 2 site [Kodukula et al., 1993]. In our study, we first mutated the postulated ω site Ser_{234} to introduce arginine and threonine, and the ω + 1 site, Gly_{235}, to introduce proline. Contrary to the expectations, we observed efficient translation as GPI-linked proteins releasable by PI-PLC in all three mutants. Particularly intriguing was the efficiency of the mutant with proline substituted for glycine at position 235. In light of previously given hierarchical values, this mutant should not yield a GPI-linked protein. Yan et al. [1995] obtained similar FR mutants and, in the case of threonine at position 234, observed a very low translation efficiency (about 20% compared to more than 80% in our mutant). However, in that study, the human fibroblast line 293 was used for transfection, and it has been shown that the efficiency of processing to a GPI-linked protein can be lower when palmitoyl groups are scarce, especially in cells of nonepithelial origin [Englund, 1993]. In our study, the high expression of FR containing the threonine or the proline most likely reflects the high efficiency of transfec-

tion, although we cannot exclude a specific role for the type of mutation.

After assessing the hierarchical value of each cleavage-attachment site of the three different mutants, we considered the possibility that FR might have alternative ω sites. The Ala-Ala-Ala sequence at position 230–232 seemed the most probable alternative cleavage/attachment site and a deleted FR construct was designed. We tested individual clones instead of the total population to better evaluate the alternative pathways that may follow the FR in the absence of GPI processing. Deletion gave raise to clones in which only a small amount of FR was detected in the total lysates but not on the membrane. Only one of 15 clones secreted the entire FR, as detected by immunoreactivity, suggesting that degradation is the most frequent event when FR is not GPI-processed. Considering the molecular weight and the amount of cellular versus secreted FR in clone ΔH12, we assume that the COOH-terminus was cleaved and that the protein underwent N-glycosylation and was secreted. The FR polypeptide of the other deletion clones was not processed but degraded and only partially retained in the ER. However, as observed with ΔF3, a small proportion of the polypeptide was further glycosylated and processed. Previous studies on other proteins have shown that misfolding causes degradation of the majority of native polypeptide in the ER, where a small proportion with the hydrophobic tail is retained [Delahunty et al., 1993]. The fraction of polypeptide that lacks the hydrophobic COOH-terminus does not enter the cis-Golgi stacks, but is N-glycosylated and then secreted [Field et al., 1994; Cross, 1990]. FR deleted clones behave similarly, suggesting that the lack of the five amino acids from Met_{233} to Gly_{237} influences the folding of the protein.

It has been reported for other GPI-linked proteins that the sequences on the NH_2-terminal side of the cleavage/attachment site do not influence the processing [Moran et al., 1991a]. In the case of FR, the deletion might alter the conformation of the protein such that the protein is not properly folded and is, therefore, unstable. Alternatively, when the stretch of small uncharged amino acids is too short as in the deleted clones, the Ala-Ala-Ala sequence at position 230–232 might not be recognized as a cleavage-attachment site due to misfolding of the polypeptide chain which impairs transamidase binding. Moran et al. [1991a] found that the GPI signal requires only a hydrophobic domain and a cleavage/attachment site consisting of a pair of small residues, positioned 10–12 residues NH_2-terminal to the hydrophobic domain. Accordingly, the GPI signal of the FR might also requires a tail of defined length between the cleavage/attachment site and the hydrophobic domain.

Our data could also serve to explain the absence of GPI processing of the FR γ isoform. Of the three human FR isoforms discovered so far, α and β, which share about 70% homology, are both GPI-linked membrane proteins [Ratnam et al., 1989; Elwood, 1989], whereas γ, which shares overall amino acid sequence homology of 71% and 79% with the α and β, respectively [Shen et al., 1995], shows no evidence of GPI processing. Based only on their NH_2-terminal sequence, all three isoforms should be able to enter the ER. Although the sequence from residues 194 to 233 is 95% homologous among the three isoforms, γ is not processed to a GPI-linked protein because the COOH-terminal tail is too short [Shen et al., 1995].

In conclusion, it appears that in the case of FR, more direct approaches than those based only on the primary sequence deduced from cDNA, are required to determine the requisites for GPI processing.

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