A Nonfunctional Sequence Converted to a Signal for Glycophosphatidylinositol Membrane Anchor Attachment

Paul Moran and Ingrid W. Caras

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

Abstract. The COOH terminus of decay-accelerating factor (DAF) contains a signal that directs glycophosphatidylinositol (GPI) membrane anchor attachment in a process involving concerted proteolytic removal of 28 COOH-terminal residues. At least two elements are required for anchor addition: a COOH-terminal hydrophobic domain and a cleavage/attachment site located NH_2-terminal to it, requiring a small amino acid as the acceptor for GPI addition. We previously showed that the last 29–37 residues of DAF, making up the COOH-terminal hydrophobic domain plus 20 residues of the adjacent serine/threonine-rich domain (including the anchor addition site), when fused to the COOH terminus of human growth hormone (hGH) will target the fusion protein to the plasma membrane via a GPI anchor. In contrast, a similar fusion protein (hGH-LDLR-DAF17, abbreviated HLD) containing a fragment of the serine/threonine-rich domain of the LDL receptor (LDLR) in place of the DAF-derived serine/threonine-rich sequences, does not become GPI anchored. We now show that this null sequence for GPI attachment can be converted to a strong GPI signal by mutating a pair of residues (valine-glutamate) in the LDLR sequence at a position corresponding to the normal cleavage/attachment site, to serine-glycine, as found in the DAF sequence. A single mutation (converting valine at the anchor addition site to serine, the normal acceptor for GPI addition in DAF) was insufficient to produce GPI anchoring, as was mutation of the valine-glutamate pair to serine-phenylalanine (a bulky residue). These results suggest that a pair of small residues (presumably flanking the cleavage point) is required for GPI attachment. By introducing the sequence serine-glycine (comprising a cleavage-attachment site for GPI addition) at different positions in the LDLR sequence of the fusion protein, HLD, we show that optimal GPI attachment requires a processing site positioned 10–12 residues NH_2-terminal to the hydrophobic domain, the efficiency anchor attachment dropping off sharply as the cleavage site is moved beyond these limits. These data suggest that the GPI signal consists solely of a hydrophobic domain combined with a processing site composed of a pair of small residues, positioned 10–12 residues NH_2-terminal to the hydrophobic domain. No other structural motifs appear necessary.

While most integral membrane proteins are anchored to the lipid bilayer by hydrophobic transmembrane sequences, a small but diverse class of cell surface proteins is held on the plasma membrane by covalent attachment of a glycophosphatidylinositol (GPI)[1] structure (for reviews see Cross, 1990; Low, 1989; Ferguson and Williams, 1988). The GPI membrane anchor, containing phosphatidylinositol, carbohydrate and ethanolamine, is apparently preassembled in the ER (Masterson et al., 1989) and then added to the COOH terminus of the protein in a processing event involving coordinated proteolytic removal of 17–31 residues from the nascent chain (Boothroyd et al., 1981; Tse et al., 1985). This processing event is thought to take place in the ER (Bangs et al., 1985, 1986; Ferguson et al., 1986) and is directed by a signal at the COOH terminus of the protein (Caras et al., 1987*a*). Although all GPI-anchored proteins are presumably processed by a common pathway, there is no primary sequence homology or obvious consensus among different GPI-anchored proteins, suggesting that the signal for GPI attachment is of a general nature. The only feature common to all GPI-anchored proteins is the presence of a short (15–20 residues) COOH-terminal hydrophobic domain (predicted by the cDNA but absent from the final GPI-anchored product). Deletion of this domain converts the GPI-anchored protein to a secreted protein suggesting that the COOH-terminal hydrophobic domain plays an important role in GPI attachment (Caras et al., 1989).

We have used the GPI-anchored protein, decay-accelerating factor (DAF) (Davitz et al., 1986; Medof et al., 1986), as a model system to analyze the signal for anchor attachment. The last 37 amino acids predicted by the DAF cDNA contain the information required for GPI addition, and when fused to the COOH terminus of a normally secreted protein will target the fusion protein to the plasma membrane by

1. *Abbreviations used in this paper*: DAF, decay-accelerating factor; GPI, glycophosphatidylinositol; LDLR, low-density lipoprotein receptor; PIPLC, phosphatidylinositol-specific phospholipase C.

means of a GPI anchor (Caras et al., 1987*a*). This DAF sequence contains at least two elements necessary for anchor attachment: a 17-residue COOH-terminal hydrophobic domain that is necessary but insufficient, and a cleavage/attachment site for the anchor located NH_2-terminal to the hydrophobic domain (Caras et al., 1989).

During normal processing, a 28-residue peptide (including the 17-residue hydrophobic domain) is removed from the DAF COOH terminus and the GPI anchor is then added to Ser319, which forms the new COOH terminus (Moran et al., 1991). Systematic replacement of Ser319 with all possible amino acids suggested that the anchor addition site of DAF (in the context of a human growth hormone [hGH]–DAF fusion protein) requires a residue with a small side chain (Ser, Gly, Ala, Asp, or Asn). A similar conclusion was reached in a study using alkaline phosphatase (Micanovic et al., 1990) although in the latter case, cysteine was also able to function as an acceptor for the GPI anchor. The six amino acids listed above, and only these, have been found at the known COOH termini of natural GPI-anchored proteins (Cross, 1990). Replacement of Ser319 with alternative amino acids having larger side chains severely impairs or abolishes anchor addition, suggesting that the requirement for a small amino acid at the attachment site is a major constraint of the GPI signal (Moran et al., 1991).

Using a GPI-anchored fusion protein containing the last 37 amino acids of DAF fused to the COOH terminus of human growth hormone (hGH-DAF37), we showed that the COOH-terminal 29 residues of DAF (comprising the attachment site serine and the 28-residue peptide that is removed during processing) are sufficient to direct anchor attachment, suggesting that sequences immediately NH_2-terminal to the attachment site are not required for GPI-anchoring (Moran et al., 1991). We now address the following question: apart from a limited specificity at the anchor addition site and the requirement for a COOH-terminal hydrophobic domain, are there additional structural or conformational features necessary for GPI anchor addition? In particular, how important are the sequences located between the anchor attachment site and the COOH-terminal hydrophobic domain? Such problems are usually approached using substitution or deletion mutagenesis to pinpoint critical residues or sequences. However, the general nature of the GPI signal suggested that the results of such a study might be ambiguous and difficult to interpret. We therefore approached the problem from the opposite direction by starting with a sequence that does not signal anchor addition and asking what changes are required to convert it to a GPI signal. We show that a null (nonfunctional) sequence for GPI attachment, consisting of a fragment of the serine/threonine-rich domain of the LDL receptor (LDLR) combined with the DAF COOH-terminal hydrophobic domain, can be converted to a strong GPI signal by the insertion of a pair of small residues (forming a cleavage/attachment site) positioned at an appropriate distance (10–12 residues) NH_2-terminal to the hydrophobic domain.

Materials and Methods

Phosphatidylinositol-specific phospholipase C (PIPLC) purified from *Bacillus thuringiensis* was provided by Dr. Martin G. Low (Columbia University, New York). Purified rabbit or goat antibodies against hGH were provided by the Medicinal Analytical Chemistry Department at Genentech, Inc. (South San Francisco, CA); IgG coupled to fluorescein was from Cappel Laboratories (Malvern, PA); [^{3}H]ethanolamine was from Amersham Corp. (Arlington Heights, IL). Oligonucleotides were provided by Mark Vasser, Parkash Jhurani, and Peter Ng (Genentech, Inc.).

Recombinant Plasmids and Fusion Proteins

hGH-DAF37 and hGH-LDLR-DAF (HLD) were constructed as previously described (Caras et al., 1989). hGH-LDLR-S^{12}DAF17 (HLD.S^{12}), hGH-LDLR-S^{12}G.DAF17 (HLD.S^{12}G) and hGH-LDLR-S^{12}F.DAF17 (HDL.S^{12}F) were constructed from HLD by oligonucleotide-directed mutagenesis using a phagemid vector (McClary et al., 1989). HLD.S^{9}G, HLD.S^{6}G, HLD.S^{4}G, HLD.S^{2}G, and HLD.S^{16}G were similarly constructed from HLD by oligonucleotide-directed mutagenesis. After mutagenesis, an 840-bp BglII-NotI fragment containing the COOH terminus of hGH together with the LDLR and DAF sequences of each HLD derivative was subcloned into a mammalian expression vector containing the NH_2-terminal portion of hGH and a cytomegalovirus enhancer/promoter. All recombinant plasmids were verified by sequencing.

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).

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 purified rabbit antibody against hGH, followed by fluorescein-conjugated goat anti-rabbit antiserum (Cappel Laboratories).

hGH-ELISA

hGH levels were measured by an ELISA, using 96-well dishes coated with goat anti-hGH IgG (2 μg/ml in 0.05 M bicarbonate buffer, pH 9.6). Following incubation with antigen, the plates were treated with peroxidase-conjugated goat anti-hGH IgG in PBS, 0.1% bovine serum albumin. Color was developed using as a peroxidase substrate, *o*-phenylenediamine tablets, 5 mg (Sigma P-6912; Sigma Chemical Co., St. Louis, MO) in 12.5 ml PBS, 0.012% H_2O_2. The reaction was stopped by adding an equal volume of 4.5 N H_2SO_4 and the optical density at 490 and 405 nm was determined. The assay detected hGH in the range 2–100 ng/ml.

Results

Conversion of a Nonfunctional Sequence for GPI Attachment to a GPI Signal

Previous work has shown that the last 29–37 residues predicted by the DAF cDNA, when fused to the COOH terminus of hGH (as in hGH-DAF37 [Fig. 1]) will target the fusion protein to the plasma membrane via a GPI anchor (Moran et al., 1991; Caras et al., 1989). This DAF sequence is comprised of a 17-residue COOH-terminal hydrophobic domain plus 12–20 residues of the adjacent serine/threonine-rich domain that both contains the anchor addition site and serves as a substrate for *O*-linked glycosylation necessary to stabilize DAF on the plasma membrane (Reddy et al., 1989). In contrast, a sequence containing the DAF hydrophobic domain combined with a 15-residue fragment of a functionally similar serine-threonine-rich domain found in the LDLR (Cummings et al., 1983; Russell et al., 1984) fails to direct anchor attachment when fused to the COOH terminus of

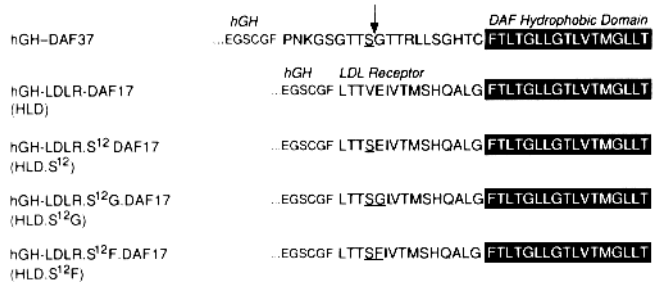

Figure 1. Schematic diagram showing the COOH-terminal sequences of hGH-DAF37 and hGH-LDLR-DAF (*HLD*) fusion proteins. (*Small print*) hGH COOH-terminus; (*boxed sequence*) DAF hydrophobic domain. The LDLR sequence of HLD is indicated. An arrow denotes the normal cleavage point in the DAF sequence of hGH-DAF37 and the anchor addition site, Ser319, is underlined. Residues in HLD.S^{12}, HLD.S^{12}G, and HLD.S^{12}F that were altered by in vitro mutagenesis are indicated by an underline.

hGH (as in the fusion protein HLD [Fig. 1]) (Caras et al., 1989). As a means to further elucidate the structural features of the GPI signal we asked the following questions: (*a*) why does the DAF-derived sequence of hGH-DAF37 direct anchor attachment while the LDLR-DAF sequence of HLD does not? (*b*) What changes are required to convert the LDLR-DAF sequence of HLD into a GPI signal?

A comparison of the hGH-DAF37 and HLD COOH-terminal sequences revealed one potentially critical difference at the position of the anchor addition site located at Ser319 in the DAF sequence, 12 residues NH_2-terminal to the hydrophobic domain (Fig. 1). HLD contains a valine at this position and since valine is not one of the six small amino acids known to be allowed at the anchor addition site (Moran et al., 1991; Micanovic et al., 1990), we mutated this valine to serine, producing a new fusion protein, HLD.S^{12} (Fig. 1). Secondly, inspection of the known cleavage sites of naturally occurring GPI-anchored proteins suggests that a small amino acid is usually found on the COOH-terminal side of the cleavage point. For example, in the DAF sequence, this position is occupied by glycine (Gly320; Fig. 1), whereas HLD contains glutamate. We therefore constructed HLD.S^{12}G (Fig. 1), converting the valine-glutamate at positions 12 and 11 (relative to the hydrophobic domain) of HLD to serine-glycine. Finally, to test the hypothesis that large or bulky amino acids may not be tolerated at the position immediately COOH-terminal to the cleavage point, we constructed HLD.S^{12}F (Fig. 1), containing phenylalanine at this position with serine at the addition site (12 residues NH_2-terminal to the hydrophobic domain).

These fusion proteins were transiently expressed in COS cells under control of the cytomegalovirus promoter. Cells were labeled with [^{35}S]methionine and hGH was immunoprecipitated from both cell extracts and culture media using a purified goat anti-hGH antibody. All of the fusion were localized primarily in the cell lysates (Fig. 2), with only trace amounts being found in the media (not shown). As has been previously reported two forms of hGH-DAF37 can be detected in cell lysates, a lower molecular weight, GPI-linked form and a larger species representing uncleaved, unprocessed fusion protein (Moran et al., 1991). Of the various HLD constructs tested only HLD.S^{12}G showed a similar

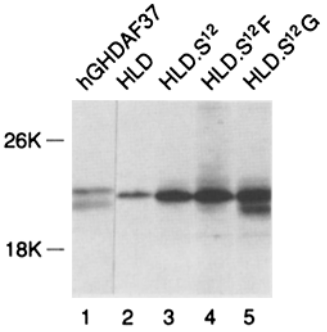

Figure 2. Immunoprecipitation of fusion proteins from [^{35}S]-methionine-labeled, transfected COS cells. COS cells were labeled with [^{35}S]methionine 24 h after transfection with DNAs encoding fusion proteins as indicated. The proteins were immunoprecipitated from cell lysates using a purified goat antibody against hGH.

doublet pattern suggesting that processing had occurred (Fig. 2, lane *5*).

To test for cell surface expression of these HLD fusions, we analyzed the cells by indirect immunofluorescence. Staining of intact cells revealed that of the HLD fusions only HLD.S^{12}G was expressed on the cell surface, as is hGH-DAF37 (Fig. 3, *left*). Immunofluorescence analysis of permeabilized cells confirmed that all of the fusion proteins are efficiently expressed and suggested that HLD, HLD.S^{12} and HLD.S^{12}F are localized in the ER as well as in a Golgi compartment–like organelle located on one side of the nucleus. In contrast, HLD.S^{12}G is present on the plasma membrane as well as in these internal organelles (Fig. 3, *right*).

To test whether the HLD.S^{12}G protein expressed on the cell surface is GPI-anchored, the cells were incubated with PIPLC from *Bacillus thuringiensis*, and the released hGH was measured by an ELISA. We observed PIPLC-dependent release of the GPI-anchored protein hGH-DAF37 and of HLD.S^{12}G (Table I), indicating that the latter is anchored by a GPI anchor. No release was observed with cells expressing HLD, HLD.S^{12} or HLD.S^{12}F confirming that these proteins are not GPI anchored on the cell surface. The above results suggest that two point mutations, resulting in the substitution of valine-glutamate with serine-glycine, at positions 12 and 11 NH_2-terminal to the hydrophobic domain, are sufficient to convert the non-GPI-anchored protein, HLD, to a GPI-linked cell surface protein. These mutations presumably create a recognizable cleavage/attachment site for the GPI anchor.

Position of the Cleavage/Attachment Site Relative to the Hydrophobic Domain

We next asked how important is the position of the serine-glycine cleavage/attachment site relative to the hydrophobic domain? To address this we introduced a series of serine-glycine substitutions in the HLD sequence, placing the serine at positions 9, 6, 4, 2, and 16 NH_2-terminal to the hydrophobic domain in HLD.S^{9}G, HLD.S^{6}G, HLD.S^{4}G, HLD.S^{2}G and HLD.S^{16}G, respectively (Fig. 4). These fusion proteins were transiently expressed in COS cells, and after labeling of the cells with [^{35}S]methionine we analyzed both cell lysates and culture media by immunoprecipitation. As observed above, all of the fusion proteins were localized primarily in the cell lysates (Fig. 5), essentially no protein being detected in the media (not shown). Of the mutant proteins tested only HLD.S^{12}G (containing serine-glycine at positions 12 and 11) showed evidence of processing, suggested by the doublet pattern upon electrophoresis (Fig. 5, lane *2*).

To test whether the cell-associated fusion proteins were on

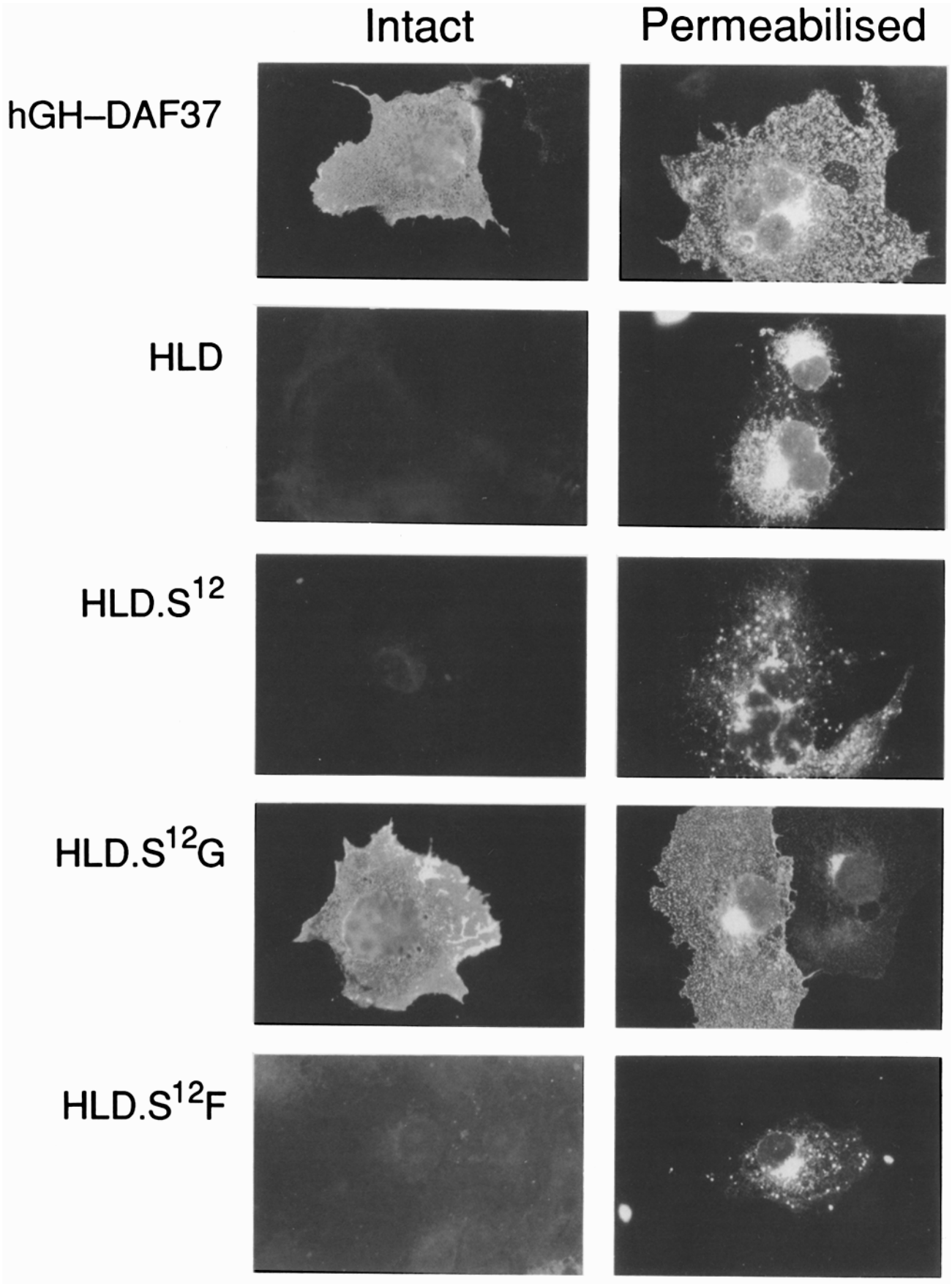

Figure 3. Immunofluorescent labeling of transfected COS cells expressing fusion proteins as indicated. (*Left*) Fixed, nonpermeabilized cells labeled as described in Materials and Methods, showing cell surface protein. (*Right*) Permeabilized cells.

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

Fusion protein	hGH −PIPLC	hGH +PIPLC
	ng/ml	
hGH-DAF37	5.2	38.0
HLD	<1.0	<1.0
HLD.S^{12}	<1.0	<1.0
HLD.S^{12}G	<1.0	15.2
HLD.S^{12}F	<1.0	<1.0

Transfected cells growth in 60-mm dishes were removed with 7 mM EDTA in PBS, washed, and resuspended in 10% FCS/PBS. Aliquots containing 10^6 cells in 100 μl were incubated in the presence or absence of PIPLC (3.9 U/ml) for 60 min at 37°C. The cells were then removed by centrifugation and hGH released into the supernatants was measured by an ELISA as described in Materials and Methods. Shown is a representative of three experiments.

the cell surface we analyzed the cells by indirect immunofluorescence. Staining of intact cells (Fig. 6, *left*) suggested that whereas HLD.S^{12}G was strongly expressed on the cell surface of many cells in the preparation, HLD.S^{9}G was present at moderate levels on fewer cells, and HLD.S^{6}G, HLD.S^{4}G, HLD.S^{2}G, and HLD.S^{16}G were essentially undetectable on the cell surface. As previously observed, analysis of permeabilized cells (Fig. 6, *right*) suggested that the latter proteins are localized in the ER and possibly the Golgi apparatus. In addition we also observed staining of vesicular structures scattered throughout the cytoplasm (Fig. 6, see HLD.S^{4}G staining pattern).

We next tested for the presence of GPI-anchored cell surface fusion proteins by incubating transfected cells with PIPLC and measuring the released hGH. We detected PIPLC-dependent release of HLD.S^{9}G (Table II) indicating that this protein is GPI-anchored on the cell surface. However, consistent with the immunofluorescence analysis, the level of released protein was approximately fourfold lower than that observed with HLD.S^{12}G, suggesting that HLD.S^{9}G is less efficiently processed relative to HLD.S^{12}G. No PIPLC-dependent release of the remaining mutant proteins was observed.

Figure 4. Schematic diagram showing the COOH-termini of HLD fusion proteins with cleavage/attachment sites (*underlined*) introduced at various positions NH_2-terminal to the hydrophobic domain. (*Boxed sequence*) DAF hydrophobic domain; (*large print*) unboxed, LDLR sequence; (*small print*) hGH COOH-terminal sequence.

Figure 5. Immunoprecipitation of HLD fusion proteins containing cleavage/attachment sites at various positions relative to the hydrophobic domain. HLD fusion proteins as indicated were immunoprecipitated from cell lysates derived from transfected COS cells after labeling with [^{35}S]methionine.

To confirm that HLD.S^{12}G and HLD.S^{9}G are GPI-anchored and to rule out the possibility that the intracellular mutant proteins contain anchors, we labeled the cells with [^{3}H]ethanolamine and analyzed them by immunoprecipitation. Of the seven fusion proteins tested only HLD.S^{12}G was strongly labeled with [^{3}H]ethanolamine (Fig. 7, lane *2*). Weak labeling of HLD.S^{9}G (lane *4*) confirmed that this protein does become GPI-linked, although considerably less efficiently than HLD.S^{12}G. No labeled bands were observed with cells expressing HLD, HLD.S^{16}G, HLD.S^{6}G, HLD.S^{4}G, or HLD.S^{2}G, confirming that these fusion proteins do not become GPI anchored. These results suggest that the efficiency of GPI anchor attachment decreases sharply as the processing site is moved closer to, or further from, the hydrophobic domain, the optimal distance being between 10 and 12 residues NH_2-terminal to the hydrophobic domain.

Discussion

GPI Attachment Requires a Pair of Small Residues Flanking the Cleavage Site

Previous reports have localized the signal for GPI anchor attachment to the COOH-terminal 29 residues of DAF and shown that this signal contains at least two critical elements: a 17-residue, COOH-terminal hydrophobic domain and a cleavage/attachment site for the anchor, located within the adjacent sequence (at Ser319, 12 residues NH_2-terminal to the hydrophobic domain) (Caras et al., 1987*a*, 1989; Moran et al., 1991). While previous work indicated that the residue at the anchor attachment site must be small (only Ser, Gly, Ala, Asp, Asn, and possibly Cys can function as acceptors for the anchor [Moran et al., 1991]), additional structural

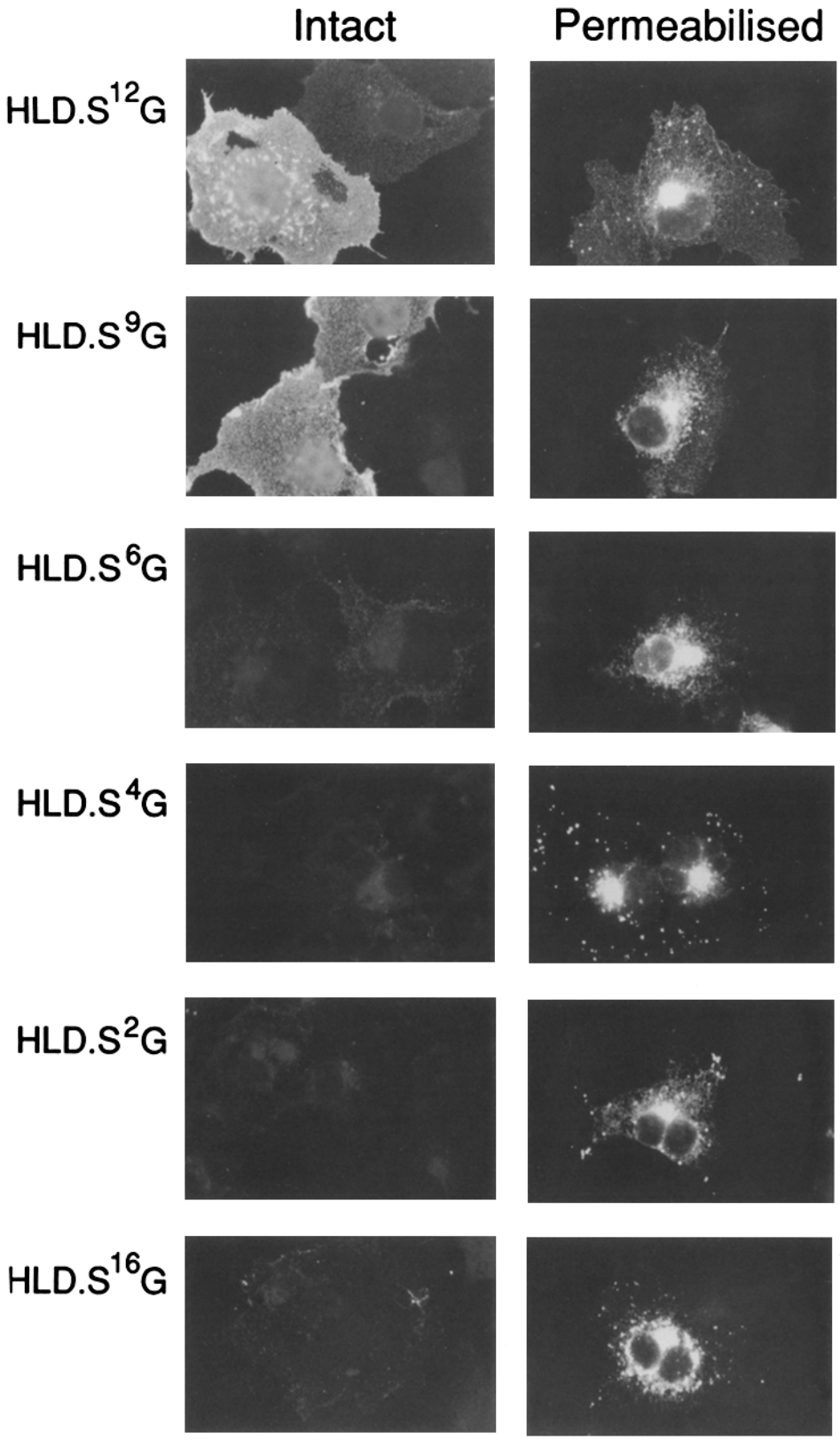

Figure 6. Immunofluorescent labeling of transfected COS cells expressing HLD fusion proteins. (*Left*) Intact (nonpermeabilized) cells showing cell-surface protein; (*right*) permeabilized cells.

features of the cleavage site or GPI signal remained unknown. In this report we addressed this issue using a non-GPI-anchored fusion protein, HLD, containing a fragment of the serine/threonine-rich domain of the LDLR in place of the DAF sequence immediately adjacent to the hydrophobic domain. (The LDLR sequence was chosen since the DAF sequence to be replaced forms part of a functionally similar serine/threonine-rich domain thought to be the site of *O*-linked glycosylation, as in the LDLR [Reddy et al., 1989].) We show that a pair of substitution mutations (con-

Table II. ELISA of hGH in Supernatants from Transfected COS Cells Incubated with or without PIPLC, Carried Out as Described in the Legend to Table I

Fusion protein	hGH	
	−PIPLC	+PIPLC
	ng/ml	
HLD	<1.0	<1.0
HLD.$S^{12}G$	<1.0	27.9
HLD.$S^{9}G$	<1.0	7.4
HLD.$S^{6}G$	<1.0	<1.0
HLD.$S^{4}G$	<1.0	<1.0
HLD.$S^{2}G$	<1.0	<1.0
HLD.$S^{16}G$	<1.0	<1.0

verting a valine-glutamate sequence to serine-glycine) at the position corresponding to the normal processing site in the DAF sequence are sufficient to convert the non-GPI-linked fusion protein, HLD, to a GPI-linked protein on the cell surface. This serine-glycine pair presumably flanks the cleavage point, with serine acting as the acceptor for anchor addition. Both mutations were necessary to achieve anchor attachment. A single substitution (valine-glutamate to serine-glutamate) failed to produce a viable GPI signal. In addition, substitution of valine-glutamate at the position of the processing site with serine-phenylalanine did not allow GPI attachment. These data suggest that in addition to a limited specificity (for small residues) at the anchor addition site, there is a similar limited specificity at the position immediately COOH-terminal to the cleavage point. Inspection of the known cleavage sites of natural GPI-anchored proteins reveals that serine, glycine, alanine, asparagine, aspartate, and arginine occur at this position (Cross, 1990). Except for arginine, this specificity is similar to that observed at the anchor addition site, suggesting that both positions require residues with small side chains. This view is supported by our observation that a bulky residue (such as phenylalanine) at this position abolishes anchor attachment.

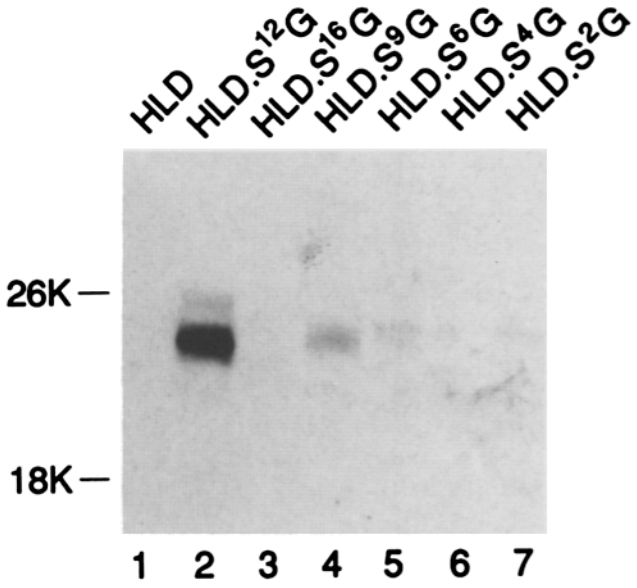

Figure 7. [^{3}H]Ethanolamine labeling and immunoprecipitation of HLD fusion proteins expressed in COS cells. Transfected COS cells were labeled with [^{3}H]ethanolamine (166 μCi/35-mm dish) for 16 h before immunoprecipitation from cell lysates using a purified goat anti-hGH antibody. Equivalent amounts of protein were loaded in all lanes.

Position of the Cleavage/Attachment Site

Analysis of natural GPI anchored proteins suggests that processing usually occurs 10 to 12 residues NH_2-terminal to the hydrophobic domain (Ferguson and Williams, 1988; Cross, 1990). Using the fusion protein, HLD, containing a null sequence for GPI attachment (lacking any potential cleavage/attachment sites) we investigated the consequences of introducing a cleavage site (comprising the sequence serine-glycine) at various positions relative to the hydrophobic domain. Our results show that positioning of this sequence with the attachment site (the serine residue) placed nine residues NH_2-terminal to the hydrophobic domain allows GPI-addition albeit with severely reduced efficiency compared to a similar protein containing the attachment site at position 12. Proteins containing attachment sites positioned 6, 4, 2, or 16 residues NH_2-terminal to the hydrophobic domain failed to become GPI anchored. These data suggest that the position of the processing site relative to the hydrophobic domain is a critical feature of the GPI signal. Although the spacial topology of these two elements allows some flexibility, our data suggest that the optimal distance for anchor attachment is probably 10–12 residues as observed in nature. As the processing site is moved further towards or away from the hydrophobic domain, the efficiency of anchor attachment drops off sharply, the GPI signal eventually becoming essentially nonfunctional.

Significance of Sequences between the Processing Site and the Hydrophobic Domain

Since the LDLR sequence in HLD and the DAF sequence that it replaced show no homology and are presumably also conformationally different, and since the insertion of a cleavage site into HLD is sufficient to induce GPI anchoring, it is not unreasonable to conclude that apart from the two (small) residues that constitute the processing site, this stretch of sequence does not contribute significantly to the GPI signal other than providing a hydrophilic spacer between the hydrophobic domain and the cleavage site. While these conclusions remain to be tested using other hydrophilic spacers, the complete absence of homology or recognizable consensus patterns within this region when different GPI anchored proteins are compared, is consistent with this view.

We conclude that the GPI signal requires only a hydrophobic domain and a cleavage/attachment site consisting of a pair of small residues, positioned 10 to 12 residues NH_2-terminal to the hydrophobic domain. These features appear to define the constraints of the GPI signal, no other structural motifs being necessary.

Received for publication 18 March 1991 and in revised form 17 May 1991.

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.

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. *Nucleic Acids Res.* 9:4735–4743.

Caras, I. W., G. N. Weddell,. M. A. Davitz, V. Nussenzweig, and D. W. Martin, Jr. 1987*a*. 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. 1987*b*. 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.

Cummings, R. D., S. Kornfeld, W. J. Schneider, K. K. Hobgood, H. Tolleshaug, M. S. Brown, and J. L. Goldstein. 1983. Biosynthesis of the N- and O-linked oligosaccharides of the low density lipoprotein receptor. *J. Biol. Chem.* 258:15261–15273.

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.

Ferguson, M. A. J., and A. F. Williams. 1988. Cell surface anchoring of proteins via glycosyl-phosphatidylinositol 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.

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

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.

McClary, J. A., Witney, F. and J. Geisselsoder. 1989. Efficient site-directed in vitro mutagenesis using phagemid vectors. *Biotechniques.* 7:282–289.

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.

Micanovic, R., L. D. Gerber, J. Berger, K. Kodukula, and S. Udenfriend. 1990. Selectivity of the cleavage/attachment site of phosphatidylinositol-glycan-anchored membrane proteins determined by site-specific mutagenesis at Asp-484 of placental alkaline phosphatase. *Proc. Natl. Acad. Sci. USA.* 87:157–161.

Moran, P., H. Raab, W. J. Kohr, and I. W. Caras. 1991. Glycophospholipid membrane anchor attachment. Molecular analysis of the cleavage/attachment site. *J. Biol. Chem.* 266:1250–1257.

Reddy, P., I. Caras, and M. Krieger. 1989. Effects of O-linked glycosylation on the cell surface expression and stability of decay accelerating factor, a glycophospholipid-anchored membrane protein. *J. Biol. Chem.* 264:17329–17336.

Russell, D. W., W. J. Schneider, T. Yamamoto, K. L. Kuskey, M. S. Brown, and J. L. Goldstein. 1984. Domain flap of the LDL receptor: sequence homology with the epidermal growth factor precursor. *Cell.* 37:577–585.

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