Proteolysis of the Carboxyl-Terminal GPI Signal Independent of GPI Modification as a Mechanism for Selective Protein Secretion†

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ABSTRACT: Variable amounts of soluble forms of a variety of glycosyl-phosphatidylinositol (GPI)-anchored proteins occur extracellularly, but the molecular mechanisms governing their release are not entirely clear. When the GPI-anchored folate receptor (FR) type β was expressed transiently in human 293 fibroblasts, there was a roughly equal distribution of [^{3}H]folic acid binding protein between the cell surface and the medium after 24 h over a wide range of expression levels of FR-β. The difference in apparent molecular masses between the soluble FR-β and the PI-PLC-treated membrane protein indicated that the former was not released from the membrane by the action of phospholipase. Brefeldin A inhibited the release of soluble FR-β from both the transfected 293 cells and stable recombinant CHO (CHO-FR-β) cells while pre-existing levels of cell surface FR were unaltered suggesting the absence of a precursor-product relationship between the membrane-associated FR-β and the soluble protein in the medium. [^{35}S]Cysteine pulse-chase analysis was consistent with this finding. Interchanging of carboxyl-terminal peptides between FR-β and FR-α revealed that the nature of the processed signal for GPI modification was responsible for the quantitative membrane anchoring of FR-α and the production of soluble FR-β. When total cell lysates were analyzed by Western blot, a diffuse band of apparent 41 kDa and three additional sharp bands of apparent 35, 33, and 29.3 kDa were seen. The 41 kDa band was identified as the PI-PLC sensitive cell surface receptor. Several mutant constructs of FR-β, in which the carboxyl-terminal signal for GPI modification was either disrupted or deleted only gave the three lower bands. The three sharp bands from the wild-type and the mutant forms of FR-β were identified as nonglycosylated (29.3 kDa) or glycosylated polypeptides in which the carboxyl-terminal peptide was at least partially proteolyzed without GPI modification. All of the mutations in the GPI signal resulted in the recovery of [^{3}H]folic acid binding protein in the media which, similar to the wild-type FR recovered from the media, were converted to the 29.3 kDa band by *N*-glycanase. The results from this study indicate that a carboxyl-terminal peptide in FR-β is efficiently proteolyzed intracellularly by a pathway that is independent of GPI signal recognition resulting in proper protein folding and secretion. Such carboxyl-terminal sequences could represent a simple adaptation for proteins whose physiologic functions reside both at the cell surface and in extracellular fluids, allowing their selective and tissue-specific release.

A large number of proteins are attached to the eukaryotic cell surface by a glycosyl-phosphatidylinositol (GPI) membrane anchor. Such proteins include a wide variety of enzymes, receptors, matrix proteins, and cell surface antigens with a diverse variety of physiologic functions (Low, 1989; Cross, 1990; McConville & Ferguson, 1993; Udenfriend & Kodukula, 1995). A common diagnostic test for the presence of the GPI modification is the sensitivity of the anchor to bacterial phosphatidylinositol-specific phospholipase C (PI-PLC). The enzyme cleaves the membrane-associated diacylglycerol moiety resulting in a soluble protein with its associated inositol phosphoglycan.

The GPI anchor is conferred in the endoplasmic reticulum following recognition of a carboxyl-terminal signal which is cleaved prior to GPI modification of the new carboxyl-terminus by a transamidation reaction (Bangs et al., 1985, 1986; Ferguson et al., 1986; Maxwell et al., 1995a,b). The components of the machinery for GPI modification in the ER have not yet been identified. However, the signal for GPI modification has been characterized in some detail and contains three parts: (i) the site (ω) of cleavage/GPI modification represented by an uncharged amino acid residue that is modified; only certain amino acids are allowed at the ω,ω+1 and ω+2 positions (Moran et al., 1991; Kodukula et al., 1993; Nuoffer et al., 1993); (ii) a moderately hydrophobic carboxyl-terminal peptide of 10−20 amino acids; and (iii) a spacer of 8−12 amino acids between the ω residue and the hydrophobic peptide (Ferguson & Williams, 1988; Cross, 1990; Moran et al., 1991). In several instances, it has been found that when mutations cause defects in the GPI signal or when GPI-anchored proteins are expressed in cells incapable of synthesizing GPI, the unmodified proteins are not targeted to the plasma membrane but instead accumulate in the cell and are eventually degraded (Moran et al., 1992; Jost et al., 1991).

Soluble counterparts of several GPI-anchored proteins have been found in cell culture media and in extracellular fluids (Ferguson, 1992); physiologic roles have been suggested for such proteins both in their membrane-anchored and soluble forms. In several instances, it has been shown that the soluble proteins are generated from the membrane proteins

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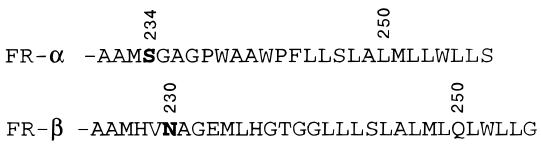

FIGURE 1: The C-terminal amino acid sequences of FR-α and FR-*β* prior to GPI modification. The sites of GPI anchor attachment are represented by bold letters.

by an endogenous membrane-associated phospholipase (Almqvist & Carlsson, 1988; He et al., 1987; Musaka et al., 1995; Brunner et al., 1994; Metz et al., 1994). Membrane-associated phospholipases would not be expected to distinguish between the different GPI-anchored proteins and thus may not explain the different proportions of the soluble proteins that may be produced from the same cells. In a few other examples, a membrane-associated protease is implicated in the processing of membrane-bound GPI proteins to soluble forms (Nemoto et al., 1996; Bazil & Strominger, 1994; Durieux et al., 1994; Elwood et al., 1991). The possibility of alternative splicing has also been noted leading to GPI anchoring, polypeptide anchoring, or secretion (Soloski et al., 1986; Seidenbecher et al., 1995; Nonaka et al., 1995).

In this report, we describe a mechanism by which a protein may be simultaneously directed toward GPI anchoring or secretion utilizing two independent pathways by which its carboxyl-terminal peptide is processed intracellularly. The model protein used in this study is the human folate receptor (FR) type *β* expressed either transiently in human 293 fibroblasts or stably in recombinant CHO (CHO-FR-*β*) cells. FR is an N-glycosylated polypeptide that mediates cellular uptake of folate coenzyme and antifolates by an endocytic mechanism (Rothberg et al., 1990; Leamon & Low, 1991; Birn et al., 1993; Turek et al., 1993). The receptor occurs as multiple homologous isoforms that are tissue specific (Lacey et al., 1989; Ratnam et al., 1989; Brigle et al., 1992; Ross et al., 1994; Shen et al., 1994, 1995). The GPI-anchored FR-α is specific for certain epithelial cells and is overexpressed in certain carcinomas (Ross et al., 1994). Placenta is a major source of the GPI-anchored FR-*β*, which is also expressed at moderate levels in spleen and thymus and elevated in certain sarcomas (Ratnam et al., 1989; Ross et al., 1994). FR-*γ* is specific for hematopoietic tissue and is constitutively secreted due to the lack of an efficient signal for GPI modification in this protein (Shen et al., 1994, 1995). The principal sites of GPI modification in FR-α and FR-*β* are Ser234 and Asn230, respectively, resulting in the cleavage of carboxyl-terminal peptides of 23 (FR-α) and 25 (FR-*β*) amino acids that also contain the signal for GPI modification (Figure 1) (Yan & Ratnam, 1995). Soluble forms of FR have been found in milk, cerebrospinal fluid, urine, amniotic fluid, and in the serum of pregnant women (Antony et al., 1982; Hansen et al., 1985, 1989; Kamen & Caston, 1975; Holm et al., 1990). The postulated physiologic roles of the soluble FR are to bind and stabilize 5-methyltetrahydrofolate, the circulating form of the vitamin, to facilitate intestinal absorption of folate from milk and to facilitate delivery of folate to the fetus via umbilical cord serum (Tani et al., 1983; Tani & Iwai, 1984; Antony et al., 1989).

In certain cultured epithelial cells, FR-α is the precursor of a soluble protein in the medium, and this has been attributed to the action of either a membrane-associated metalloprotease or an endogenous phospholipase (Elwood et al., 1991; Luhrs & Slomiany, 1989). The present study is based on our initial findings that when expressed in fibroblasts, FR-*β*, but not FR-α, was produced in a soluble form released into the medium. The studies reveal an intracellular proteolytic mechanism that is independent of the GPI modification pathway and that could result in the selective and efficient secretion of soluble forms of certain GPI-anchored proteins.

MATERIALS AND METHODS

Reagents and Antibodies. Lipofectamine and opti-MEM I reduced serum medium were from Gibco BRL. Minimal essential medium (MEM), folate-free RPMI (FFRPMI) medium, and fetal bovine serum were from Irvine Scientific. [^{3}H]Folic acid (specific radioactivity 26 Ci/mmol) was from Moravek Biochemicals. [^{35}S]Cysteine (specific radioactivity 1000 Ci/mmol) was from Amersham Life Sciences. Anti-rabbit IgG (Fc) AP conjugate was from Promega. PI-PLC was from Boehringer Mannheim. *N*-Glycanase was from Genzyme. Brefeldin A and the substrates for immunoblotting, nitro blue tetrazolium (NBT), and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were from Sigma Chemical Co. Rabbit antiserum against FR purified from human placenta was obtained and affinity purified using immobilized FR as described previously (Ratnam et al., 1989). ImmunoPure immobilized protein A (protein A-agarose) was purchased from Pierce.

cDNA Constructs. The cDNAs for FR-*β*, FR-β_{N230Q}, and FR-β_{A246D} were constructed and inserted into the expression plasmid, pcDNAI/Neo (Invitrogen) as described previously (Yan & Ratnam, 1995). The other mutants were also constructed similarly using the polymerase chain reaction (PCR). The reactions were performed using Vent (New England Biolabs) or Taq DNA polymerase (Gibco BRL) with the cDNA for FR-α or FR-*β* as template. The synthetic oligonucleotides used as the PCR primers were designed either to encompass the mutated region or to contain the mutated sites. If there were no appropriate restriction sites within the amplified region, the appropriate restriction sites were introduced into either the upstream primer or the downstream primer or both without changing the encoded amino acids. The PCR products were then isolated by agarose gel electrophoresis and purified with the Geneclean kit (Bio101, Inc.). The purified DNA fragments were digested with the appropriate restriction enzymes and inserted into the polylinker region of the plasmid pcDNAI/Neo. For each mutant, the entire cDNA sequence was verified by dideoxy sequencing using Sequenase, version 2.0 (USB). The synthetic oligonucleotides used in generating the mutants not reported previously are listed in Table 1.

Cell Culture and Transfection. Human 293 cells were grown in MEM supplemented with fetal bovine serum (10% v/v), penicillin (100 units/mL), streptomycin (100 mg/mL), and L-glutamine (2 mM). Transfections with the cDNA constructs were carried out in six-well plates (Corning) using lipofectamine (Gibco BRL), according to the manufacturer's protocol. Recombinant CHO cells stably expressing FR-*β* (CHO-FR-*β*) were grown in FFRPMI medium supplemented with fetal bovine serum (10% v/v), penicillin (100 units/mL), streptomycin (100 mg/mL) and L-glutamine (2 mM).

PI-PLC Treatment. The cells (1×10^6) were washed twice with 1 mL of PBS (10 mM sodium phosphate, pH

Table 1: Synthetic Oligonucleotides Used to Generate Some Mutant and Chimeric Constructs of FR-β

FR-$\beta\Delta10$[a]	CGACTTAAGCTTGGCTGGATCTATTGCCAAC
	TTGCAGCATCTAGACCTAACTGAGCAG
FR-$\beta\Delta24$[a]	CGACTTAAGCTTGGCTGGATCTATTGCCAAC
	AGGAGACCTCTAGATCAATGAAGCATC
FR-$\beta\Delta24$-(CHO^-)[b]	AAGGTCAGCCAGTACAGCGA
	TCGGCTGTACTGGCTGACCTT
	CAGCAGGTGCAGCAGACGTGG
	CCACGTCTGCTGCACCTGC
FR-α/β[c] and	CGAGGGAGCGGCCGCTGCATCCAG
FR-β/α[d]	CTGGATGCAGCGGCCGCTCCCTCG
	GGCAGGGATTTCTAGATATCAG
	CCCTCAAAGCTTAGACATGGCTAGCGGATGAC

[a] FR-$\beta\Delta10$ and FR-$\beta\Delta24$ indicate deletion of 10 and 20 amino acids, respectively, from the carboxyl-terminus in FR-β. [b] FR-$\beta\Delta24(CHO^-)$ represents FR-β containing a 24 amino acid carboxyl-terminal deletion and N to Q substitutions at all of the potential sites for N-glycosylation. [c] FR-α/β represents a chimera in which the entire C-terminal GPI signal in FR-α is substituted by that of FR-β. [d] FR-β/α represents a chimera in which the entire C-terminal GPI signal in FR-β is substituted by that of FR-α.

7.5, and 150 mM sodium chloride) followed by 1 mL of PI-PLC buffer (25 mM Tris-HCl, pH 7.5, 250 mM sucrose, 10 mM glucose, and 1% bovine serum albumin). Then the cells were incubated at 37 °C for 1 h with either PI-PLC buffer alone or with the buffer containing 0.3 U PI-PLC.

[^{3}H]Folic Acid Binding Assays. (1) Assay of cell surface binding: the cells (1×10^6) in six-well tissue culture plates were washed with 1 mL of ice-cold PBS, followed by 1 mL of ice-cold acid buffer (10 mM sodium acetate, pH 3.5, and 150 mM sodium chloride) to remove endogenous bound folate and once again with PBS. Then to each well, 1 mL of PBS containing 25 pmol of [^{3}H]folic acid was added and incubated at 4 °C for 30 min. The cells were then washed twice with 1 mL of ice-cold PBS. Finally, 1 mL of acid buffer was added to extract the cell-surface bound [^{3}H]folic acid, and the radioactivity was measured in a liquid scintillation counter. Specificity of [^{3}H]folic acid binding was determined by competition with excess (1 mM) unlabeled folic acid. 293 cells transfected with the vector alone were used as the negative control. (2) Assay of the folate binding proteins in cell culture media: the media were collected and any suspended cells were removed by centrifuging at 10000*g* for 15 min. To 250 mL of media, 25 mL of 100 mM sodium acetate, pH 3.5, and 25 mL of 10% Triton X-100 were added. After vortexing, 300 mL of an ice-cold suspension of Norit A charcoal (80 mg/mL; Baker) in 10 mM sodium acetate containing 1% Triton X-100 was added in order to remove the free and bound endogenous folate. The samples were then centrifuged at 10000*g* for 15 min at 4 °C. To the supernatants, 1/10 volume of 0.5 M sodium phosphate buffer (pH 7.5) was added and the pH adjusted to 7.5 using NaOH. To each 50 mL of the treated media, 450 mL of PBS containing 1% Triton X-100 was added, followed by the addition of 3 pmol of [^{3}H]folic acid and incubation at 37 °C for 1 h. Then, 500 mL of an ice-cold suspension of Norit A charcoal (80 mg/mL) in PBS containing 1% Triton X-100 was mixed with the samples followed by centrifugation at 10000*g* for 15 min at 4 °C. The radioactivity in the supernatant was measured in a liquid scintillation counter.

Preparation of Cell Lysates. The cells (1×10^6) were scraped off the six-well plates with a rubber policeman. The cells were then lysed at 37 °C for 30 min in 30 mL of PBS containing 1% Triton X-100 and 1 mM PMSF. After sedimenting the insoluble material by centrifugation at 10000*g* for 10 min, the supernatants (cell lysates) were collected for *N*-glycanase treatment and immunoblotting.

N-Glycanase Treatment. For the cell lysates, the samples were denatured by boiling for 5 min in PBS containing 0.5% SDS and 50 mM β-mercaptoethanol. To each 10 mL aliquot, 5 mL of 7.5% NP-40 and 0.3 unit of *N*-glycanase were added and incubated at 37 °C for at least 18 h. The samples were then electrophoresed on SDS−polyacrylamide gels. For the cell culture media, 12.5 mL of 10% SDS, 12.5 mL of deionized water, and 25 mL of 500 mM β-mercaptoethanol were added to 200 mL of the media and the sample was boiled for 5 min. NP-40 was added to a final concentration of 1.5% followed by 1 unit of *N*-glycanase, and the samples were incubated at 37 °C for at least 18 h. After the incubation, 12.5% trichloroacetic acid (TCA) was added to precipitate the proteins. Residual TCA was removed by washing with ethanol. The precipitate was then dissolved in the sample buffer for SDS−polyacrylamide gel electrophoresis.

Immunoblotting. The samples were electrophoresed on 12% SDS−polyacrylamide gels and transferred to nitrocellulose filters. The blots were probed with affinity-purified anti-FR antibodies using an anti-rabbit IgG (Fc) AP conjugate (Promega) as the secondary antibody and developed as described previously (Wang et al., 1992).

[^{35}S]Cysteine Pulse Chase. The cells were incubated with cysteine-free MEM supplemented with 70 mCi/mL [^{35}S]-cysteine for 15 min at 37 °C. The medium was then changed to MEM and the cells were incubated for a further period of 10 min, 1 h, 2 h, and 4 h. This was followed by immunoprecipitation of both the cell lysates and the media.

Immunoprecipitation. Protein A-agarose (Pierce) was washed twice with PBS containing 1% Triton X-100, and a 1:1 suspension of the gel was prepared in the same buffer. For each sample, 40 mL of the protein A-agarose suspension was mixed with 80 mL of affinity-purified anti-FR-β antibody and 80 mL of the buffer. The gel suspension was incubated at room temperature on a rotary mixer for 4 h. Unbound antibody was removed by washing twice with 200 mL of the above buffer, and the gel was resuspended in an equal volume of the buffer. To each sample (300 mL) of cell lysates and media, 40 mL of the antibody-bound protein A-agarose suspension was added and incubated at 4 °C overnight on a rotary mixer. The protein A-agarose was then washed three times with 300 mL of the buffer. The proteins bound to the gel were solubilized by incubation in the sample buffer for SDS−polyacrylamide gel electrophoresis at 37 °C for 30 min. The samples were then separated by electrophoresis. The gels were then treated with Entensify (Dupont) followed by autoradiography.

Brefeldin A Treatment. The transfected 293 cells or CHO-FR-β cells were incubated either in the presence or in the absence of Brefeldin A (50 mg/mL; Sigma) in serum-free media for 6 h at 37 °C. Then, the media were collected for immunoblotting.

RESULTS

Production of a Soluble Folate Binding Protein by Cells Expressing FR-β. Human 293 fibroblasts that were transiently transfected with the cDNA for FR-β produced a

Table 2: Distribution of Mature FR-β between the Cell Surface and the Medium[a]

[^{3}H]folic acid binding protein from 10^6 cells[b]			
transfected cDNA (μg)	total (pmol)	cell surface (percent)	medium (percent)
2	1.42	58.7	41.3
1	0.4	57	43
0.4	0.16	52	48
0.2	0.09	52.7	47.3
0.1	0.04	55	45

[a] Human 293 fibroblasts were transiently transfected with the cDNA for FR-β. After 24 h transfection, specific [^{3}H]folic acid binding protein in the medium and on the cell surface was estimated as described in the Materials and Methods. The experiments were repeated at least three times and concordant results were obtained. [b] Percent error <10.

soluble form of FR-β in the culture medium. When the soluble protein was quantitated in terms of [^{3}H]folic acid binding, it was found to represent about 40−50% of the total [^{3}H]folic acid binding protein expressed by the cells 24 h post-transfection (Table 2). The proportion of the total FR-β that was recovered from the medium was not significantly dependent upon the expression level of the protein ranging from the optimal level of transient expression of FR-β (transfection with 2 μg of cDNA per 10^6 cells) to approximately its lowest detectable level (transfection with 0.1 μg of cDNA per 10^6 cells). The level of soluble FR-β in the medium was not altered by growing the cells in serum-free media supplemented with insulin and transferrin (results not shown) indicating that the production of the soluble protein is a cellular phenomenon independent of serum components including proteases and phospholipases. It was also found that the soluble FR-β was produced in the medium even when extracellular folate was depleted (results not shown).

Relationship between Membrane-Anchored and Soluble FR-β. Several experiments were designed to test a possible precursor-product relationship between the GPI anchored cell surface FR-β and the soluble FR-β in the medium and they are described below.

(i) Comparison of FR-β in the Medium with the PI-PLC Released Membrane Receptor. Cells expressing FR-β were treated with PI-PLC, and the soluble proteins that were released were deglycosylated with *N*-glycanase and subjected to Western blot analysis (Figure 2). PI-PLC treatment is expected to cleave the diacylglycerol moiety from GPI, resulting in a soluble protein containing the residual inositol phosphoglycan portion of GPI, which should influence its electrophoretic mobility. Comparison of the PI-PLC released FR-β with the soluble FR-β obtained from the media showed a distinct difference in electrophoretic mobility after treatment with *N*-glycanase. Further, the soluble FR-β, after deglycosylation, comigrated with a mutant construct of FR-β (termed FR-$\beta\Delta$24CHO$^-$) in which the two N-glycosylation sites were eliminated by Asn-Gln substitutions and in which the carboxyl-terminal 24 amino acids that serve as the processed signal for GPI modification were deleted (Figure 2). These results suggest the absence of inositol phosphoglycan in the soluble FR-β. This would imply that the soluble protein was not produced from the membrane protein due to any phospholipase activity either in the cell membrane or in the medium.

(ii) Exchanging the Signals for GPI Modification between FR-α and FR-β. Direct evidence for the absence of a precursor-product relationship between membrane-associated and soluble forms of FR-β was obtained by transfecting 293 cells with a chimeric cDNA construct (FR-β/α), in which a carboxyl-terminal peptide (228−255) containing the entire GPI signal in FR-β was replaced by that of FR-α (peptide 234−257). The cells expressing either FR-α or the chimera produced predominantly GPI-anchored [^{3}H]folic acid binding protein on the cell surface (Table 3). Because the GPI signal is processed prior to GPI modification in the endoplasmic reticulum, the mature GPI-anchored FR-β on the cell surface should be identical whether the GPI signal is derived from FR-α or from FR-β. The result points to a fundamental difference in the fate of FR-β that is dependent upon the nature of the GPI signal and further demonstrates that the soluble FR-β is not derived from the plasma membrane associated receptor. Consistent with this conclusion, a second chimera (FR-α/β) containing a substitution of the GPI signal of FR-α (peptide 234−257) with that of FR-β (peptide

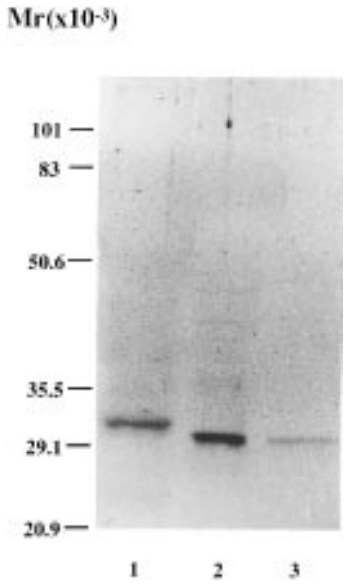

FIGURE 2: Western blot comparing soluble FR-β in the medium vs FR-β released from the cell surface by PI-PLC. Lane 1, FR-β released from the cell surface by PI-PLC and treated with *N*-glycanase; lane 2, FR-β in the medium after treatment with *N*-glycanase; lane 3, lysate from cells expressing FR$\beta\Delta$24(CHO$^-$). Transfected 293 cells were used as described in the Materials and Methods. PI-PLC and *N*-glycanase treatments and immunoblotting using anti-FR-β antibody were carried out as described in the Materials and Methods.

Table 3: Distribution of Mature [^{3}H]Folic Acid Binding Protein between the Cell Surface and the Medium[a]

	[^{3}H]folic acid binding protein from 10^6 cells[b]	
protein	cell surface (percent)	medium (percent)
FR-β	50.9	49.1
FR-α	100	0
FR-α/β	68.6	31.4
FR-β/α	100	0
FR-β_{N230Q}	14.8	85.2
FR-β_{A246D}	0	100
FR-$\beta\Delta$10	0	100
FR-$\beta\Delta$24	0	100

[a] Human 293 fibroblasts were transiently transfected with 2 μg of the appropriate cDNA. After 24 h transfection, specific [^{3}H]folic acid binding protein in the medium and on the cell surface was estimated as described in the Materials and Methods. The experiments were repeated at least three times and concordant results were obtained. [b] Percent error <10.

228−255) resulted in a significant release of [^{3}H]folic acid binding protein into the media (Table 3).

(iii) Effect of Brefeldin A on the Production of Soluble FR-β. Brefeldin A is an inhibitor of the secretory pathway, causing fusion of the Golgi compartments with the endoplasmic reticulum. The amount of Brefeldin A required to block the secretory pathway in 293 cells and in CHO cells was first determined by using various amounts of the reagent to treat the cells expressing a truncated version of FR-β (FR-$\beta\Delta$24) in which the carboxyl-terminal GPI signal was deleted. FR-$\beta\Delta$24 served as a control construct that should be constitutively released via the vesicular secretory pathway. It was determined in both transiently transfected 293 cells and in stable recombinant CHO cells that while no significant inhibition of secretion of FR-$\beta\Delta$24 occurred at 5 μg/mL of Brefeldin A, the secretion was virtually completely blocked with 50 μg/mL of the reagent (results not shown). Accordingly, 293 cells transiently expressing FR-β and recombinant CHO-FR-β cells were treated with Brefeldin A (50 μg/mL). The treatment prevented the appearance of a significant amount of soluble FR-β in the medium (Figure 3A). During the 6 h incubation with Brefeldin A, there was no appreciable decrease in the pre-existing level of membrane associated FR-β determined by both [^{3}H]folic acid binding (Figure 3B) and Western blot (Figure 3C). These results indicate that the production of the soluble form of FR-β in the medium of cells expressing the membrane-associated protein occurs via the secretory pathway and that the soluble protein is not derived from the membrane-anchored form.

(iv) [^{35}S]Cysteine Pulse Chase Analysis. Metabolic pulse labeling of FR-β with [^{35}S]cysteine followed by various periods of chase with unlabeled cysteine and immunoprecipitation of the labeled protein was employed to monitor the fate of *de novo* synthesized FR-β both in cell lysates and in the extracellular media (Figure 4). A 41 kDa band, identified below as the mature GPI-anchored cell surface FR, first appeared in the cell lysates at 0.5 h of the pulse-chase reaching its maximum intensity at 2 h and remaining until 4 h (Figure 4A). The FR in the medium first appeared at 1 h and remained at a constant level between 2 and 4 h (Figure 4B). These results indicate the absence of a temporal relationship between the 41 kDa band and the FR in the medium consistent with a mechanism for the production of soluble FR-β that is independent of the plasma membrane-associated form of the protein. On the other hand, the results were consistent with a possible temporal relationship between two bands of lower apparent molecular mass in the cell lysate and the FR in the medium. The reduction/disappearance of those bands from the cell lysate coincided with the release of the soluble FR-β (Figure 4, panels A and B). The results suggest the possibility that the lower molecular mass bands in the cell lysate may be precursors of a secreted form of FR-β.

FR-β on the Cell Surface and in Intracellular Compartments. When human 293 fibroblasts expressing FR-β were solubilized by incubation at 37 °C in the presence of Triton X-100 and the total cell lysate was analyzed by Western blot, multiple protein bands were observed (Figure 5A); the apparent molecular masses of those bands are 41, 35, 33, and 29.3 kDa. A similar analysis of cell lysates after prior treatment of the cells with PI-PLC resulted in the disappearance of the 41 kDa band with no change in the remaining bands (Figure 5A). Thus, the 41 kDa band was identified

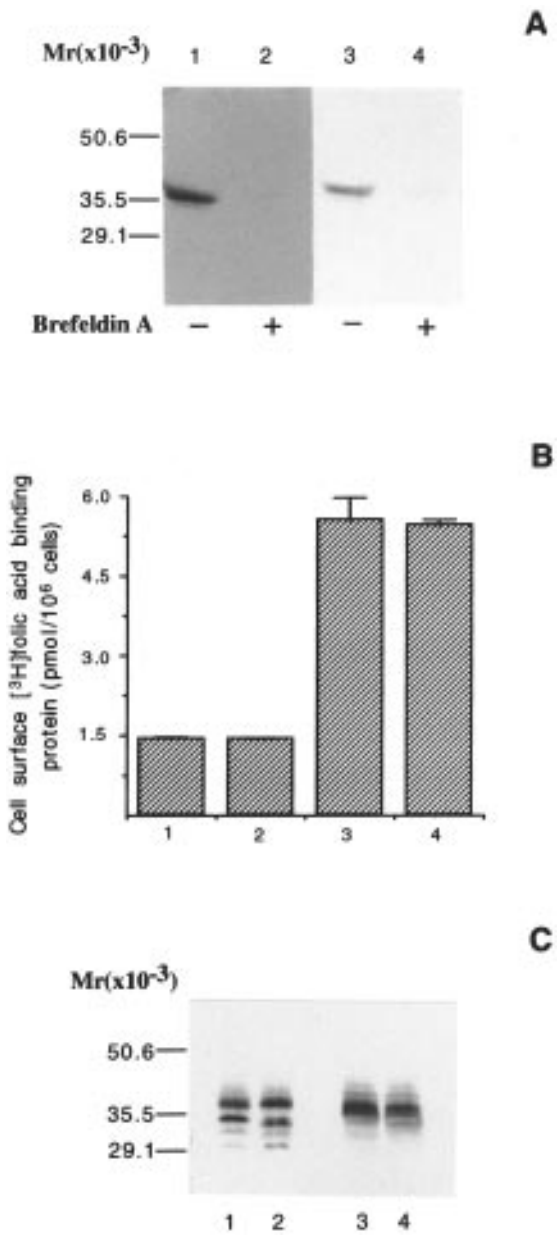

FIGURE 3: The effect of Brefeldin A on the release of FR-β. (A) 293 cells (lanes 1 and 2) or CHO-FR-β cells (lanes 3 and 4) were incubated in the presence or absence of Brefeldin A (50 μg/mL) in serum-free media for 6 h. TCA precipitated proteins from the media were analyzed by Western blot using affinity-purified rabbit antibodies to FR as described in the Materials and Methods. (B) Cell surface [^{3}H]folic acid binding before and after incubation with Brefeldin A; 293 cells transiently expressing FR-β (lanes 1 and 2) or CHO-FR-β cells (lanes 3 and 4) prior to (lanes 1 and 3) or after (lanes 2 and 4) 6 h incubation with Brefeldin A (50 μg/mL). The cell surface FR-β was measured by the [^{3}H]folic acid binding assay as described in the Materials and Methods. (C) After the ligand binding assay in Figure 3B, the cells were harvested, lysed, and 20 μg of total protein was subjected to Western blot analysis as described in the Materials and Methods. Lanes 1−4 correspond to the samples 1−4 in Figure 3B.

as the GPI-anchored cell surface protein that is sensitive to PI-PLC. The 29.3 kDa band comigrated with the mutant construct of FR-β, termed FR-$\beta\Delta$24 (CHO$^-$), lacking the sites of N-linked glycosylation and with a deletion of the carboxyl-terminal GPI signal (Figure 5A). Treatment of the cell lysate with *N*-glycanase resulted in two sharp bands with apparent molecular masses of 31 and 29.3 kDa (Figure 6). Treatment of the cells with PI-PLC prior to the preparation

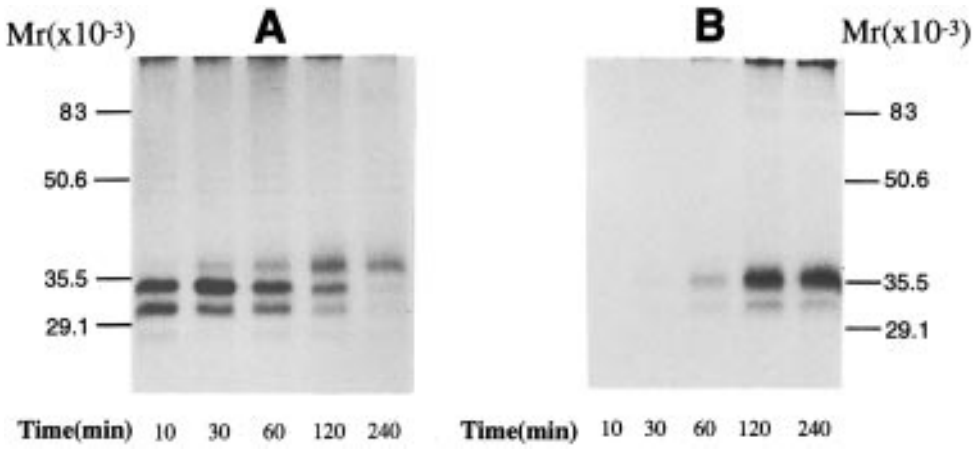

FIGURE 4: [^{35}S]Cysteine pulse-chase analysis of FR-β. Human 293 cells transiently transfected with the cDNA for FR-β were labeled with [^{35}S]cysteine (70 μCi/mL) for 20 min followed by chase with unlabeled cysteine for the indicated periods as described in the Materials and Methods. The labeled protein in the cell lysates (A) and in the media (B) was immunoprecipitated using anti-FR antibodies and analyzed by SDS−polyacrylamide gel electrophoresis and autoradiography as described in the Materials and Methods.

of the cell lysate and *N*-glycanase treatment resulted in the disappearance of the 31 kDa band (Figure 6). These results indicate that the 29.3 kDa band in the cells expressing FR-β represents the receptor that has not been N-glycosylated and which further lacks both a GPI anchor and the carboxyl-terminal signal for GPI modification. It should be noted that had the carboxyl-terminal peptide been retained in the 29.3 kDa band, its mobility would have been distinct from that of the nonglycosylated and truncated mutant construct under the electrophoretic conditions in Figure 5A. This is demonstrated by an additional control shown in Figure 7 in which a mutant construct termed FR-β' with an internal deletion of 20 amino acids (residues 143−163) migrated with a significantly greater mobility. The PI-PLC sensitive 31 kDa band in Figure 6 is presumably derived by deglycosylation of the mature GPI anchored FR-β on the plasma membrane. Thus, the lower mobility of the 31 kDa band compared to the 29.3 kDa band may be ascribed to GPI modification. The conversion of the 33 and 35 kDa bands in cells expressing FR-β (Figure 5) to a 29.3 kDa band upon *N*-glycanase treatment (Figure 6) indicates that those bands represent FR-β with different degrees of N-glycosylation and lack both the carboxyl-terminal GPI signal and GPI modification.

Relationship between GPI Signal Recognition and Carboxyl-Terminal Processing of FR-β. The apparent proteolysis of the carboxyl-terminal GPI signal without GPI modification in a significant proportion of FR-β obtained from the cell lysate led to the obvious question of whether such a proteolysis represented a partial sequence of the GPI modification reaction, i.e., recognition and cleavage of the GPI signal without addition of the GPI anchor. If this were true, it may be expected that one or more mutations in the GPI signal that will prevent its recognition for cleavage and GPI modification may result in the intracellular accumulation of FR-β with an uncleaved GPI signal. To test this possibility, several mutant constructs of FR-β were used. In one mutant, the primary site of GPI modification, Asn 230, was replaced by Gln, which cannot be modified by GPI. In such a construct, GPI modification occurs with a low efficiency at an alternate site (Yan & Ratnam, 1995). In another construct, the hydrophobicity of the GPI signal was altered by substituting Ala at position 246 with Asp. Deletion mutants were also used in which the GPI signal was deleted either entirely (24 residues) (FR-$\beta\Delta$24) or partially (10 residues) (FR-$\beta\Delta$10) from the carboxyl-terminus. The above mutations in the GPI signal have been previously shown to disrupt GPI modification and cell surface expression of FR-β (Yan & Ratnam, 1995). As seen in Figure 5, panels A and B, lysates from cells expressing all of the mutant proteins showed primarily the 29.3, 33, and the 35 kDa bands, all of which were insensitive to PI-PLC treatment of whole cells; treatment with *N*-glycanase resulted in a single sharp band of apparent 29.3 kDa indicating proteolysis of at least a major portion of their carboxyl-terminal peptides except for FR-$\beta\Delta$24 in which the GPI signal was previously removed (Figure 7). These results suggest that proteolysis of the GPI signal in the fraction of FR-β that occurred without GPI modification may not require recognition of its carboxyl-terminal peptide as a GPI signal.

FR-β *in the Extracellular Medium of Cells Expressing Wild-Type and Mutant Constructs.* There was a roughly equal distribution of [^{3}H]folic acid binding protein between the cell surface and the media in cells expressing wild-type FR-β, 24 h post-transfection, while the majority of the protein was recovered in the medium from cells expressing FR with disruptive mutations of the GPI signal (Table 3). Western blot analysis of the media from cultures expressing FR-β, as well as the receptor containing the various mutations described above in its carboxyl-terminal region that disrupted the GPI signal, showed the presence of FR-β (Figure 8A). Upon deglycosylation with *N*-glycanase, the protein was converted to an apparent 29.3 kDa band in every case (Figure 8B), corresponding to the nonglycosylated protein observed above in the cell lysates that lacked both GPI modification and an unprocessed carboxyl-terminus. These results suggest that the soluble FR-β in the medium is the mature form of the glycosylation intermediates observed above in the cell lysates of both the wild-type and the mutant forms of FR-β in which the carboxyl-terminus was processed without GPI modification. This conclusion is consistent with the observations made above by [^{35}S]cysteine pulse-chase analysis (Figure 4).

DISCUSSION

The results of this study demonstrate that a significant proportion of FR-β expressed in fibroblasts is present in the

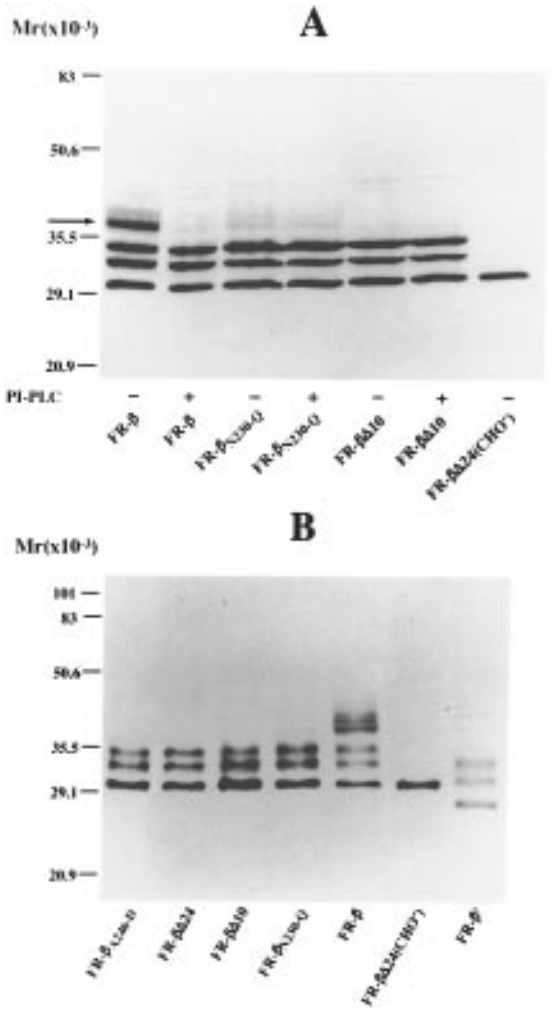

FIGURE 5: Western blot of cell lysates from transfected 293 cells expressing wild-type FR-β and its mutants. After 24 h transfection with cDNA for either the wild-type or the mutant FR-β constructs, the cells were incubated with or without PI-PLC at 37 °C for 1 h. The cell lysates were subjected to immunoblotting using anti-FR antibodies as described in the Materials and Methods. Panel A shows FR-β and its mutated forms with an Asn-Gln substitution at position 230 (FR-β_{N230-Q}), a carboxyl-terminal deletion of 10 amino acids (FR-$\beta\Delta10$) and a carboxyl-terminal deletion of 24 amino acids plus elimination of N-glycosylation sites by Asn-Gln substitution [FR-$\beta\Delta24(CHO^-)$]. Panel B includes additional mutants in which 24 carboxyl-terminal amino acids were deleted from FR-β (FR-$\beta\Delta24$), Ala at position 246 was replaced by Asp (FR-β_{A246-D}) or 20 amino acids were deleted from FR-β internally (FR-β').

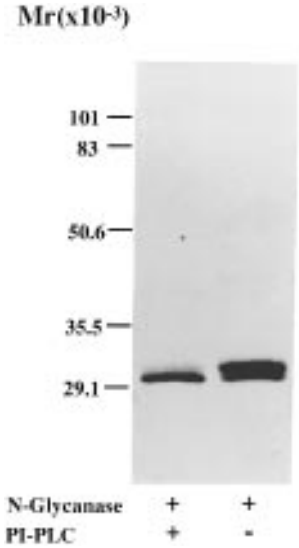

FIGURE 6: PI-PLC and *N*-glycanase treatment of cell lysates from transfected 293 cells expressing FR-β. The cells were incubated with or without PI-PLC at 37 °C for 1 h. Then, the cell lysates were prepared and incubated with *N*-glycanase at 37 °C for 18 h followed by Western blot analysis using anti-FR antibodies as described in the Materials and Methods.

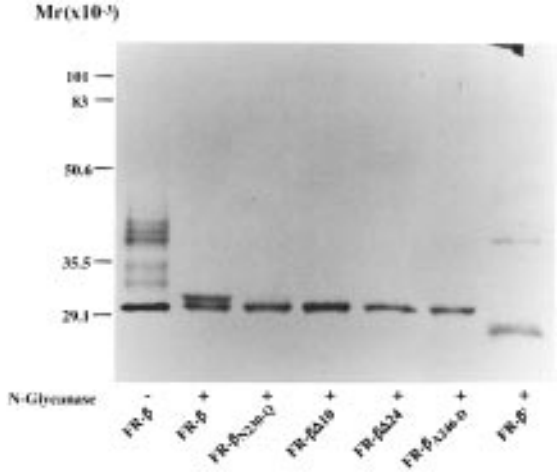

FIGURE 7: *N*-Glycanase treatment of cell lysates from transfected 293 cells expressing FR-β and its mutants. The mutant constructs are described in the legend to Figure 5. The cell lysates were incubated with *N*-glycanase at 37 °C for 18 h and subjected to Western blot analysis using anti-FR antibodies as described in the Materials and Methods.

extracellular media as a soluble folate binding protein. The production of soluble FR-β is folate independent and is clearly not an artifact of overexpression of the protein in transiently transfected human 293 fibroblasts because the distribution of the protein between the cell surface and the extracellular media is independent of overall expression levels of FR-β in these cells and also because recombinant CHO cells expressing a moderate level of FR-β (3.4×10^5 receptors per cell) produce the soluble protein.

In several instances, the plasma membrane origin of soluble forms of GPI-anchored proteins has been documented. Among these, cleavage of the GPI anchor by a membrane-associated phospholipase appears to be one common mechanism. The examples include decay accelerating factor (DAF) in HeLa cells (Metz et al., 1994), basic fibroblast growth factor-heparan sulfate proteoglycan in bone marrow cultures (Brunner et al., 1994), neural cell adhesion molecule (NCAM) in neural and skeletal muscle cell lines (He et al., 1987; Musaka et al., 1995) and a hydrophilic form of Thy-1 purified from cerebrospinal fluid (Almqvist & Carlsson, 1988). However, the action of membrane phospholipases on the GPI anchor cannot be expected to distinguish between the various GPI-linked proteins and cannot account for the selective release observed for certain proteins such as ADP-ribosyltransferase in activated T-cells (Nemoto et al., 1996) and CD-antigens in stimulated human granulocytes (Bazil & Strominger, 1994). In those examples and a few others, a proteolytic mechanism appears to be the likely means for the release of the soluble proteins. Indeed, a membrane-associated metalloprotease implicated in the release of soluble FR-α from epithelial cells was recently isolated (Yang et al., 1996). In addition, one of two soluble forms of CD14 is reported to arise from proteolysis of the

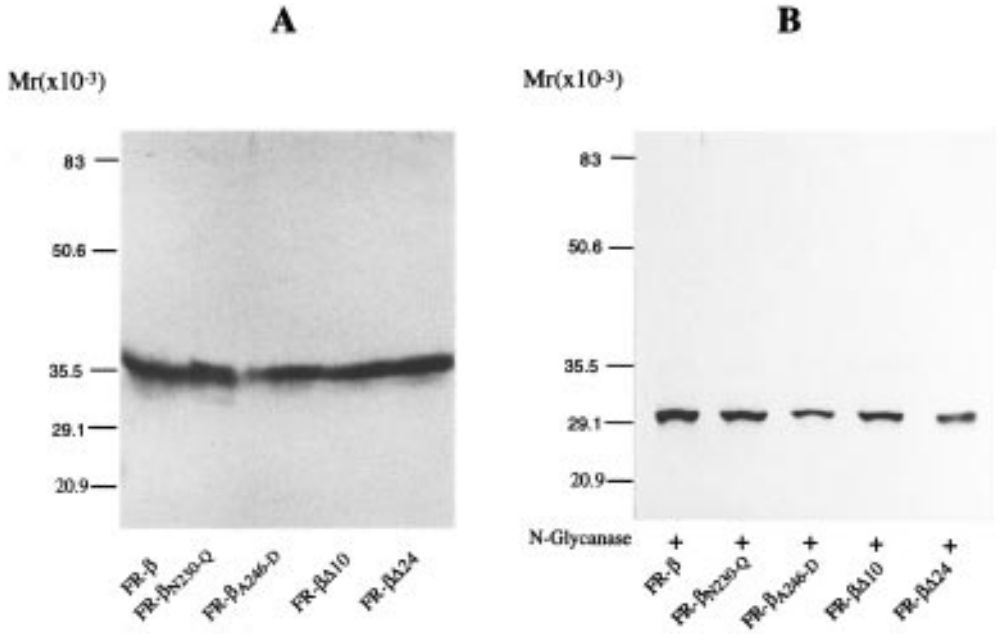

FIGURE 8: Western blot of proteins in the media of transfected 293 cells expressing FR-β and its mutants. (A) After 24 h transfection, the media were collected and the proteins precipitated with TCA. The pellets were dissolved in SDS sample loading buffer and subjected to immunoblotting using anti-FR antibodies as described in the Materials and Methods. The mutant constructs are described in the legend to Figure 5. (B) After 24 h transfection, the media were incubated with *N*-glycanase for 18 h at 37 °C. The proteins in the samples were then precipitated with TCA, dissolved in SDS sample loading buffer and analyzed by Western blot using anti-FR antibodies as described in the Materials and Methods. The mutant constructs are described in the legend to Figure 5.

GPI-anchored plasma membrane protein during a cycle of endocytosis followed by exocytosis (Durieux et al., 1994).

The production of the soluble FR-β in the medium occurred independently of serum factors. On the other hand, several independent lines of evidence discount the possibility that it is derived from the GPI anchored plasma membrane-associated receptor either due to the action of plasma membrane or lysosomal proteases or due to a membrane phospholipase. [^{35}S]Cysteine pulse-chase experiments failed to demonstrate a temporal relationship between the membrane-associated and soluble forms of FR-β. The possible involvement of an endogenous phospholipase in the release of a soluble FR-β from the membrane-bound receptor may be ruled out because of the smaller size of the protein from the media compared to FR-β released from the membrane by PI-PLC treatment. Evidence that the GPI-anchored membrane receptor is not the precursor of the soluble FR-β is offered by the finding that after inhibition of vesicular trafficking by Brefeldin A, the pre-existing cell surface FR-β was not processed to yield soluble protein. Direct evidence for this view is also provided by the finding that the appearance of the soluble protein in the medium is dependent upon the nature of the processed carboxyl-terminal signal for GPI modification. Thus, it could be demonstrated that FR-β may be engineered to reside predominantly as a GPI-anchored cell surface protein similar to FR-α simply by substituting its GPI signal with that of FR-α. Conversely, a significant proportion of FR-α may be released into the medium as a soluble protein by substituting its GPI signal with that of FR-β. These results indicate a fundamental role for the GPI signal in determining the ultimate destination of the protein. Since the recognition and cleavage of the GPI signal occurs prior to GPI modification in the endoplasmic reticulum, the above results also indicate that the cellular event(s) that determine the production of soluble FR-β must be initiated at this early stage of processing of the newly synthesized protein. Indeed, the inhibition of the release of soluble FR-β into the medium by Brefeldin A provides strong support for the intracellular generation of soluble FR-β and its passage through the vesicular secretory pathway.

In contrast to FR-β, there are examples in the literature in which proteins containing GPI modification signals retain their carboxyl-terminal sequences in the absence of GPI modification either due to suboptimal modification of the protein or due to a disruptive mutation in the GPI signal (Delahunty et al., 1993; Moran & Caras, 1992). Uncleaved GPI signals may also be retained due to the inability of the cell to synthesize GPI (Jost et al., 1991). In those cases, the unmodified proteins accumulated in intracellular compartments (Moran et al., 1992; Jost et al., 1991).

A major question pertaining to the molecular mechanism for the intracellular processing of a secreted FR-β is whether it is the result of the initial events leading to GPI anchor attachment, i.e., the recognition of the carboxyl-terminal GPI signal peptide and its cleavage, without completion of the transamidation reaction required for the modification of the new carboxyl-terminal amino acid. It was recently reported that in a small fraction (10%) of a truncated form of placental alkaline phosphatase, cleavage of the GPI signal occurs without anchor addition (Maxwell et al., 1995b). It was postulated that the presence of Ser at the ω position in the recombinant enzyme led to the formation of a partially stabilized six-membered ring in a transamidase−GPI-substrate complex which may have sufficient time to hydrolyze as a minor side reaction leading to a small fraction of cleaved but unmodified protein. It is thus conceivable that the GPI signal in a fraction of the total FR-β is recognized and cleaved by the cellular machinery without completion of the reaction sequence for GPI modification. To test this possibility, the effects of several mutations of the GPI signal in FR-β were studied. If the signal cleavage step of the GPI modification pathway caused the carboxyl-

terminal processing and secretion of a significant proportion of FR-β, then mutations of the GPI signal that are known to abolish GPI modification may result in the accumulation of the protein with unprocessed carboxyl-terminal peptides, as observed for other proteins (Jost et al., 1991; Moran et al., 1992). The mutations included (i) substitution of Asn230, which is the site of cleavage/GPI modification in FR-β (Yan & Ratnam, 1995), to Gln, which cannot be modified; (ii) substitution of Ala246 in the hydrophobic region of FR-β with the negatively charged Asp; (iii) deletion of the ten carboxyl-terminal amino acids in FR-β; and (iv) deletion of the carboxyl-terminal 24 amino acids which constitute the entire GPI signal in FR-β. While all of these mutations prevented attachment of the GPI membrane anchor, they resulted in secreted forms of FR-β that were indistinguishable from each other and from the secreted FR-β from cells expressing the wild-type protein. Further, the lysates from cells expressing the mutant constructs showed intracellular protein with different degrees of N-glycosylation, indistinguishable from the intracellular protein in cells expressing wild-type FR-β. After deglycosylation with *N*-glycanase, it was clear that the carboxyl-terminal peptides of the proteins from the cell lysates were all processed to an extent that rendered their electrophoretic mobility indistinguishable from that of the FR-β polypeptide with a 24 amino acid carboxyl-terminal deletion. These results strongly suggest the occurrence of intracellular carboxyl-terminal proteolytic processing in FR-β, independent of its recognition by the GPI modification machinery. One possible interpretation of these results is that the carboxyl-terminal peptide of the protein with an intact signal is quantitatively proteolyzed by the GPI modification pathway without efficient anchor addition while in the mutant proteins, which are not recognized for GPI modification, the carboxyl-termini are proteolyzed by a different mechanism(s). However, this possibility is deemed unlikely because of the variety of mutations that render the GPI signal unrecognizable leading to carboxyl-terminal processing and protein secretion. Furthermore, it is unlikely that in 293 cells the signal cleavage step of the GPI modification pathway occurs more efficiently than anchor addition because of the quantitative membrane anchoring observed when the GPI modification signal of FR-β was substituted with that of FR-α. Yet another possibility is that anchor addition is slower than signal cleavage during GPI modification of FR-β because of the nature of the modified amino acid (Asn). However, this is also unlikely because it has been previously shown that the efficiency of GPI modification of Asn is as high or higher than that of any other amino acid residue (Micanovic et al., 1990). It may also be noted that the postulated intermediate of the transmidation reaction for Ser at the ω site is not applicable to Asn (Maxwell et al., 1995b).

An alternate possibility in the production of secreted FR-β may be that a portion of the GPI modified FR-β is proteolytically cleaved in intracellular compartments shortly after its synthesis. This possibility is discounted by the production of secreted FR-β from the mutant forms of the protein discussed above that cannot be GPI modified. Direct evidence against intracellular proteolysis of GPI-modified FR-β is provided by the observation discussed above that secretion vs membrane anchoring of either FR-β or FR-α is dependent upon the nature of the carboxyl-terminal signal for GPI modification.

A functionally diverse variety of GPI-anchored proteins is present in soluble form in various extracellular fluids. In several instances, a single mechanism does not appear to account entirely for the production of the soluble proteins. In some cases, the reported data suggest either a major or a supplemental role of the secretory pathway. Thus, the GPI-linked form of hyaluronidase remains intact on the surface of mouse sperm while a soluble form of the enzyme is present in the acrosomal vesicle and is released during acrosomal exocytosis (Thaler & Cardullo, 1995). As another example, CD14, which occurs as a GPI-anchored protein in leukocytes, appears to be the precursor of only one of two soluble forms of the protein; a second soluble form of CD14 has a lower (by ~2000) apparent molecular mass, indicative of proteolytic processing, and is secreted even after deletion of its carboxyl-terminal eight amino acids (Durieux et al., 1994). Furthermore, the soluble CD14 is also released in the plasma of PNH patients that are deficient in GPI synthesis (Durieux et al., 1994). In several studies that have suggested a plasma membrane origin for the soluble forms of GPI-anchored proteins, a quantitative analysis of the relative contribution of such a mechanism to the production of the soluble proteins has not been reported. Much less is known about the origin of truncated soluble forms of GPI-anchored proteins in biological fluids *in vivo*. For example, a soluble form of decay accelerating factor (DAF) was shown to be derived from a GPI-anchored precursor of a similar size due to the action of a plasma membrane phospholipase in HeLa cells (Metz et al., 1994); on the other hand, both larger and smaller sizes have been noted for the protein in biological fluids. Thus, the physiologic mechanisms for the generation of hydrophilic extracellular forms of GPI-anchored proteins may vary depending upon the protein and the tissue of origin.

The results of this study have revealed an intracellular mechanism for the production of soluble counterparts of GPI-anchored proteins. They suggest the presence of two competing pathways for the carboxyl-terminal processing of FR-β in the ER. The fraction of the total protein that escapes GPI modification may instead be proteolytically processed at the carboxyl-terminus by a separate mechanism that may lead to its proper folding and secretion as a functional protein. The exact carboxyl-terminal peptide sequences of other GPI-modified proteins may determine their suitability as substrates for the latter proteolytic pathway. Even if only a small proportion of the total protein is secreted in this manner, it could accumulate in extracellular fluids reaching steady state levels determined by its rate of turnover, which may be significantly slower than that of its membrane counterpart. Since the carboxyl-terminal GPI signals of various proteins have considerable amino acid sequence diversity, the above mechanism could enable the selective and variable secretion of certain proteins while the unmodified forms of other proteins are retained intracellularly and degraded by cellular salvage mechanisms. The proteolytic processing and secretion may be tissue specific. Further, such a mechanism may also be utilized in the regulated secretion of certain proteins, which may be temporarily stored in secretory vesicles. Thus, the divergence of the carboxyl-terminal signal for GPI modification may provide an adaptive advantage when there is a need for the functional protein both at the cell surface and in the extracellular environment.

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