An Internally Positioned Signal Can Direct Attachment of a Glycophospholipid Membrane Anchor

Ingrid W. Caras

Department of Immunobiology, Genentech, Inc., South San Francisco, California 94080

Abstract. All known glycophosphatidylinositol (GPI)-anchored membrane proteins contain a COOH-terminal hydrophobic domain necessary for signalling anchor attachment. To examine the requirement that this signal be at the COOH terminus of the protein, we constructed a chimeric protein, DAFhGH, in which human growth hormone (hGH) was fused to the COOH terminus of decay accelerating factor (DAF) (a GPI-anchored protein), thereby placing the GPI signal in the middle of the chimeric protein. We show that the fusion protein appears to be processed at the normal DAF processing site in COS cells, producing GPI-anchored DAF on the cell surface. This result indicates that the GPI signal does not have to be at the COOH terminus to direct anchor addition, suggesting that the absence of a hydrophilic COOH-terminal extension (beyond the hydrophobic domain) is not a necessary requirement for GPI anchoring. A similar DAFhGH fusion, containing an internal GPI signal in which the DAF hydrophobic domain was replaced with the signal peptide of hGH, also produced GPI-anchored cell surface DAF. The signal for GPI attachment thus exhibits neither position specificity nor sequence specificity. In addition, mutant DAF or DAFhGH constructs lacking an NH₂-terminal signal peptide failed to produce GPI-anchored protein, suggesting that membrane translocation is necessary for anchor addition.

A diverse class of integral membrane proteins is now known to be anchored to the plasma membrane by a glycophosphatidylinositol (GPI)[1] structure covalently attached to the COOH terminus of the protein (for reviews see Cross, 1990; Low, 1989; Ferguson and Williams, 1988; Low and Saltiel, 1988). The GPI anchor contains phosphatidylinositol, carbohydrate, and ethanolamine, and is thought to be preassembled in the ER (Masterson et al., 1989). Attachment to the protein is directed by a signal at the COOH terminus of the protein (Caras et al., 1987a) and involves a processing event in which 17–31 COOH-terminal residues are removed from the nascent chain immediately before or concomitant with anchor addition (Boothroyd et al., 1981; Tse et al., 1985), the GPI anchor being added to the new COOH terminus. Anchor addition apparently takes place in the ER (Bangs et al., 1985, 1986; Ferguson et al., 1986), after which the protein is transported to the cell surface via the Golgi apparatus.

Although all GPI-anchored proteins presumably contain a signal for anchor attachment that is recognized by a common pathway, there is little or no primary sequence homology. The only commonly observed feature is the presence of a short hydrophobic domain (15–20 residues) at the COOH terminus of the protein (Low, 1989; Ferguson and Williams, 1988). This hydrophobic domain is absolutely necessary (although insufficient) for anchor attachment, deletion or shortening of the hydrophobic stretch leading to abolition of anchor attachment and secretion of the protein (Caras et al.,

1989; Berger et al., 1988). Using the GPI-anchored protein decay accelerating factor (DAF)[1] (Davitz et al., 1986; Medof et al., 1986) as a model system to analyze the signal for anchor attachment, we have previously shown that replacement of the COOH-terminal hydrophobic domain either with a signal peptide that normally functions in protein translocation, or with a random hydrophobic sequence, results in efficient and correct processing, producing GPI-anchored DAF on the cell surface (Caras and Weddell, 1989). These observations suggest that the function of the COOH-terminal hydrophobic domain in anchor attachment depends on its hydrophobicity rather than its precise sequence. In addition to the hydrophobic domain, anchor attachment requires a second element believed to be the cleavage/attachment site for the anchor (Caras et al., 1989), generally located 10–12 residues NH₂-terminal to the hydrophobic domain. Recent experiments have shown that Ser-319 (occurring 12 residues NH₂-terminal to the hydrophobic domain) is the GPI-linked residue of DAF, indicating that 28 residues are removed from the nascent chain during processing (Moran et al., 1991).

It has been suggested that a third feature is important for GPI attachment: the absence of a hydrophilic tail COOH-terminal to the hydrophobic domain (Berger et al., 1989;

---

1. *Abbreviations used in this paper:* DAF, decay accelerating factor; hGH, human growth hormone; GPI, glycophosphatidylinositol; PIPLC, phosphatidylinositol-specific phospholipase C.

Cross, 1990). This report focuses on the question of position specificity of the GPI signal and asks, by adding a long hydrophilic extension to the COOH terminus of DAF, whether an internally positioned (as opposed to COOH-terminal) GPI signal can function in anchor attachment. We also examine whether translocation into the ER is necessary for anchor addition and ask if the COOH-terminal hydrophobic domain can function as a translocation signal in the absence of an NH_2-terminal signal peptide.

Materials and Methods

Materials

mAbs against DAF were obtained from Dr. V. Nussenzweig of New York University (New York); purified rabbit antibody against human growth hormone (hGH) was provided by the Medicinal Analytical Chemistry Department (Genentech, Inc., South San Francisco, CA); IgG coupled to fluorescein was from Cappel Laboratories (Cochranville, PA); [^{3}H]ethanolamine was from Amersham Corp. (Arlington Heights, IL). Phosphatidylinositol-specific phospholipase C (PIPLC) purified from *Bacillus thuringiensis* was provided by Dr. M. G. Low of Columbia University (New York). Oligonucleotides were provided by M. Vasser, P. Jhurani, and P. Ng (Genentech, Inc.).

Recombinant Plasmids

A DAFhGH fusion was constructed by cloning a full-length DAF cDNA (Caras et al., 1987 *b*) and a full-length hGH cDNA (DeNoto et al., 1981) in tandem into the M13 vector mp 18. Deletion mutagenesis (Zoller and Smith, 1982) was then used to delete unwanted 3' sequence (i.e., the 3' untranslated region) from the DAF cDNA and unwanted 5' sequence from the hGH cDNA, thereby creating an in-frame fusion in which the last residue of DAF was fused to the first residue of mature hGH. To construct DAF-Sig2hGH, a cDNA encoding Δ1 DAF, which lacks the COOH-terminal hydrophobic domain of DAF (Caras et al., 1989), was used in place of wild-type DAF and the last residue of Δ1 DAF was fused to the first residue of the signal peptide of pre-hGH. DAF-Sig1hGH was constructed by fusing the previously described protein DAF-Sig1 (Caras and Weddell, 1989), containing residues −26 to −6 of the hGH signal peptide in place of the DAF COOH-terminal hydrophobic domain, to mature hGH as described above. The recombinant DNAs were verified by sequencing before being inserted into a mammalian expression vector between a cytomegalovirus enhancer-promoter and an SV40 polyadenylation sequence (Eaton et al., 1986). Sig⁻ mutants, lacking the NH_2-terminal signal peptide of DAF, were constructed by deletion mutagenesis in M13, verified by sequencing, and cloned into a mammalian expression vector as above.

Transfections, Metabolic Labeling, and Immunoprecipitation

COS cells were transfected using the DEAE dextran method as described by Selden (1987) using 2 µg of plasmid DNA per 35-mm dish and DEAE-Dextran at 400 µg/ml. Metabolic labeling of cells with [^{35}S]methionine and analysis of proteins by immunoprecipitation was as previously described (Caras et al., 1989; Caras and Weddell, 1989).

Immunofluorescent Labeling of Cells

Immunofluorescent labeling of intact cells (cell surface labeling) or permeabilized cells (internal labeling) was carried out essentially as described (Caras et al., 1987*b*) except that 0.5% Triton X-100/PBS was used to permeabilize the cells. Cells were incubated with a mAb against DAF or a purified rabbit antibody against hGH, followed by fluorescein-conjugated goat anti-mouse or -rabbit antiserum (Cappel Laboratories).

Results

All GPI-anchored membrane proteins contain a COOH-terminal hydrophobic domain required for anchor addition
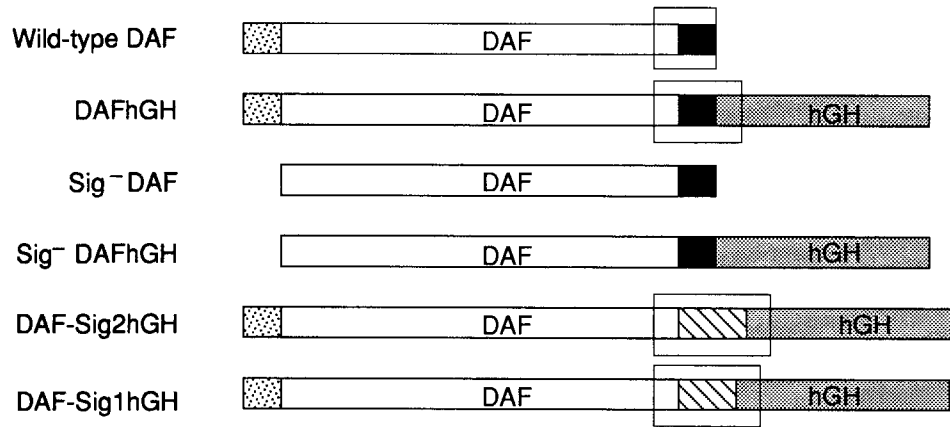
(Caras et al., 1989; Caras and Weddell, 1989; Berger et al., 1988). To investigate whether this hydrophobic domain must be at, or very near, the COOH terminus, we constructed a fusion protein, DAFhGH, containing mature hGH fused in-frame to the COOH terminus of DAF (Fig. 1). This fusion effectively repositions the COOH-terminal hydrophobic domain of DAF, placing it in the middle of the fusion protein between two large hydrophilic domains. The position of the cleavage/attachment site for the anchor (Ser-319) (Moran et al., 1991), relative to the hydrophobic domain, remains unchanged. In addition, we constructed two deletion mutants, Sig⁻ DAF and Sig⁻DAFhGH (Fig. 1) in which the NH_2-terminal signal peptide of DAF was removed from both wild-type DAF and the DAFhGH fusion protein. These deletion mutants address the questions: (*a*) Can the COOH-terminal hydrophobic domain serve as a membrane translocation signal in addition to, or simultaneous with, triggering GPI anchor attachment; and (*b*) is translocation across the membrane of the ER necessary for GPI attachment?

Immunoprecipitation Analysis

The cDNAs encoding these proteins were transiently expressed in COS cells under control of the cytomegalovirus promoter. The cells were labeled with [^{35}S]cysteine and the expressed proteins were immunoprecipitated from both the cell extracts and culture media using antibodies against either DAF or hGH. As previously reported (Caras et al., 1989), wild-type DAF is localized primarily in the cell lysate as a heterogenous, extensively glycosylated ∼70-kD mature form, and two ∼40-kD, partially glycosylated, precursor forms (Fig. 2, lane 2). In addition, the culture medium contains an ∼65-kD shed form (Fig. 2, lane 11). The cell lysate from cells transfected with the DAFhGH expression vector contained the ∼40- and ∼70-kD DAF species, both of which immunoprecipitated with the anti-DAF antibody but not with the anti-hGH antibody (Fig. 2, lanes 4 and 6), suggesting cleavage of the fusion protein and release of the NH_2-terminal DAF portion. The lysate also contained an ∼63-kD species that immunoprecipitated with both antibodies (anti-DAF and anti-hGH) and presumably represents uncleaved, partially or unglycosylated fusion protein (predicted molecular weight = 66 kD). A larger, ∼80-kD species, probably a glycosylated form of the uncleaved fusion protein, was secreted into the culture medium and coprecipitated with antibodies against both DAF and hGH (Fig. 2, lanes 13 and 15). In addition, low levels of an ∼27-kD hGH fragment, presumably the cleaved COOH-terminal portion of the fusion protein, was detected in the cell lysate using anti-hGH antiserum (lane 6). (The estimated molecular weight of this fragment, which should contain mature hGH plus 28 COOH-terminal residues of DAF, is ∼26-kD). These data suggest that the DAFhGH fusion protein was in part cleaved to yield an NH_2-terminal DAF fragment and a COOH-terminal hGH fragment, both of which remained cell associated, and in part secreted as an intact fusion protein.

Cells expressing Sig⁻DAFhGH, which lacks an NH_2-terminal signal peptide, contained low levels of a 60-kD protein in the cell lysate. Although the molecular weight suggests that this species represents uncleaved fusion protein, it was detectable only with the anti-hGH antibody (lane 7). The slightly lower molecular weight of this 60-kD species relative to the 63-kD species generated by the DAFhGH fu-
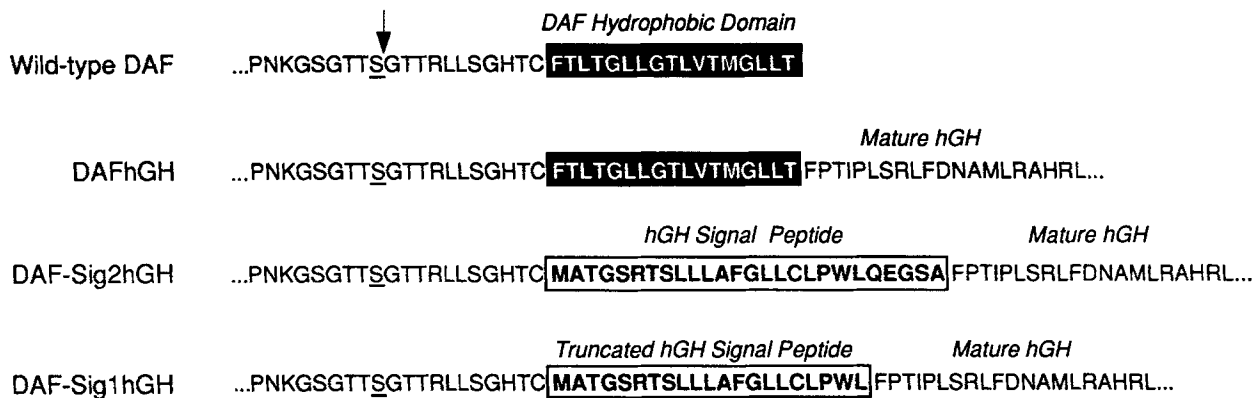
A

Wild-type DAF   DAF ▪

DAFhGH   DAF ▪ hGH

Sig⁻DAF   DAF ▪

Sig⁻ DAFhGH   DAF ▪ hGH

DAF-Sig2hGH   DAF ▨ hGH

DAF-Sig1hGH   DAF ▨ hGH

B

Wild-type DAF   *DAF Hydrophobic Domain*
...PNKGSGTTSGTTRLLSGHTC FTLTGLLGTLVTMGLLT

DAFhGH   *Mature hGH*
...PNKGSGTTSGTTRLLSGHTC FTLTGLLGTLVTMGLLT FPTIPLSRLFDNAMLRAHRL...

DAF-Sig2hGH   *hGH Signal Peptide*   *Mature hGH*
...PNKGSGTTSGTTRLLSGHTC MATGSRTSLLLAFGLLCLPWLQEGSA FPTIPLSRLFDNAMLRAHRL...

DAF-Sig1hGH   *Truncated hGH Signal Peptide*   *Mature hGH*
...PNKGSGTTSGTTRLLSGHTC MATGSRTSLLLAFGLLCLPWL FPTIPLSRLFDNAMLRAHRL...

Figure 1. (*A*) Schematic diagram showing DAFhGH fusion proteins and Sig⁻ mutants. The unshaded areas represent the DAF coding sequence. Shaded areas are as follows: (▨) signal peptide of DAF (residues −34 to −1); (▪) COOH-terminal hydrophobic domain of DAF (residues 331–347); (▨) mature hGH coding sequence (residues 1–191); (▨) signal peptide of hGH (residues −26 to −1 in DAF-Sig2hGH). DAF-Sig1hGH is similar to DAF-Sig2hGH but contains a truncated hGH signal peptide (residues −26 to −6) lacking the signal peptidase cleavage site. The boxed regions are expanded in *B* to show the actual sequences across the fusion junctions. (*B*) Expansion of the boxed areas in *A*, showing the amino acid sequence of the DAF COOH terminus and of the fusion junctions in the DAFhGH, DAF-Sig2hGH, and DAF-Sig1hGH fusion proteins. The cleavage/attachment site of DAF (Ser-319) is indicated by an arrow.

sion (lane 6) implies modification by glycosylation of the latter species only, in turn suggesting that the product of the Sig⁻DAFhGH construct was not translocated across the ER membrane. In addition, there was no evidence for secretion of this protein into the medium (lane 16), again suggesting that the internally positioned hydrophobic domain did not function as a translocation signal. No DAF protein was detectable in cells transfected with the Sig⁻DAF construct suggesting that if this protein was indeed expressed, it may have been rapidly degraded in the cytoplasm. Alternatively, incorrect folding may have precluded recognition by the anti-DAF antibody, which is known to be conformation dependent and does not recognize reduced DAF. Such conformation dependence of the anti-DAF antibody might also explain why the 60-kD Sig⁻DAFhGH fusion protein was recognized only by the anti-hGH antibody and not by the anti-DAF antibody.

Lanes 8 and 17 show a control transfection with the gene for chloramphenicol acetyl transferase (CAT), a cytoplasmic protein. The CAT protein was present exclusively in the cell lysate, indicating that there is no leakage of cytoplasmic protein to the medium. A control transfection with the hGH gene (lanes 9 and 18) shows a secreted protein of lower molecular weight than the COOH-terminal hGH fragment produced by cleavage of the DAFhGH fusion (lane 6), suggesting that the DAFhGH fusion protein was cleaved within the DAF sequence (possibly at the normal anchor addition site) rather than at the fusion junction. (Mature hGH has a molecular weight of ∼23 kD and electrophoreses with the dye front in this gel system).

Immunofluorescence Microscopy

To determine the cellular localization of protein(s) encoded by the DAFhGH expression plasmid, the cells were exam-
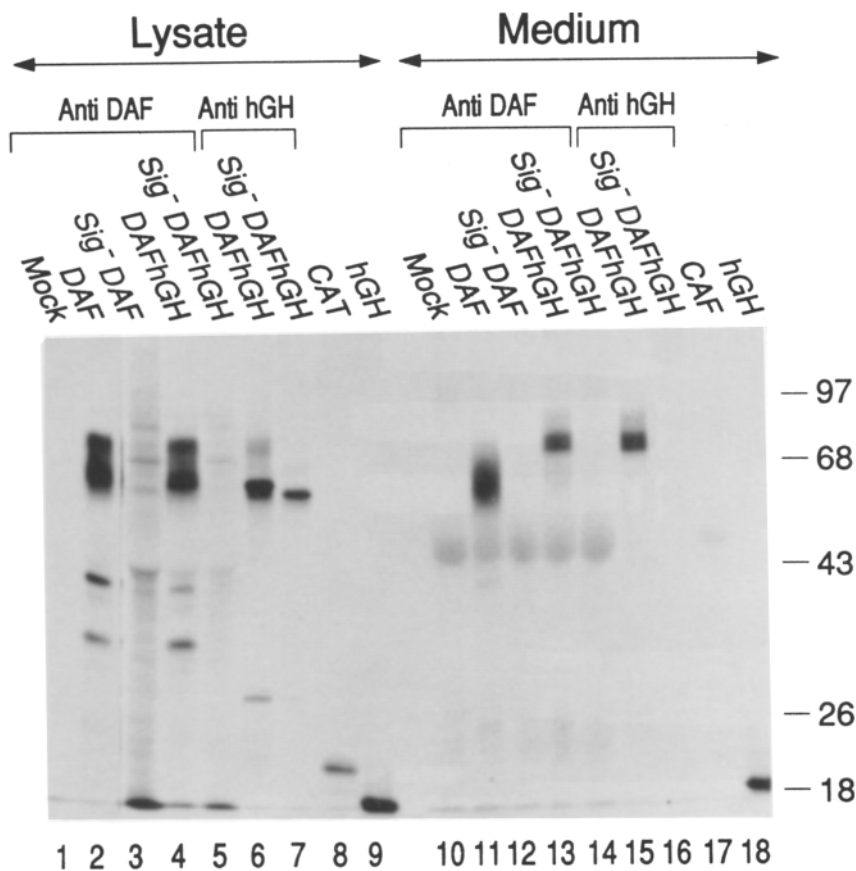
Figure 2. Immunoprecipitation of proteins from [³⁵S]cysteine-labeled transfected COS cells using anti-DAF and anti-hGH antibodies. COS cells were labeled with [³⁵S]cysteine for 6 h, 24 h after transfection. Proteins were immunoprecipitated from both cell lysates (lanes *1–9*) and the corresponding culture media (lanes *10–18*) using either anti-DAF or anti-hGH antibodies as indicated. Lysate and media fractions thus represent equal numbers of cells and the exposure times were equivalent. The DNAs used for transfection are indicated above each lane; DNA was omitted from mock transfected control cultures. Additional controls included transfection with RSV.CAT (to control for leakage of cytoplasmic proteins into the medium) followed by immunoprecipitation with an anti-CAT antibody (lanes *8* and *17*), and transfection with an hGH expression plasmid, followed by immunoprecipitation with anti-hGH (lanes *9* and *18*).

ined by immunofluorescence microscopy using antibodies directed against either DAF or hGH. Cell-surface labeling of intact cells indicated that DAF derived from the DAFhGH fusion is on the cell surface (Fig. 3 *b*) as is wild-type DAF (Fig. 3 *a*). HGH was not detected on the cell surface (data not shown). Staining of permeabilized cells with the anti-hGH antibody showed intense labeling of the ER and Golgi apparatus but not the plasma membrane (Fig. 3 *e*), whereas the anti-DAF antibody stained both the cell boundaries (plasma membrane) and internal organelles (Fig. 3, *c* and *d*). These data suggest that the cleaved DAF fragment produced from the DAFhGH fusion is targeted to the cell surface while the uncleaved fusion protein is confined to the organelles of the secretory pathway. No significant staining of either DAF or hGH could be detected in cells transfected with Sig⁻DAF or Sig⁻DAFhGH (data not shown), suggesting either low expression levels (possibly because of instability of non-translocated proteins) or poor recognition by the antibodies due to incorrect folding in the cytoplasm.

PIPLC Release Indicating the Presence of a GPI Anchor

To determine whether the cell surface DAF produced from the DAFhGH fusion is anchored by a GPI anchor, transfected COS cells were incubated with purified PIPLC, and the levels of DAF in the incubation supernatants were measured by an ELISA assay. PIPLC treatment of cells transfected with the DAFhGH expression vector resulted in a PIPLC-dependent release of DAF from the cell surface (Ta-

ble I), indicating that DAF molecules produced from the DAFhGH fusion are attached to the plasma membrane by a GPI membrane anchor. Shown for reference are the results obtained with control cells expressing either wild-type DAF, similarly showing PIPLC-dependent release, or a secreted form of DAF (Δ DAF; Caras et al., 1989) lacking a COOH-terminal hydrophobic domain, showing PIPLC-independent secretion into the incubation supernatant. Cells transfected with DAFhGH produced approximately fivefold less PIPLC-releasable DAF than cells expressing wild-type DAF. Although transient transfection efficiencies are variable (depending on factors such as the quality and purity of the DNA used), this result suggests that the internal GPI signal is recognized less efficiently than the COOH-terminal signal. As expected, no DAF was detected in supernatants from cells transfected with the Sig⁻ deletion mutants, Sig⁻DAF, and Sig⁻DAFhGH (not shown).

[³H]Ethanolamine Labeling

To verify that the cell surface DAF produced from the DAFhGH fusion is anchored by a GPI membrane anchor, transfected COS cell were metabolically labeled with [³H]ethanolamine and analyzed by immunoprecipitation using an anti-DAF antibody. Cell lysates from cells transfected with DAFhGH contained a labeled ∼70-kD doublet (Fig. 4, lane *4*) that comigrated with authentic DAF (Fig. 4, lane *2*), confirming that correctly processed GPI-anchored DAF is produced from the DAFhGH fusion. In addition, the media from cells expressing either DAF or DAFhGH con-
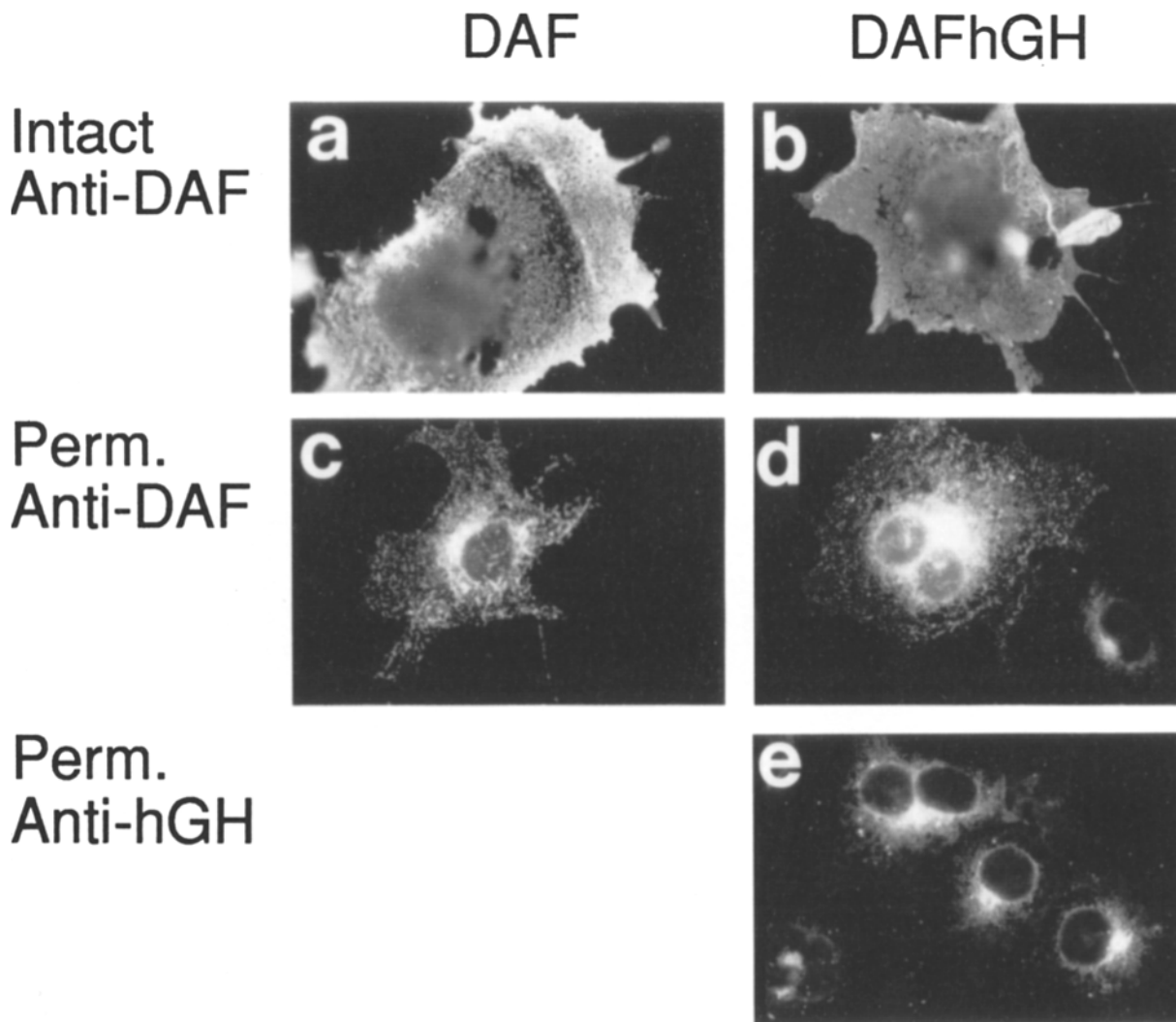
DAF

DAFhGH

Intact Anti-DAF

Perm. Anti-DAF

Perm. Anti-hGH

Figure 3. Immunofluorescent labeling of transfected COS cells. Cells were transfected with DNAs encoding either wild-type DAF (*a* and *c*) or the DAFhGH fusion (*b*, *d*, and *e*). To detect cell surface expression (*a* and *b*), cells were fixed but not permeabilized, and labeled as described in Materials and Methods using an anti-DAF antibody. To detect intracellular protein (*c*, *d*, and *e*), cells were fixed and permeabilized as described in Materials and Methods labeled with either an anti-DAF or anti-hGH antibody as indicated.

tained an ∿68-kD ethanolamine-labeled species, believed to represent a released form of DAF resulting from a cleavage within the GPI anchor (Caras et al., 1989). The ∿80-kD uncleaved hGHDAF fusion protein secreted to the medium

Table I. DAF Levels Measured by ELISA in Supernatants from Transfected COS Cells Incubated With or Without PIPLC

Transfected DNA	Addition	
	None	PIPLC
	DAF (ng/ml)	
None	<0.09	<0.09
Wild-type DAF	10	252
DAFhGH	4	48
Δ1 DAF*	22	28

* Δ1 DAF is a deletion mutant of DAF that lacks a COOH-terminal hydrophobic domain, fails to become GPI-anchored, and is secreted (Caras et al., 1989).

(Fig. 2) was not labeled with [^{3}H]ethanolamine, nor were any ethanolamine-labeled bands detected in cells expressing Sig⁻DAF or Sig⁻DAFhGH.

An Internally Positioned Signal Peptide Can Direct GPI Attachment

We have previously shown that a signal for protein secretion can direct GPI anchor attachment when positioned at the COOH terminus of DAF, in place of the DAF hydrophobic domain (Caras and Weddell, 1989). To determine whether a signal peptide located internally within a protein can similarly direct GPI attachment, we constructed the fusion protein DAF-Sig2hGH (Fig. 1) in which a DAF sequence lacking the COOH-terminal hydrophobic domain was fused to pre-hGH. The NH₂-terminal signal peptide of hGH thus replaces the COOH-terminal hydrophobic domain of DAF and is followed by the complete mature hGH coding sequence. The position of the cleavage/attachment site relative to the hydrophobic signalling domain, remains unchanged.
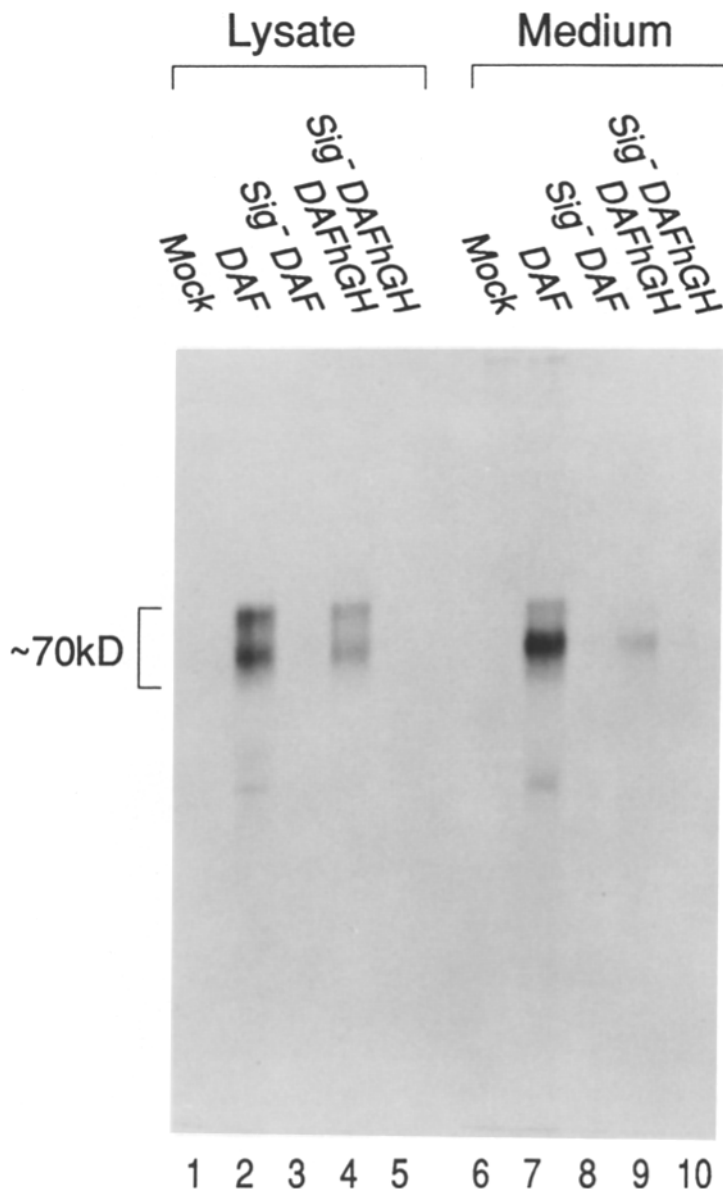
Lysate   Medium

Mock
Sig⁻ DAF
DAF
Sig⁻ DAFhGH
DAFhGH
Mock
Sig⁻ DAF
DAF
Sig⁻ DAFhGH
DAFhGH

~70kD

1 2 3 4 5   6 7 8 9 10

Figure 4. [³H]Ethanolamine labeling and immunoprecipitation of proteins in transfected COS cells. COS cells transfected with DNAs as indicated were labeled overnight with [³H]ethanolamine (200 μCi per 35-mm dish). Cell lysates (lanes *1–5*) and media (lanes *6–10*) were analyzed by immunoprecipitation using an anti-DAF antibody. DNA was omitted from mock-transfected controls.

To eliminate potential complications caused by cleavage of the internal signal peptide by signal peptidase, a similar fusion, DAF-Sig1hGH (Fig. 1), was constructed in which the signal peptidase cleavage site (residues −5 to −1) was deleted from the hGH signal peptide, thus preventing possible cleavage of the fusion protein near the fusion junction. This fusion protein is analogous to the previously described protein DAF-Sig1, containing a truncated hGH signal peptide in place of the DAF COOH-terminal hydrophobic domain (Caras and Weddell, 1989), but the entire hGH coding region has now been added as a COOH-terminal extension. COS cells were transfected with expression plasmids encoding DAF-Sig2hGH or DAF-Sig1hGH, and then labeled with [³⁵S]cysteine. The proteins were analyzed by immunoprecipitation, revealing a pattern similar to that observed with the DAFhGH fusion (data not shown), suggesting that, like DAFhGH, these fusion proteins were in part cleaved to yield both DAF and hGH fragments, and in part secreted as intact fusion proteins. There was no significant difference in the polypeptides produced from the DAF-Sig1hGH and DAF-Sig2hGH fusions, and the absence of a fragment corresponding to mature hGH in either the cell lysate or culture medium suggests that the internally positioned hGH signal peptide was not cleaved by signal peptidase.
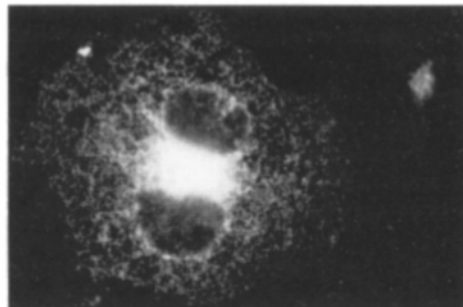
Immunofluorescent staining of nonpermeabilized COS cells transfected with DAF-Sig2hGH or DAF-Sig1hGH revealed that DAF (but not hGH) was expressed on the cell surface (Fig. 5, *top*), data shown only for DAF-Sig2hGH). Staining of permeabilized cells indicated that hGH was present in the secretory pathway (the ER and Golgi apparatus were intensely stained; Fig. 5, *bottom*), but not on the plasma membrane whereas DAF could be detected on the plasma membrane as well as in these internal organelles (Fig. 5, middle).

To determine whether cleavage of DAF-Sig1hGH or DAF-Sig2hGH produces GPI-anchored DAF, transfected COS cells were labeled with [³H]ethanolamine and then analyzed by immunoprecipitation. The DAF-Sig1hGH and DAF-

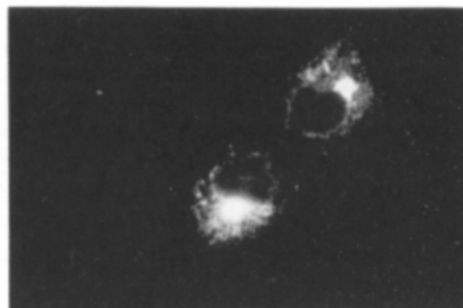# Intact Anti-DAF
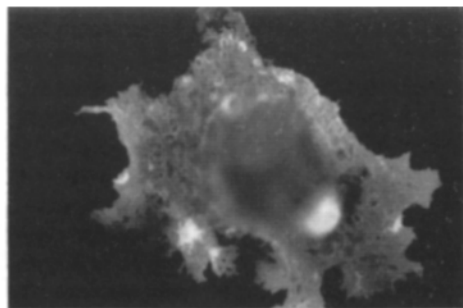
Perm. Anti-DAF

Perm. Anti-hGH

Figure 5. Immunofluorescent labeling of COS cells transfected with DAF-Sig2hGH. Intact cells (fixed but not permeabilized) were labeled with an anti-DAF antibody (*top*); permeabilized cells were labeled with either anti-DAF (*middle*) or anti-hGh antibodies (*bottom*). Procedures were as described in Materials and Methods.

Sig2hGH products (Fig. 6, lanes 2 and 3) were compared with authentic DAF (lane 4) and DAF-Sig2 (lane 5), containing a COOH-terminal hGH signal peptide in place of the DAF hydrophobic domain (Caras and Weddell, 1989). All the cell lysates contained similar [³H]ethanolamine-labeled bands corresponding to the 40-kD precursor and ~70-kD mature form of authentic DAF, indicating that the DAF-Sig1hGH and DAF-Sig2hGH fusions were processed at or near the normal DAF processing site, giving rise to GPI-anchored cell surface DAF. These results indicate that an internally positioned signal peptide can function in GPI attachment, suggesting that the requirement for a COOH-terminal hydrophobic domain is neither sequence specific nor position specific.

Discussion

The Signal for GPI Attachment Does Not Have To Be at the COOH Terminus

Previous reports have shown that GPI attachment is directed by a COOH-terminal signal consisting of two elements: a hydrophobic domain and a cleavage attachment site, located ~10–12 residues NH₂-terminal to the hydrophobic domain (Caras et al., 1989). In addition, it has been suggested that

the absence of a hydrophilic (cytoplasmic) tail may be an important feature of the GPI signal (Berger et al., 1980; Cross 1990). In this report, we examined the requirement that the GPI signal be at the COOH terminus of the protein. Our results show that repositioning of the GPI signal of DAF to the middle of a large hydrophilic fusion protein allows correct processing, apparently at or near the normal processing site, producing authentic GPI-anchored DAF on the cell surface. The question as to whether the absence of a hydrophilic COOH-terminal tail is an important feature of the GPI signal, has been controversial. The presence or absence of the 21-amino acid cytoplasmic tail present in the transmembrane but not the GPI-anchored form of the human IgG Fc receptor (FcγRIII) is apparently not the critical feature that determines GPI anchoring, and molecules containing this short, COOH-terminal extension can become GPI anchored (Lanier et al., 1980; Kurosaki and Ravetch, 1989). On the other hand, replacement of the COOH-terminal hydrophobic domain of placental alkaline phosphatase with the transmembrane-cytoplasmic domains of vesicular stomatitis virus glycoprotein completely abolished GPI attachment, leading to the suggestion that the absence of a cytoplasmic tail may be important for GPI anchoring (Berger et al., 1989). Our data now indicate that despite the presence of a long hydrophilic extension of 191 amino acids, DAF molecules can be targeted to the plasma membrane via a GPI anchor, sug-
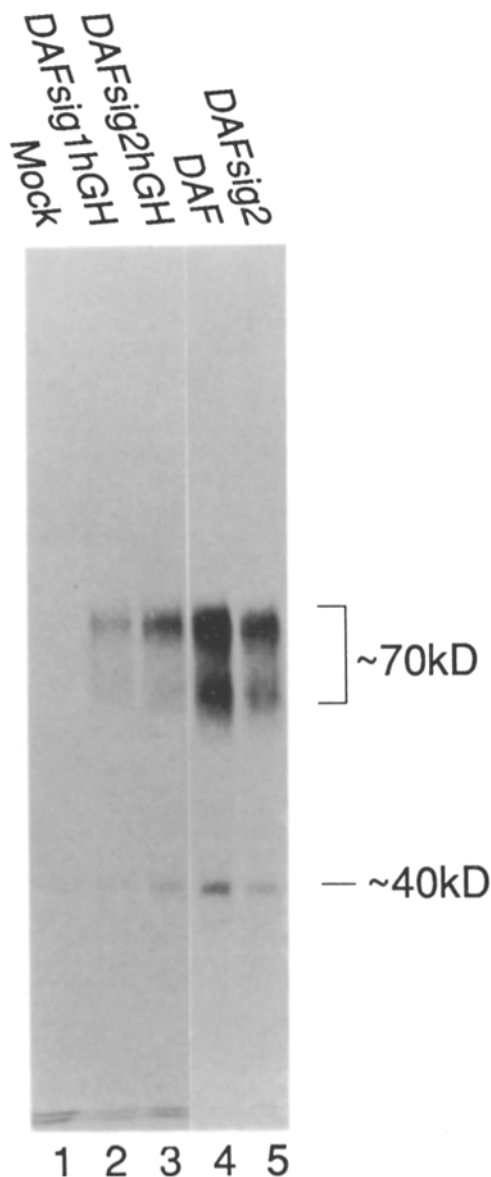
Mock
DAFsig1hGH
DAFsig2hGH
DAF
DAFsig2

~70kD

~40kD

1 2 3 4 5

Figure 6. [³H]Ethanolamine labeling of COS cells transfected with DAF-Sig1hGH or DAF-Sig2hGH. Cells were labeled with [³H]-ethanolamine and analyzed by immunoprecipitation using an anti-DAF antibody as described in Materials and Methods. The DNAs used for transfection are: DAF-Sig1hGH, similar to DAF-Sig2hGH but containing a truncated hGH signal peptide lacking the signal peptidase cleavage site (lane *2*); DAF-Sig2hGH (lane *3*); wild-type DAF (lane *4*); and DAF-Sig2, containing the signal peptide of hGH in place of the DAF COOH-terminal hydrophobic domain (lane *5*). DNA was omitted from the mock-transfected control.

results suggest that this may represent an evolutionary adaptation rather than a strict functional constraint. Since the COOH-terminus of the nascent polypeptide is removed (and presumably degraded) during anchor addition, there may be little advantage to synthesizing more than the minimum COOH-terminal sequence required to contain the GPI signal.

The absence of any detectable staining of the plasma membrane when permeabilized cells were stained with an anti-hGH antibody suggests that intact fusion molecules were not held on the cell surface in a non-GPI-anchored form. This observation that uncleaved fusion molecules are secreted rather than held in the plasma membrane as transmembrane proteins, suggests that the COOH-terminal hydrophobic domain of DAF (17 amino acids) does not function as a membrane anchor, being either insufficiently hydrophobic, or lacking additional stop-transfer signals. This implies that the hydrophobic domain functions as an actual recognition signal for GPI attachment rather than as a transient membrane anchor, as has been suggested.

An Internally Positioned Signal Peptide Can Direct GPI Attachment

We have previously shown that the COOH-terminal hydrophobic domain of DAF can be replaced with unrelated hydrophobic sequences such as the hGH membrane translocation signal peptide, without affecting anchor addition (Caras and Weddell, 1989). Our present results show that this is also true for the internally positioned GPI signal. The hGH signal peptide for protein secretion effectively replaced the DAF hydrophobic domain when positioned in the middle of the hydrophilic DAFhGH fusion protein, indicating that the requirement for a hydrophobic domain in GPI attachment is neither sequence specific nor position specific. It is not unreasonable to speculate that any internal hydrophobic domain has the potential to act as a signal for GPI addition, one constraint being the requirement for a recognizable cleavage/attachment site located ∿10–12 residues NH₂-terminal to the hydrophobic domain. It is conceivable that the accumulation of point mutations creating or deleting a GPI cleavage/attachment site could interconvert genes encoding transmembrane or GPI-linked proteins. The closely related genes for two forms of the FcγRIII receptor (encoding a GPI-linked protein on neutrophils and a transmembrane protein on natural killer cells and macrophages) could represent such a case. Here, a single amino acid change near the COOH terminus has been shown to determine the mode of anchorage of the protein (Lanier et al., 1989; Kurosaki and Ravetch, 1989).

Membrane Translocation Appears To Be A Prerequisite for GPI Attachment

DAF or DAFhGH proteins that lacked an NH₂-terminal signal peptide failed to produce GPI-anchored cell surface DAF. Deletion of the NH₂-terminal signal peptide from DAF or the DAFhGH fusion apparently prevented membrane translocation and curtailed the expression level of these proteins in COS cells, possibly because failure to be translocated resulted in rapid degradation in the cytoplasm. In addition, there was no evidence for incorporation of a GPI anchor (e.g., by [³H]ethanolamine labeling). These results suggest that the DAF hydrophobic domain, positioned either

gesting that the absence of a hydrophilic tail is not a critical feature of the GPI signal. A fraction of these altered DAF molecules escape processing however and are secreted as intact fusion protein, suggesting that an internal GPI signal may be recognized less efficiently than a COOH-terminal signal.

All known GPI-anchored proteins examined to date contain hydrophilic extensions (beyond the COOH-terminal hydrophobic domain) of no more than a few amino acids. Our

internally or at the COOH terminus, did not function as a translocation signal. Secondly, although negative results preclude a firm conclusion, it seems likely that membrane translocation is required for GPI anchor attachment.

References

Bangs, J. D., D. Hereld, J. L. Krakow, G. W. Hart, and P. T. Englund. 1985. Rapid processing of the carboxyl terminus of a trypanosome variant surface glycoprotein. *Proc. Natl. Acad. Sci. USA.* 82:3207-3211.

Bangs, J. D., N. W. Andrews, G. W. Hart, and P. T. Englund. 1986. Posttranslational modification and intracellular transport of a trypanosome variant surface glycoprotein. *J. Cell Biol.* 103:255-263.

Berger, J., A. D. Howard, L. Brink, L. Gerber, J. Hauber, B. R. Cullen, and S. Udenfriend. 1988. COOH-terminal requirements for the correct processing of a phosphatidylinositol-glycan anchored membrane protein. *J. Biol. Chem.* 263:10016-10021.

Berger, J., R. Micanovic, R. J. Greenspan, and S. Udenfriend. 1989. Conversion of placental alkaline phosphatase from a phosphatidylinositol-glycananchored protein to an integral transmembrane protein. *Proc. Natl. Acad. Sci. USA.* 86:1457-1460.

Boothroyd, J. C., C. A. Paynter, G. A. M. Cross, A. Bernards, and P. Borst. 1981. Variant surface glycoproteins of *Trypanosoma brucei* are synthesized with cleavable hydrophobic sequences at the carboxy and amino termini. *Nucl. Acids Res.* 9:4735-4743.

Caras, I. W., and G. N. Weddell. 1989. Signal peptide for protein secretion directing glycophospholipid membrane anchor attachment. *Science (Wash. DC).* 243:1196-1198.

Caras, I. W., G. N. Weddell, M. A. Davitz, V. Nussenzweig, and D. W. Martin, Jr. 1987a. Signal for attachment of a phospholipid membrane anchor in decay accelerating factor. *Science (Wash. DC).* 238:1280-1283.

Caras, I. W., M. A. Davitz, L. Rhee, G. Weddell, D. W. Martin, Jr., and V. Nussenzweig. 1987b. Cloning of decay-accelerating factor suggests novel use of splicing to generate two proteins. *Nature (Lond.).* 325:545-549.

Caras, I. W., G. N. Weddell, and S. R. Williams. 1989. Analysis of the signal for attachment of a glycophospholipid membrane anchor. *J. Cell Biol.* 108:1387-1396.

Cross, G. A. M. 1990. Glycolipid anchoring of plasma membrane proteins. *Annu. Rev. Cell Biol.* 6:1-39.

Davitz, M. A., M. G. Low, and V. Nussenzweig. 1986. Release of DAF from the cell membrane by PI-specific PIPLC: selective modification of a complement regulatory protein. *J. Exp. Med.* 163:1150-1161.

DeNoto, F. M., D. D. Moore, and H. M. Goodman. 1981. Human growth hormone DNA sequence and mRNA structure: possible alternative splicing. *Nucl. Acids Res.* 9:3719-3730.

Eaton, D. L., W. I. Wood, D. Eaton, P. E. Hass, P. Hollingshead, K. Wion, J. Mather, R. M. Lawn, G. A. Vehar, and C. Gorman. 1986. Construction and characterization of an active factor VIII variant lacking the central one-third of the molecule. *Biochemistry.* 25:8343-8347.

Ferguson, M. A. J., and A. F. Williams. 1988. Cell surface anchoring of proteins via glycosylphosphatidylinositol structures. *Annu. Rev. Biochem.* 57:285-320.

Ferguson, M. A. J., M. Duszenko, G. S. Lamont, P. Overath, and G. A. M. Cross. 1986. Biosynthesis of *Trypanosoma brucei* variant surface glycoproteins. N-glycosylation and addition of a phosphatidylinositol membrane anchor. *J. Biol. Chem.* 261:356-362.

Kurosaki, T., and J. V. Ravetch. 1989. A single amino acid in the glycosyl phosphatidylinositol attachment domain determines the membrane topology of FcγRIII. *Nature (Lond.).* 342:805-807.

Lanier, L. L., S. Cwirla, G. Yu, R. Testi, and J. H. Phillips. 1989. Membrane anchoring of a human IgG Fc receptor (CD16) determined by a single amino acid. *Science (Wash. DC).* 246:1611-1613.

Low, M. G. 1989. Glycosyl-phosphatidylinositol: A versatile anchor for cell surface proteins. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 3:1600-1608.

Low, M. G., and A. R. Saltiel. 1988. Structural and functional roles of glycosylphosphatidylinositol in membranes. *Science (Wash. DC).* 239:268-275.

Masterson, W., T. L. Doering, G. W. Hart, and P. T. Englund. 1989. A novel pathway for glycan assembly: biosynthesis of the glycosyl-phosphatidylinositol anchor of the trypanosome variant surface glycoprotein. *Cell.* 56:793-800.

Medof, M. E., E. I. Walter, W. L. Roberts, R. Haas, and T. L. Rosenberry. 1986. Decay accelerating factor of complement is anchored to cells by a C-terminal glycolipid. *Biochemistry.* 25:6740-6747.

Moran, P., H. Raab, W. J. Kohr, and I. W. Caras. 1991. Glycophospholipid membrane anchor attachment: analysis of the cleavage/attachment site. *J. Biol. Chem.* 226:1250-1257.

Selden, R. F. 1987. Transfection using DEAE-Dextran. *In* Current Protocols in Molecular Biology, Vol. 1. Ausubel, F. N., R. Brent, R. E. Kingston, Moore, J. G. Seidman, J. A. Smith, and K. Struhl, editors. Greene Publishing Associates and Wiley-Interscience, John Wiley & Sons Inc., New York. 9.2.1-9.2.6.

Tse, A. G. D., A. N. Barclay, A. Watts, and A. F. Williams. 1985. A glycophospholipid tail at the carboxyl terminus of the Thy-1 glycoprotein of neurons and thymocytes. *Science (Wash. DC).* 230:1003-1008.

Zoller, M. J., and M. Smith. 1982. Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any fragment of DNA. *Nucl. Acids Res.* 10:6487-6500.