

Analysis of the Signal for Attachment of a Glycophospholipid Membrane Anchor

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Abstract. The COOH terminus of decay accelerating factor (DAF) contains a signal that directs attachment of a glycophospholipid (GPI) membrane anchor. To define this signal we deleted portions of the DAF COOH terminus and expressed the mutant cDNAs in CV1 origin-deficient SV-40 cells. Our results show that the COOH-terminal hydrophobic domain (17 residues) is absolutely required for GPI anchor attachment. However, when fused to the COOH terminus of a

secreted protein this hydrophobic domain is insufficient to direct attachment of a GPI anchor. Additional specific information located within the adjacent 20 residues appears to be necessary. We speculate that by analogy with signal sequences for membrane translocation, GPI anchor attachment requires both a COOH-terminal hydrophobic domain (the GPI signal) as well as a suitable cleavage/attachment site located NH₂ terminal to the signal.

A novel mechanism for anchoring proteins to the plasma membrane has recently been described involving covalent attachment of a complex structure containing phosphatidylinositol, carbohydrate, and ethanolamine to the COOH terminus of the protein (for reviews see Low, 1987; Low and Saltiel, 1988; Ferguson and Williams, 1988). Attachment of this glycophospholipidinositol (GPI)¹ anchor is thought to occur in the endoplasmic reticulum (Bangs et al., 1985, 1986; Ferguson et al., 1986) after proteolytic removal of 17–31 COOH-terminal residues including a hydrophobic domain (Boothroyd et al., 1980; Tse et al., 1985). A functionally diverse group of ~30 proteins is currently known to be anchored in this way (Low and Saltiel, 1988), including Thy-1 (Low and Kincade, 1985; Tse et al., 1985), the variant surface glycoproteins of African trypanosomes (Ferguson et al., 1985), acetylcholinesterase (Futerman et al., 1985; Roberts and Rosenberry, 1986), the neural cell adhesion molecule (Hemperly et al., 1986), Qa-2 (Stroynowski et al., 1987; Wanneck et al., 1988), the Scrapie prion protein (Stahl et al., 1987), and decay accelerating factor (DAF) (Davitz et al., 1986; Medof et al., 1986).

DAF is a complement regulatory protein that binds activated complement fragments, C3b and C4b, thereby inhibiting amplification of the complement cascade on host cell membranes (Kinoshita et al., 1986; Medof et al., 1984; Nicholson-Weller et al., 1982). We recently identified two classes of DAF mRNA in HeLa cells, one apparently related to the other by a splicing event that causes a coding frame-

shift near the COOH terminus (Caras et al., 1987a). Two DAF proteins are therefore possible, having divergent COOH-terminal domains. The spliced DAF mRNA predicts a hydrophobic COOH terminus and generates GPI-anchored, membrane-bound DAF. The protein encoded by the unspliced cDNA has a hydrophilic COOH terminus and is secreted. These observations suggest that the COOH terminus of membrane DAF contains information required for attachment of a GPI anchor. We confirmed this by showing that the last 37 amino acids of membrane DAF, when fused to a secreted protein, are sufficient to target the fusion protein to the plasma membrane by means of a GPI anchor (Caras et al., 1987b). Similar experiments involving the construction of hybrid proteins by exon switching indicated that the COOH-terminal domain of the Qa-2 antigen can produce a GPI-anchored H-2 antigen (a molecule normally anchored by a transmembrane domain near the COOH terminus). Conversely, a fusion containing the NH₂-terminal portion of Qa-2 and the COOH-terminal domain of an H-2 antigen was not GPI anchored (Stroynowski et al., 1987; Wanneck et al., 1988).

The nature of the signal that directs processing and attachment of a GPI anchor is not known. Inspection of the predicted COOH-terminal amino acid sequences available for GPI-anchored proteins has revealed no obvious homology, the only common feature being a short (15–20 residues) hydrophobic domain of variable sequence at the COOH terminus. This hydrophobic region is thought to be removed during processing and replaced with the GPI anchor. In this report we describe experiments aimed at deciphering the GPI signal of membrane DAF. We took two different approaches. First, we constructed mutants of membrane DAF having altered COOH termini and studied their expression in CV1 origin-deficient SV-40 (COS) cells. Second, we ana-

1. *Abbreviations used in this paper:* COS, CV1 origin-deficient SV-40; DAF, decay accelerating factor; gD, glycoprotein D; GPI, glycophospholipidinositol; hGH, human growth hormone; IRMA, immunoradiometric assay; LDLR, low density lipoprotein receptor; PIPLC, phosphatidylinositol-specific phospholipase C.

lyzed fusion proteins containing portions of the DAF COOH terminus fused to a normally secreted protein, human growth hormone (hGH). Our results suggest that GPI anchor attachment requires the COOH-terminal hydrophobic domain of 17 amino acids as well as additional information (possibly a suitable cleavage/attachment site for the anchor) located within the adjacent 20 amino acids.

Materials and Methods

Materials

Phosphatidylinositol-specific phospholipase C (PIPLC) purified from *Bacillus thuringiensis* was generously provided by Dr. Martin G. Low of Columbia University. Monoclonal antibodies against DAF were obtained from Dr. V. Nussenzweig of NYU; purified rabbit antibody against hGH was provided by the Medicinal Analytical Chemistry Department at Genentech, Inc. (South San Francisco, CA); anti-HSV-1 was from Dako Corp. (Santa Barbara, CA); IgG coupled to either fluorescein or rhodamine was obtained from Cappel Laboratories (Malvern, PA); [³H]ethanolamine was from Amersham Corp. (Arlington Heights, IL). Oligonucleotides were provided by Mark Vasser, Parkash Jhurani, and Peter Ng of Genentech, Inc.

Recombinant Plasmids, Mutagenesis, and Fusion Proteins

Deletion or substitution mutants of membrane DAF were constructed by oligonucleotide-directed mutagenesis of an M13-DAF vector essentially as described (Zoller and Smith, 1982). To construct hGH-DAF 17, an XbaI-KpnI fragment of the hGH gene was cloned into the M13 vector mp 19. Insertional mutagenesis was carried out (Zoller and Smith, 1982) using a 79-bp synthetic oligonucleotide encoding the last 17 amino acids of membrane DAF. This created an in-frame fusion between hGH and the COOH-terminal hydrophobic domain of DAF. hGH-DAF 27, hGH-DAF 37, hGH-LDLR-DAF, hGH-DAF, and hGH-Δ4 DAF were constructed by insertional mutagenesis of hGH-DAF 17 in an M13 vector, using synthetic oligonucleotides. gD-1-DAF 17 was constructed by oligonucleotide-directed deletion mutagenesis of gD-1-DAF (Caras et al., 1987b) cloned into an M13 vector. All of the recombinant plasmids were verified by sequencing. Recombinant DAF, hGH-DAF, or gD-1-DAF cDNAs were inserted into a mammalian expression vector between a cytomegalovirus enhancer/promoter and an SV-40 polyadenylation sequence (Eaton et al., 1986).

Transfections

Cells were transfected by the calcium phosphate coprecipitation method as described by Wigler et al. (1979) using 3–10 μg of plasmid DNA. Cells were incubated with the calcium phosphate-DNA precipitates for 3 h and then treated with 15% glycerol for 30 s.

Metabolic Labeling and Immunoprecipitation

Metabolic labeling of cells with [³⁵S]methionine or [³⁵S]cysteine (Amersham Corp., Arlington Heights, IL) (166 μCi/35-mm dish) was carried out in methionine- or cysteine-free Dulbecco's minimal essential medium for 4–6 h. [³H]Ethanolamine labeling (166 μCi/35-mm dish) was carried out overnight in a 1:1 mixture of F12 and Dulbecco's minimal essential medium supplemented with 10% FBS. After radiolabeling, the culture medium was removed and cleared by centrifugation. The cells were harvested by scraping and lysed with 1% NP-40 in the presence of a protease inhibitor (1 mM PMSF). Immunoprecipitations were carried out as described by Anderson and Blobel (1983).

Immunofluorescent Labeling of Cells

Immunofluorescent labeling of intact cells (cell surface labeling) or permeabilized cells (internal labeling) was carried out as described (Caras et al., 1987b).

Immunoradiometric Assay (IRMA) for hGH

hGH was assayed by a solid-phase two-site IRMA as described (Hybritech, Inc., San Diego, CA).

PIPLC Digestions

Transfected cells were washed in PBS and resuspended in PBS containing 2% heat-inactivated FBS with or without PIPLC, 4 μg/ml. Incubation was for 60 min at 37°C.

Results

Mutagenesis of the DAF COOH Terminus and Effect on GPI Anchor Attachment

We previously localized the GPI signal of membrane DAF to the last 37 amino acids predicted by the cDNA. To further define the GPI signal, we constructed three deletion mutants in which portions of this COOH-terminal region were removed from membrane DAF (Fig. 1 a). The Δ1 deletion removed the last 17 residues predicted by the DAF cDNA (residues 331–347; comprising the COOH-terminal hydrophobic domain), replacing them with a termination codon (TAG). Deletions Δ3 and Δ4 are internal deletions removing 8 and 20 residues, respectively (323–330 and 311–330), immediately NH₂ terminal to the hydrophobic domain. This upstream region presumably contains the proposed processing site. In addition, we constructed two point mutants, Ala³³⁰ and Ser³³⁰, in which Cys³³⁰ had been changed to Ala or Ser, respectively. Cys³³⁰ was specifically chosen for site-directed mutagenesis since the anchor of Thy-1 is known to be attached to a Cys residue after removal of 31 residues, including a hydrophobic region, from the COOH terminus (Tse et al., 1985).

The wild-type and mutant DAF cDNAs were transiently expressed in COS cells under control of the cytomegalovirus promoter. The cells were labeled with [³⁵S]cysteine and DAF was immunoprecipitated from the cell extracts and culture media. Wild-type DAF was localized primarily in the cell lysate as an ~40-kD unglycosylated precursor and an ~70-kD mature form, both of which electrophoresed as doublets (Fig. 1 b, lane 1). In addition, the culture medium contained a minor soluble form of ~68 kD (Fig. 1 b, lane 7). Pulse-chase experiments suggest that the soluble form is derived from membrane DAF by a cleavage within the GPI anchor (possibly by a phospholipase) which leaves [³H]ethanolamine attached to the protein but removes [³H]palmitate (data not shown). The patterns of expression of Δ3, Δ4, Ala³³⁰, and Ser³³⁰ DAF were similar to wild type. Both the unglycosylated and mature DAF species were localized in the cell lysates (Fig. 1 b, lanes 3–6), although the relative amounts of soluble (released) DAF in the medium appeared greater than was observed with wild-type DAF (Fig. 1 b, lanes 9–12). In contrast, the expression pattern for Δ1 DAF was dramatically different. The cell lysate contained only the unglycosylated form, while all of the mature protein was secreted into the culture medium (Fig. 1 b, lanes 2 and 8).

To determine whether the cell-associated DAF was on the cell surface, we analyzed the cells by immunofluorescence. Cell surface staining of intact cells revealed that, with the exception of Δ1 DAF, all the mutant DAF proteins were expressed on the cell surface of transfected COS cells (Fig. 2). Analysis of permeabilized cells showed localization of these proteins in a perinuclear region (possibly endoplasmic reticulum) as well as in a Golgi-like organelle located on one side of the nucleus (data not shown). Permeabilized cells expressing Δ1 DAF showed a more diffuse staining pattern charac-

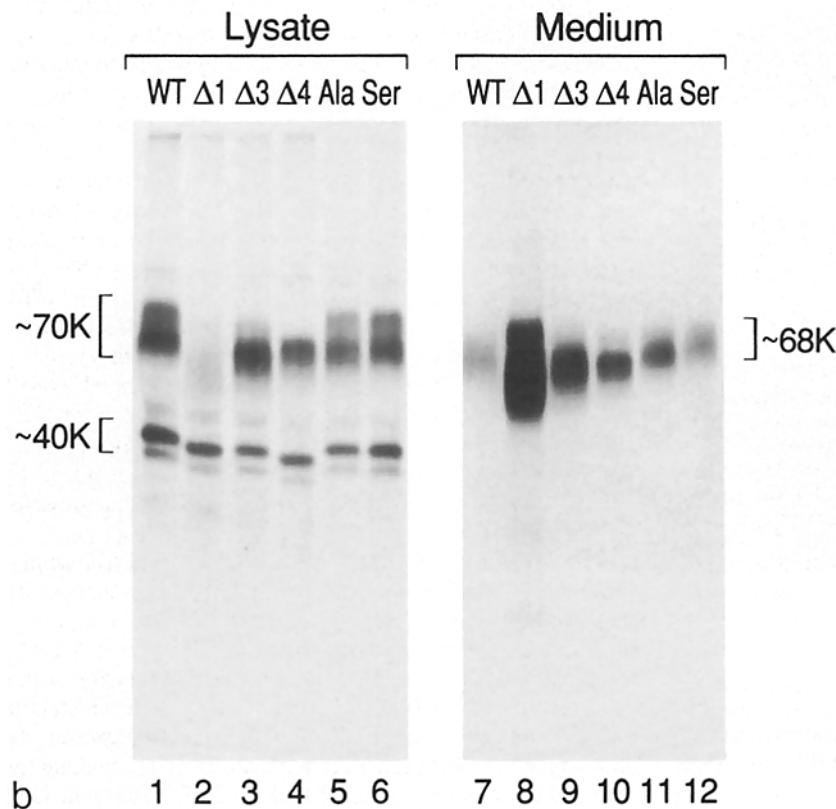
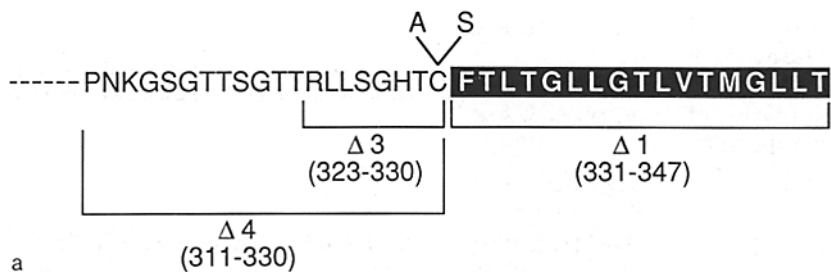


Figure 1. (a) Schematic diagram showing deletions ($\Delta 1$, $\Delta 3$, $\Delta 4$) and amino acid deletions (Ala^{330} , A; Ser^{330} , S) at the DAF COOH terminus. The COOH-terminal hydrophobic domain (17 residues) is shown in black. (b) immunoprecipitation of mutant DAF proteins from [³⁵S]cysteine-labeled transfected COS cells. COS cells transfected with mutant DAF cDNAs were labeled with [³⁵S]cysteine as described in Materials and Methods. DAF was immunoprecipitated from cell lysates (lanes 1-6) or culture media (lanes 7-12). Lanes as indicated show the expression of wild-type (WT), deletion mutant ($\Delta 1$, $\Delta 3$, and $\Delta 4$), and substitution mutant (Ala^{330} and Ser^{330}) DAF described in a.

teristic of secreted proteins (our unpublished observations). These data suggest that the COOH-terminal hydrophobic domain (deleted from $\Delta 1$ DAF) is required for expression of DAF on the cell surface, whereas deletion of sequences adjacent to this region, which presumably contains the cleavage/attachment site, do not affect targeting to the cell surface, although their absence may enhance release.

We next determined whether the mutant DAF proteins expressed on the cell surface are anchored by means of a GPI anchor. [³⁵S]Cysteine-labeled cells were incubated with PIPLC from *Bacillus thuringiensis*, and residual cell-bound as well as released DAF was analyzed by immunoprecipitation. After incubation with PIPLC the levels of 70-kD mature DAF in the cell lysates were significantly decreased both for wild-type DAF and all the mutants except $\Delta 1$ DAF (Fig. 3 a). The levels of 40-kD unglycosylated DAF, presumably an intracellular protein, were unaffected by PIPLC. The PIPLC-released DAF was specifically recovered in the supernatants from the incubations in all cases except $\Delta 1$ DAF (Fig. 3 b). These results suggest that both wild-type DAF and

the mutant DAFs with the exception of $\Delta 1$ DAF are anchored on the plasma membrane by a GPI anchor.

To confirm this, we immunoprecipitated DAF from cells labeled metabolically with [³H]ethanolamine, a component of the GPI anchor. Labeled species corresponding to both unglycosylated and mature DAF were detected in cell lysates from cells expressing either wild-type or mutant DAF proteins, with the exception of $\Delta 1$ DAF (Fig. 3 c). The ~68-kD soluble form of DAF in the culture media from these cells was also labeled with [³H]ethanolamine, suggesting that this form is derived from membrane DAF by a nonproteolytic release mechanism (Fig. 3 c). In contrast, neither the intracellular unglycosylated form nor the secreted mature form of $\Delta 1$ DAF was labeled with [³H]ethanolamine, indicating that this mutant does not contain a GPI anchor (Fig. 3 c, lanes 2 and 8).

These results suggest that the 17-residue hydrophobic domain at the COOH terminus of membrane DAF is critical for attachment of a GPI anchor, whereas the 20 residues immediately NH₂ terminal to this region do not appear to play

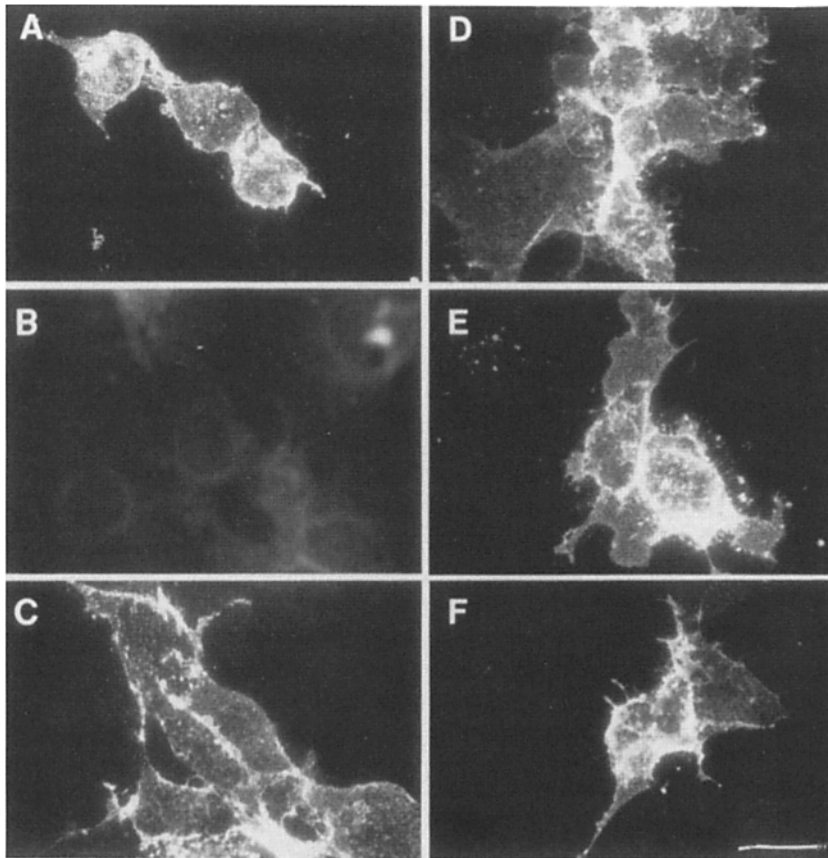


Figure 2. Immunofluorescent labeling of wild-type and mutant DAF proteins on the cell surface of transfected COS cells. Fixed, nonpermeabilized COS cells were labeled as described in Materials and Methods, 24 h after transfection. (A) Wild-type DAF; (B) $\Delta 1$ DAF; (C) $\Delta 3$ DAF; (D) $\Delta 4$ DAF; (E) Ala³³⁰ DAF; (F) Ser³³⁰ DAF. Bar, 10 μm .

an essential role. Cys³³⁰ (a candidate for the site of anchor attachment by analogy with Thy-1) is not critical since it can be replaced by Ser or Ala without affecting GPI anchorage of DAF.

Construction of Fusion Proteins to Probe the GPI Anchor Signal

To determine whether the COOH-terminal hydrophobic domain alone is sufficient to signal the attachment of a GPI anchor we used gene manipulation to construct a fusion protein, hGH-DAF 17, in which the COOH-terminal hydrophobic domain of DAF (17 amino acids) was fused in frame to the COOH terminus of hGH, a secreted protein (Fig. 4 *a*). When expressed in COS cells, this fusion protein was not a substrate for GPI anchor attachment and was targeted for secretion (see below). We considered two possible explanations for this result: (*a*) the 17-residue hydrophobic domain of DAF contains insufficient information to direct GPI anchor attachment; or (*b*) the DAF hydrophobic domain was positioned too close to the possibly folded COOH terminus of the normally secreted hGH protein, preventing access of the processing enzyme (the residue third from the COOH terminus of hGH is a cysteine known to be involved in a disulfide bond). To test these possibilities, we modified the hGH-DAF 17 fusion protein by inserting additional in-frame sequences between hGH and the DAF hydrophobic domain and asked what is required to retarget the secreted fusion protein to the plasma membrane by means of a GPI anchor.

We constructed hGH-DAF 27 and hGH-DAF 37 which contain an additional 10 or 20 residues of DAF sequence, respectively, immediately NH₂ terminal to the 17-residue hydrophobic domain (Fig. 4, *b* and *c*). This additional DAF sequence corresponds to the sequence deleted in mutant $\Delta 4$ DAF described above and forms part of the 76-residue serine/threonine-rich domain of DAF (Caras et al., 1987*a*). We also constructed hGH-LDLR-DAF 17 which contains a 15-residue segment (738-752) from the serine/threonine-rich domain of the low density lipoprotein receptor (LDLR) (Cummings et al., 1983; Russell et al., 1984) inserted between the hGH sequence and the hydrophobic domain of DAF (Fig. 4 *d*); and hGH-syn-DAF 17 which contains a synthetic octapeptide (designed to be hydrophilic) similarly inserted between hGH and the DAF hydrophobic domain (Fig. 4 *e*). The latter two constructions were designed to test the hypothesis that steric hindrance prevents correct processing of hGH-DAF 17, in which case insertion of a random "spacer" sequence might allow GPI anchor attachment to occur.

Expression of hGH-DAF Fusion Proteins in COS Cells

The hGH-DAF fusion proteins were transiently expressed in COS cells under control of the cytomegalovirus promoter and localized by [³⁵S]methionine labeling followed by immunoprecipitation using a purified rabbit anti-hGH antibody. The hGH-DAF 17 and hGH-syn-DAF 17 expression

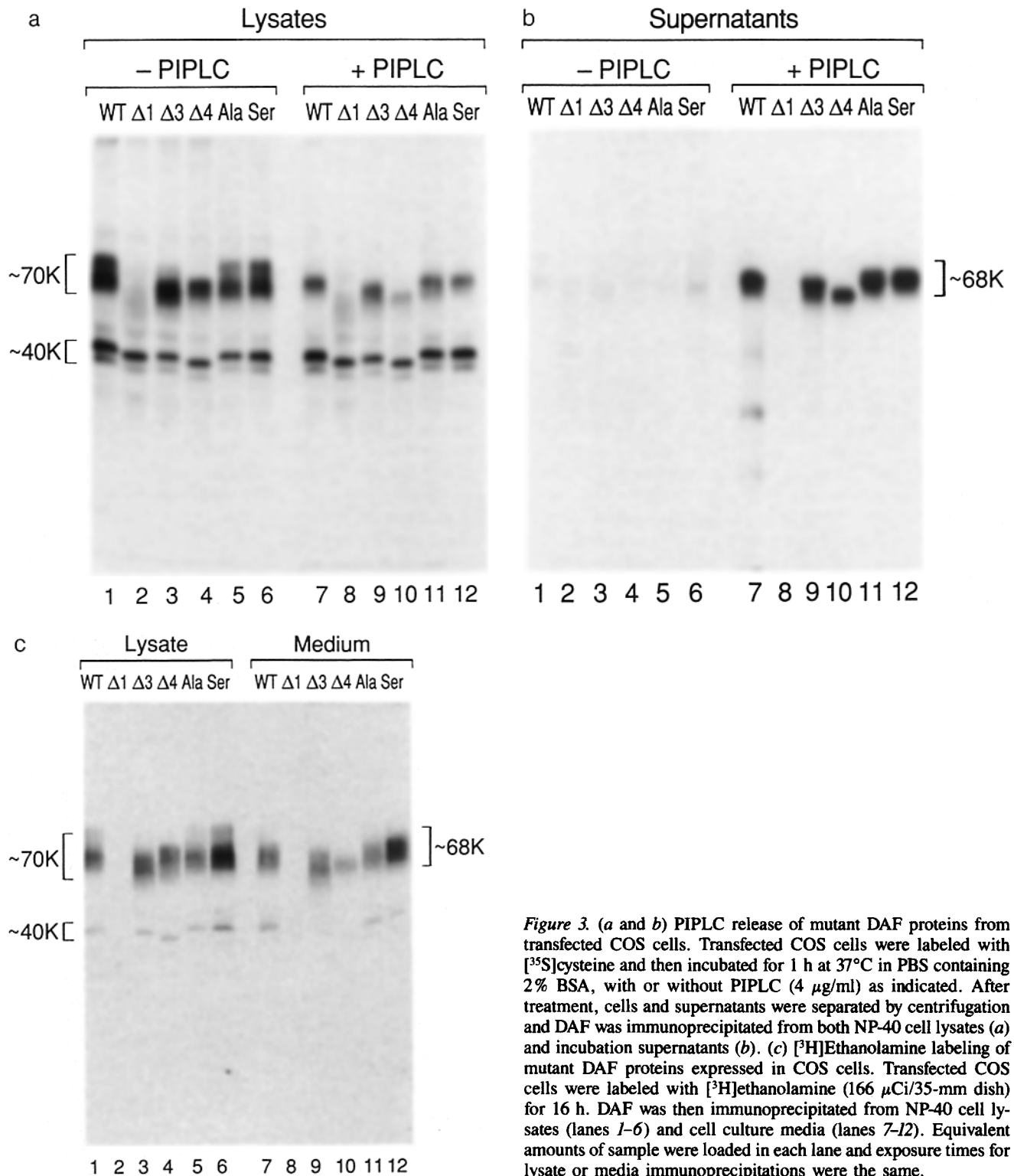


Figure 3. (a and b) PIPLC release of mutant DAF proteins from transfected COS cells. Transfected COS cells were labeled with [³⁵S]cysteine and then incubated for 1 h at 37°C in PBS containing 2% BSA, with or without PIPLC (4 μg/ml) as indicated. After treatment, cells and supernatants were separated by centrifugation and DAF was immunoprecipitated from both NP-40 cell lysates (a) and incubation supernatants (b). (c) [³H]ethanolamine labeling of mutant DAF proteins expressed in COS cells. Transfected COS cells were labeled with [³H]ethanolamine (166 μCi/35-mm dish) for 16 h. DAF was then immunoprecipitated from NP-40 cell lysates (lanes 1–6) and cell culture media (lanes 7–12). Equivalent amounts of sample were loaded in each lane and exposure times for lysate or media immunoprecipitations were the same.

products were secreted into the culture medium as unprocessed fusion proteins (Fig. 5 a, lanes 6 and 10). This was deduced by comparing their apparent molecular weights with that of native hGH (not shown). The fusion proteins were secreted less efficiently than native hGH since significant amounts of fusion protein remained cell associated (Fig.

5 a, lanes 1 and 5) whereas <10% of mature hGH expressed under similar conditions was cell associated (not shown). This suggests that an unprocessed DAF COOH-terminal hydrophobic domain, although unable to act as a membrane anchor, may retard secretion. The fusion proteins hGH-DAF 27, hGH-DAF 37, and hGH-LDLR-DAF 17 were cell as-

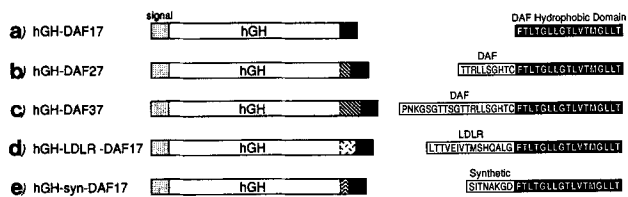


Figure 4. Schematic diagram showing hGH-DAF fusion proteins. Shaded areas are as follows: (■) COOH-terminal hydrophobic domain of DAF (residues 331-347); (▨) DAF sequence immediately NH₂ terminal to the hydrophobic domain (residues 321-330 in hGH-DAF 27 and 311-330 in hGH-DAF 37); (▩) residues 738-752 from the serine/threonine-rich domain of the LDLR; (⊞) synthetic octapeptide; (☉) hGH signal peptide.

sociated (Fig. 5 a, lanes 2-4) with only trace amounts of protein appearing in the culture medium (Fig. 5 a, lanes 7-9).

To determine whether the cell-associated fusion proteins were on the cell surface we analyzed the cells by indirect immunofluorescence. Although analyses of permeabilized cells showed strong fluorescence in populations transfected with each of the five hGH-DAF cDNAs (data not shown), surface immunofluorescence of intact cells was observed only with cells expressing hGH-DAF 37 (Fig. 5 b). The hGH-DAF 27

Table I. IRMA of hGH in Supernatants from Transfected COS Cells Incubated with or without PIPLC

Fusion protein	Addition	
	None	PIPLC
	hGH (ng/ml)	
hGH-DAF 17	22.0	29.0
hGH-DAF 27	2.9	3.6
hGH-DAF 37	4.5	33.0
hGH-LDLR-DAF 17	3.9	3.8
hGH-Syn-DAF 17	15.5	16.2

Numbers shown represent the average of two experiments.

and hGH-LDLR-DAF 17 fusions, although cell associated, were undetectable on the cell surface by immunofluorescence.

We next tested for the presence of GPI-anchored cell surface fusion proteins by incubating transfected COS cells with PIPLC. The levels of hGH in the incubation supernatants were measured using an IRMA (Hybritech, Inc.). Significant levels of hGH were detected in the absence of PIPLC in incubation supernatants from cells transfected with hGH-DAF 17 or hGH-syn-DAF, confirming that these proteins

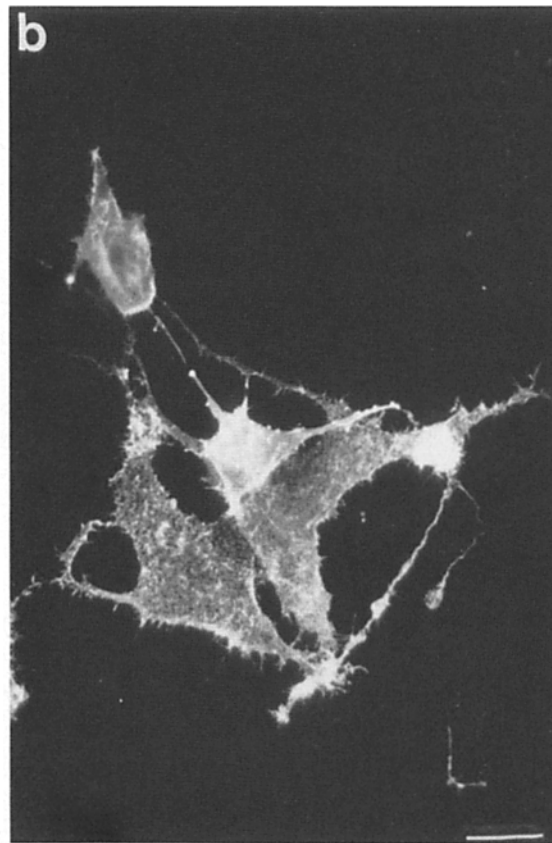
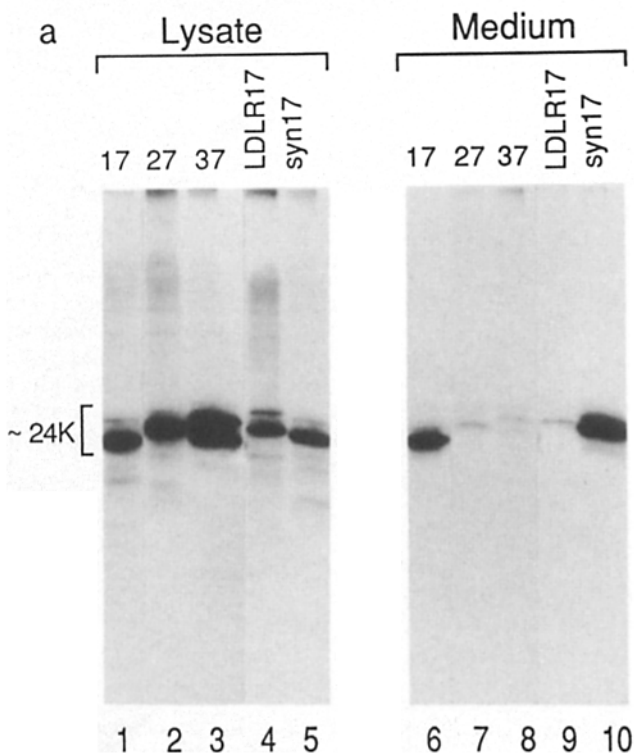


Figure 5. (a) Immunoprecipitation of hGH-DAF fusion proteins from [³⁵S]methionine-labeled transfected COS cells. COS cells were labeled with [³⁵S]methionine 24 h after transfection with DNAs encoding hGH-DAF fusion proteins as indicated. The hGH-DAF fusion proteins were immunoprecipitated from cell lysates (lanes 1-5) and culture media (lanes 6-10) using a purified rabbit antibody against hGH. (b) Immunofluorescent labeling of hGH-DAF 37 on the cell surface of transfected COS cells. Fixed, nonpermeabilized COS cells expressing hGH-DAF 37 were labeled as described in Materials and Methods. Bar, 5 μm.

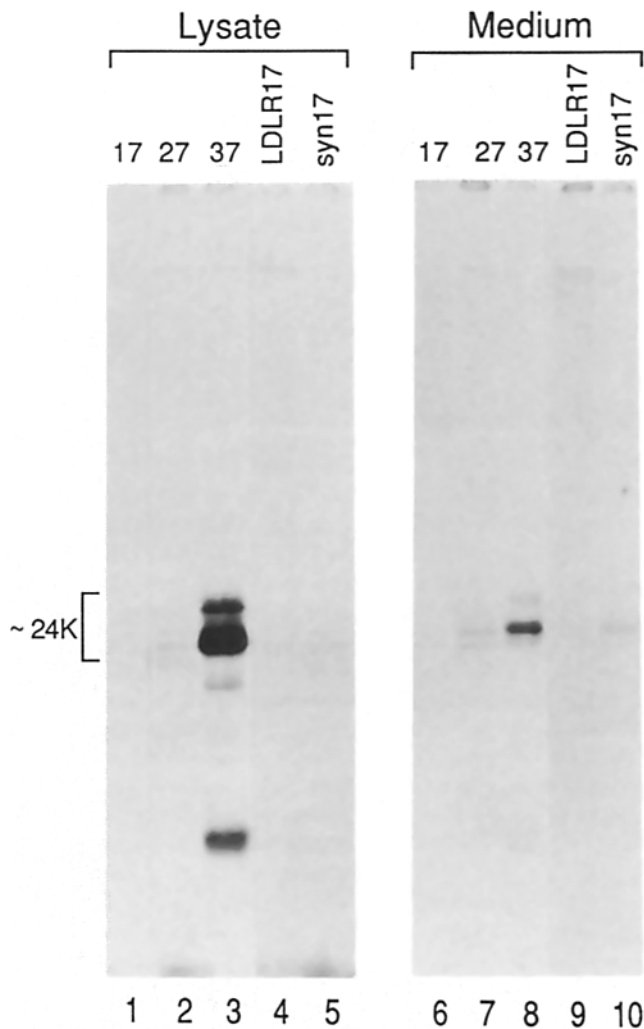


Figure 6. [^3H]Ethanolamine labeling of hGH-DAF fusion proteins in transfected COS cells. After overnight labeling of transfected COS cells with [^3H]ethanolamine, hGH-DAF fusion proteins as indicated were immunoprecipitated from cell lysates (lanes 1-5) and culture media (lanes 6-10) using an anti-hGH antibody.

are secreted (Table I). In contrast, release of the hGH-DAF 37 fusion protein is PIPLC dependent, indicating that this protein is anchored to the plasma membrane by a GPI anchor. Insignificant levels of hGH were detected in incubation supernatants from cells expressing hGH-DAF 27 or hGH-LDLR-DAF, consistent with the observation that these fusion are neither secreted nor expressed on the cell surface, but may be trapped intracellularly.

It is possible that the nonsecreted fusion proteins contained GPI anchors. To examine this we labeled transfected COS cells with [^3H]ethanolamine and analyzed the labeled proteins by immunoprecipitation. Of the five fusion proteins analyzed, only hGH-DAF 37 was significantly labeled with [^3H]ethanolamine (Fig. 6, lane 3), suggesting that this is the only fusion protein that is correctly processed and targeted to the cell surface by means of a GPI anchor. The significance of the minor labeled species visible in the remaining lanes is unclear. A soluble species of [^3H]ethanolamine-labeled hGH-DAF 37 was detected in the culture medium

(Fig. 6, lane 8), presumably derived from the membrane form by a nonproteolytic release mechanism.

The above results demonstrate that the 17-residue hydrophobic domain of DAF plus the adjacent 20 residues (37 residues in total) will target hGH to the plasma membrane via a GPI anchor. The 17-residue hydrophobic domain alone appears to be insufficient, as is the hydrophobic domain with only 10 additional residues of DAF sequence or with non-specific "spacer" sequences.

gD-1-DAF Fusion Protein

To strengthen the argument that the COOH-terminal hydrophobic domain alone is insufficient to direct GPI anchor attachment, we used a different secreted protein to test the putative GPI signal. We modified a previously described gD-1-DAF fusion protein (Caras et al., 1987b) which contains the last 37 residues of DAF (residues 311-347) fused to the COOH terminus of a truncated, secreted form of glycoprotein D (gD-1) from herpes simplex virus type 1. This fusion protein is expressed on the cell surface of mammalian cells as a GPI-anchored protein (Caras et al., 1987b). We constructed by deletion mutagenesis of gD-1-DAF a new fusion, gD-1-DAF 17, in which residues 311-330 of the DAF sequence were removed, leaving only the COOH-terminal hydrophobic domain of DAF (17 residues) fused directly to the COOH terminus of the truncated gD-1 protein. Immunoprecipitation of [^{35}S]methionine-labeled gD-1-DAF 17 from transfected COS cells indicated that this protein is secreted into the culture medium as a heterogeneous (presumably glycosylated) 40-50-kD protein (Fig. 7, lane 4), while the cell lysate contains only the 37-kD unglycosylated form (Fig. 7, lane 2). In addition, we were unable to detect cell surface gD-1-DAF 17 by immunofluorescent labeling of intact, transfected COS cells, although COS cells expressing gD-1-DAF (the GPI-anchored fusion) were strongly positive (Caras et al., 1987b). These data are consistent with the above results obtained with the hGH-DAF 17 fusion and suggest that, whereas the last 37 residues of DAF predicted by the cDNA can direct attachment of a GPI membrane anchor, the 17-residue COOH-terminal hydrophobic domain alone is insufficient.

Redundant Element in DAF

The above results indicate that, in the context of a fusion protein, the 20 amino acids NH_2 terminal to the hydrophobic domain (residues 311-330) are essential for directing attachment of a GPI membrane anchor. However, these same residues can be deleted from the DAF protein (as in $\Delta 4$ DAF) without affecting GPI anchor attachment. To explain this apparent contradiction we hypothesized that the essential information contained within the 311-330 region might be repeated in the DAF molecule such that deletion of residues 311-330 brings a redundant or cryptic element into juxtaposition with the hydrophobic domain, creating a viable signal for GPI anchor attachment. To test this hypothesis we constructed a fusion protein, hGH- $\Delta 4$ DAF, containing a 37-residue segment from the COOH terminus of $\Delta 4$ DAF fused in-frame to the COOH terminus of hGH. This 37-residue segment of $\Delta 4$ DAF includes the COOH-terminal hydrophobic domain (residues 331-347) juxtaposed with 20 residues

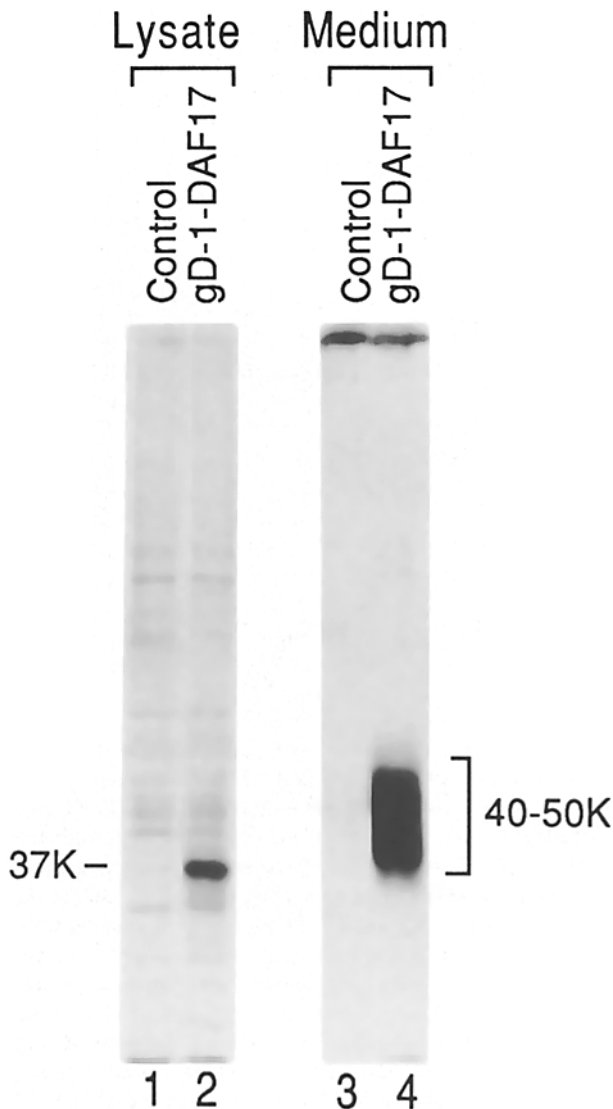


Figure 7. Immunoprecipitation of gD-1-DAF 17 from [³⁵S]methionine-labeled transfected COS cells. Transfected COS cells were labeled with [³⁵S]methionine and gD-1-DAF 17 was immunoprecipitated from the cell lysate and culture medium using a rabbit polyclonal antibody to HSV-1. (Lanes 1 and 3) Mock transfected COS cells; (lanes 2 and 4) cells transfected with gD-1-DAF 17.

(291–310) NH₂ terminal to the 311–330 region that was deleted from Δ4 DAF (Fig. 8 a).

When expressed in COS cells, the hGH-Δ4 DAF fusion protein was localized on the cell surface as indicated by immunofluorescent staining of the transfected cells (Fig. 8 b). Immunoprecipitation of [³⁵S]methionine-labeled proteins from cell lysates and culture media confirmed that hGH-Δ4 DAF is cell associated (data not shown). To test for the presence of a GPI anchor, we incubated transfected COS cells with PIPLC and measured hGH levels in the incubation supernatants. Incubation with PIPLC resulted in a significant release of hGH-Δ4 DAF from the cell surface (Table II), indicating that this protein, like hGH-DAF 37, is linked to the plasma membrane via a GPI anchor. The effect of PIPLC on the release of hGH-DAF 37 and hGH-DAF 17 was measured

in the same experiment and is shown for comparison (Table II). The PIPLC-independent release of hGH-DAF 37 and hGH-Δ4 DAF (Table II) presumably represents protein released from the cell surface by an endogenous phospholipase or protease.

We conclude that DAF contains a second element (located between residues 291 and 310) that can substitute for the 311–330 region and, in conjunction with the COOH-terminal hydrophobic domain, will direct a normally secreted protein to the plasma membrane by means of a GPI membrane anchor.

Discussion

A COOH-Terminal Hydrophobic Domain Is Critical for GPI Anchor Attachment

In a previous report we showed that the last 37 residues of membrane DAF are sufficient to direct a secreted protein to the plasma membrane by means of a GPI anchor (Caras et al., 1987b). In the present study we selectively removed portions of this region from membrane DAF to further define the GPI signal. As criteria for cell surface expression and GPI anchorage we used immunofluorescent labeling of the protein on intact cells, its release by PIPLC, and specific labeling by [³H]ethanolamine, a component of the GPI anchor. Removal of the last 17 residues (the hydrophobic COOH-terminal domain) predicted by the DAF cDNA resulted in lack of attachment of a GPI anchor and secretion of the DAF protein, suggesting that a COOH-terminal hydrophobic domain is critical for GPI anchor attachment. A similar conclusion was reached by Berger et al. (1988) who showed that mutants of placental alkaline phosphatase containing 13 or fewer hydrophobic residues at the COOH terminus failed to become GPI anchored and were secreted. Consistent with this, all known GPI-anchored proteins are synthesized with a 10–20-residue hydrophobic domain of variable sequence at the COOH terminus (Low, 1987; Low and Saltiel, 1988).

Importance of the Region NH₂ Terminal to the Hydrophobic Domain

Deletion of up to 20 residues immediately NH₂ terminal to the hydrophobic domain did not affect GPI anchorage of DAF suggesting that this region, which presumably contains the cleavage/attachment site, is not essential and implying that the COOH-terminal hydrophobic domain alone might constitute the GPI signal. However, fusion of the DAF

Table II. PIPLC Release of hGH-Δ4 DAF from Transfected COS Cells

Fusion protein	Addition	
	None	PIPLC
	hGH (ng/ml)	
None	1.2	1.2
hGH-Δ4 DAF	24.0	286.0
hGH-DAF 17	9.6	11.3
hGH-DAF 37	77.0	215.0

After incubation of transfected cells either with or without PIPLC, hGH levels in the supernatants were determined by an IRMA. Numbers shown represent the average of two experiments.

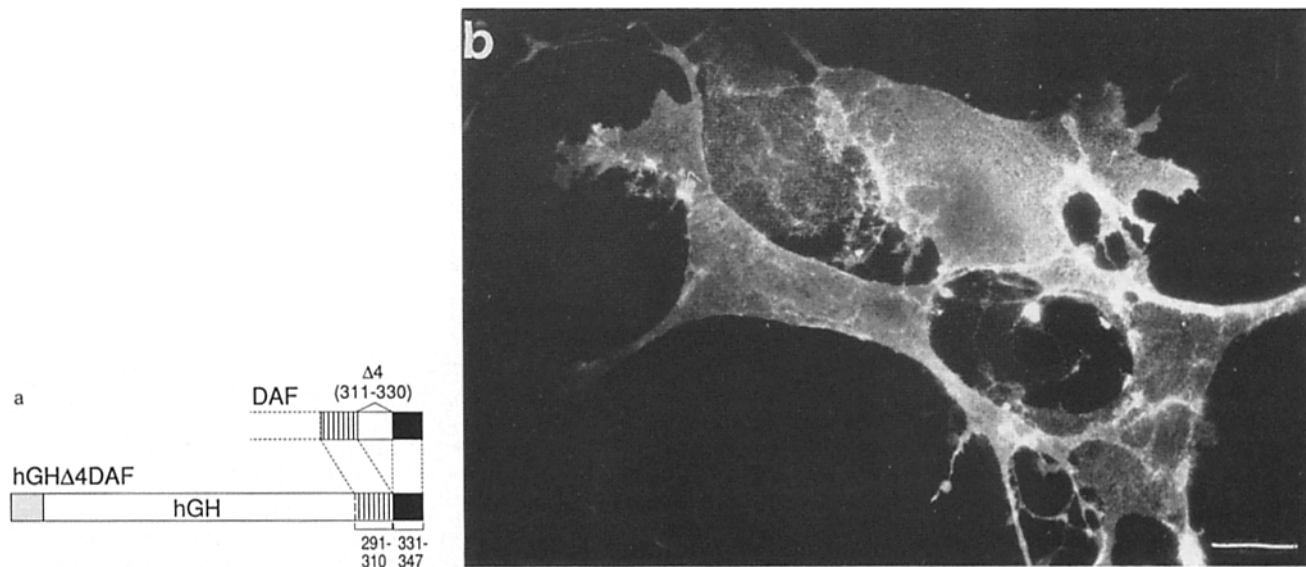


Figure 8. (a) Schematic diagram showing the hGH- Δ 4 DAF fusion protein. Shaded areas are as follows: (■) COOH-terminal hydrophobic domain of DAF (residues 331-347); (▨) DAF sequence (residues 291-310) NH₂ terminal to the Δ 4 (311-330) deletion; (▩) hGH signal peptide. (b) Immunofluorescent labeling of hGH- Δ 4 DAF on the cell surface of transfected COS cells. Fixed, nonpermeabilized COS cells expressing hGH- Δ 4 DAF were labeled as described in Materials and Methods. Bar, 5 μ m.

COOH-terminal hydrophobic domain to the COOH terminus of two different secreted proteins, hGH or a truncated form of gD-1, failed to produce a GPI-anchored protein. We considered two possible explanations for this result: (a) GPI anchor attachment requires a COOH-terminal hydrophobic domain plus additional specific information (acting as a component of the signal or as a substrate for processing) which was lacking in these fusions; or (b) correct processing was prevented by steric hindrance resulting from the direct fusion of a hydrophobic domain to the possibly folded COOH terminus of a normally secreted protein. To distinguish between these possibilities we inserted either specific (the adjacent DAF sequence) or nonspecific sequences between hGH and the DAF COOH-terminal hydrophobic domain. The nonspecific sequences chosen were a synthetic octapeptide composed primarily of hydrophilic amino acids, or a portion of the serine/threonine-rich region of the LDLR (a similar serine/threonine-rich domain is located NH₂ terminal to the hydrophobic domain in DAF). The inclusion of nonspecific "spacer" sequences between hGH and the COOH-terminal hydrophobic domain failed to produce a GPI-anchored protein. In contrast, both hGH-DAF and gD-1-DAF fusions containing 37 residues of DAF sequence (the COOH-terminal hydrophobic domain plus the adjacent 20 residues) were expressed on the cell surface as GPI-anchored proteins. This result suggests that specific information required for GPI anchor attachment is located in the region NH₂ terminal to the hydrophobic domain. The inclusion of only 10 residues of DAF sequence proximal to the hydrophobic domain did not direct GPI anchor attachment, indicating that at least some of the information required lies in the region between 10 and 20 residues NH₂ terminal to the hydrophobic domain. Deletion analysis of DAF suggested that this region is not essential for GPI anchor attachment. To resolve this apparent contradiction we proposed that redundant information resides in the 76-residue serine/threonine-rich do-

main of DAF. If this is the case, deletion of a portion of this region (as in Δ 3 DAF and Δ 4 DAF) would not be expected to significantly affect GPI anchor attachment. To test this hypothesis we constructed the hGH- Δ 4 DAF fusion, containing a 37-residue segment from the COOH terminus of Δ 4 DAF fused to the COOH terminus of hGH. This segment includes the COOH-terminal hydrophobic domain of DAF but not the adjacent 20 residues. Instead, the 20 residues immediately NH₂ terminal to the Δ 4 deletion are juxtaposed with the hydrophobic domain. The hGH- Δ 4 DAF fusion was expressed on the cell surface as a GPI-anchored protein, confirming the presence of a second element present in the DAF protein that is able to direct GPI anchor attachment in conjunction with the hydrophobic domain.

Of the four hGH-DAF fusion proteins which were not processed, two were secreted (hGH-DAF 17 and hGH-syn-DAF 17) and two were apparently trapped intracellularly without a GPI anchor (hGH-DAF 27 and hGH-LDLR-DAF 17). This difference might be related to O-linked glycosylation of the latter two proteins at sites within the DAF or LDLR segments of the protein. Alternatively, improper folding of the latter fusions may have prevented transport through the secretory pathway (Lodish, 1988).

Cleavage/Attachment Site

Although the COOH-terminal residue of mature DAF has not yet been determined, a proteolytic processing event that removes the COOH-terminal hydrophobic domain is presumed to occur before or simultaneous with GPI anchor attachment. It is possible that whereas the COOH-terminal hydrophobic domain acts as the signal for GPI anchor attachment (analogous to hydrophobic signal sequences in membrane translocation), an appropriate cleavage/attachment site (analogous to the signal peptidase site) is also required. Known cleavage points for GPI-anchored proteins occur

10–12 residues NH₂ terminal to a COOH-terminal hydrophobic domain (Boothroyd et al., 1980; Tse et al., 1985). It is precisely this region of the DAF sequence (between 10 and 20 residues NH₂ terminal to the hydrophobic domain) that appears to be necessary for GPI anchorage of a hGH-DAF fusion protein. The GPI attachment sites have been determined for Thy-1 (Tse et al., 1985), placental alkaline phosphatase (Micanovic et al., 1988), and the VSGs of *Trypanosoma brucei* (Holder and Cross, 1981; Boothroyd et al., 1980), but they as yet reveal no recognizable sequence similarity which might allow correct prediction of the cleavage site for DAF.

It is reasonable to speculate that hGH-DAF 37 and gD-1-DAF contain both a GPI signal (the 17-residue COOH-terminal domain) and a suitable cleavage/attachment site (located between 10 and 20 residues NH₂ terminal to the hydrophobic domain) and are therefore targeted to the cell surface by means of a GPI anchor. In contrast, fusion proteins containing the GPI signal alone (hGH-DAF 17 and gD-1-DAF 17) or in combination with sequences which presumably lack a suitable processing site (hGH-DAF 27, hGH-LDLR-DAF 17, and hGH-syn-DAF 17) fail to become cleaved or GPI anchored. The serine/threonine-rich region of DAF is somewhat repetitive and might contain several cryptic cleavage sites which substitute for the normal cleavage site in the deletion mutants $\Delta 3$ DAF and $\Delta 4$ DAF, and in the hGH- $\Delta 4$ DAF fusion, thereby allowing these proteins to be processed normally. Further analysis of the GPI signal will require both saturation mutagenesis of the 20 residues proximal to the hydrophobic domain of DAF (in the context of a fusion protein) and knowledge of the cleavage/attachment site.

We thank David Martin, Jr. for support, encouragement, and many helpful discussions.

Received for publication 26 July 1988 and in revised form 2 December 1988.

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