Fusion of Sequence Elements from Non-anchored Proteins
to Generate a Fully Functional Signal for Glycophosphatidylinositol
Membrane Anchor Attachment

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Abstract. Glycophosphatidylinositol (GPI) membrane anchor attachment is directed by a cleavable signal at the COOH terminus of the protein. The complete lack of homology among different GPI-anchored proteins suggests that this signal is of a general nature. Previous analysis of the GPI signal of decay accelerating factor (DAF) suggests that the minimal requirements for GPI attachment are (a) a hydrophobic domain and (b) a cleavage/attachment site consisting of a pair of small residues positioned 10–12 residues NH₂-terminal to a hydrophobic domain. As an ultimate test of these rules we constructed four synthetic GPI signals, meeting these requirements but assembled entirely from sequence elements not normally involved in GPI attachment. We show that these synthetic signals are able to direct human growth hormone (hGH), a secreted protein, to the plasma membrane via a GPI anchor. Our results indicate that different hydrophobic sequences, derived from either the prolactin or hGH NH₂-terminal signal peptide, can be linked to different cleavage sites via different hydrophilic spacers to produce a functional GPI signal. These data confirm that the only requirements for GPI-anchoring are a pair of small residues positioned 10–12 residues NH₂ terminal to a hydrophobic domain, no other structural motifs being necessary.

A growing and diverse class of integral membrane proteins is now known to be held on the plasma membrane not by a transmembrane domain but by a glycophosphatidylinositol (GPI) anchor covalently attached to the COOH-terminus of the protein (for review see Cross, 1990; Low, 1989; Ferguson and Williams, 1988). The GPI membrane anchor contains phosphatidylinositol, carbohydrate, and ethanolamine and is apparently preassembled before being added to proteins (Masterson et al., 1989). Anchor addition involves a coordinated processing event in which 22–31 residues are removed from the COOH terminus of the nascent chain following which the anchor is attached to the new COOH terminus (Boothroyd et al., 1981; Tse et al., 1985). This 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., 1987a). Although all GPI-anchored proteins are presumably processed via a common pathway, the complete lack of primary sequence homology among different GPI-linked proteins indicates that this signal is of a general nature.

Using the GPI-anchored protein, decay accelerating factor (DAF), as a model system to analyze the signal for GPI attachment we previously showed that the COOH-terminal 29 residues of DAF, when fused to the COOH terminus of a secreted protein, are sufficient to direct the fusion protein to the cell surface by means of a GPI anchor (Moran et al., 1991). This 29-residue sequence comprises a 17-residue COOH-terminal hydrophobic domain plus 12 hydrophilic residues containing the processing site for anchor addition. Further analysis of this signal suggests that the critical elements for GPI attachment are (a) a hydrophobic domain and (b) a cleavage/attachment site consisting of a pair of small residues positioned 10–12 residues NH₂ terminal to the hydrophobic domain (Caras et al., 1989; Moran and Caras, 1991). Only Ser, Gly, Ala, Asp, Asn and possibly Cys at the cleavage/attachment site can function as acceptors for GPI addition (Moran et al., 1991; Micanovic et al., 1990). The position on the COOH-terminal side of the cleavage point appears to exhibit a similar requirement for small residues, though a systematic replacement of this position with all possible amino acids has not yet been carried out. Hydrophilic sequences on either side of the cleavage site appear not to play a role in anchor attachment (Moran and Caras, 1991). The COOH-terminal hydrophobic domain, while absolutely necessary, can be replaced with alternative hydrophobic sequences, indicating that the overall hydrophobicity rather than the primary sequence of this domain is important for its functioning (Caras and Weddell, 1989).

The above requirements for GPI attachment were deduced

1. Abbreviations used in this paper: DAF, decay accelerating factor; GPI, glycophosphatidylinositol; hGH, human growth hormone; LDLR, low density lipoprotein receptor; PIPLC, phosphatidylinositol-specific phospholipase C.
either by deletion mutagenesis or by systematic replacement of individual components of the DAF GPI signal with alternative sequences. As a final test of the generality of these rules we constructed a series of synthetic GPI signals that meet these requirements. These synthetic signals are composed entirely of sequence elements that are unrelated to each other and do not normally function in GPI attachment.

We show that these synthetic signals are fully functional, directing a secreted protein to the cell surface via a GPI anchor.

Materials and Methods

Phosphatidylinositol-specific phospholipase C (PI-PLC) purified from Bacillus thuringiensis was provided by Dr. Martin G. Low of 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); [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 and Fusion Proteins

HGH-DAF37, HLD, and HLD.S12G were constructed as previously described (Caras et al., 1989; Moran and Caras, 1991). To construct HL.Psig2 and HL.Hsig2, we first modified the HLD.S12G plasmid, deleting the 17-residue COOH-terminal hydrophobic domain of DAF by oligonucleotide-directed mutagenesis (McClyr et al., 1989), and replacing it with a COOH-terminal polynucleotide, supplying unique Nhel and HindIII sites. Four overlapping synthetic oligonucleotides (25–62 nucleotides in length), encoding the complete hGH or prolactin signal peptide, were then cloned into this modified HLD.S12G plasmid using the Nhel and HindIII sites in the COOH-terminal polynucleotide. HL.Psig2 was similarly constructed using two synthetic oligonucleotides encoding a portion of the prolactin signal peptide (residues -19 to -1) lacking the charged NH2-terminal region (residues -30 to -20) (Sasavage et al., 1982). These were cloned into the modified HLD.S12G plasmid using a BspEI site in the low density lipoprotein receptor (LDLR) sequence and the HindIII site in the polynucleotide. HL.Hsig2 was constructed from HL.Hsig2 by deletion of the NH2-terminal hydrophilic portion of the hGH signal peptide (residues -26 to -19) (DeNoto et al., 1982) using the polymerase chain reaction (Saiki et al., 1988). A 625-bp fragment was synthesized by polymerase chain reaction and digested with HindIII and BspEI to yield a 96-bp fragment. The latter was cloned into the modified HLD.S12G plasmid using the BspEI and HindIII sites. All plasmids were verified by sequencing. A mammalian expression vector was used both for cloning and expression, and contained the cytomegalovirus enhancer/promoter and an SV40 poly(A) sequence (Eaton et al., 1986).

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 [35S]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., 1987b) except that 0.5% Triton X-100/PBS was used to permeabilize the cells. Cells were incubated with a purified rabbit antibody against human growth hormone (hGH), followed by fluorescein-conjugated goat antirabbit antisemirum (Cappel Laboratories).

hGH-ELISA

hGH levels were measured by an ELISA as previously described (Moran and Caras, 1991).
processed, GPI-linked forms of HLPsigl and HLHsigl would be identical to GPI-linked HLD.S\(^{3\text{rd}G}\), all three proteins being cleaved at the same processing site (Ser-Gly), while HLPsig2 and HLHsig2 if cleaved at the Ala-Ser sequence, would be \(\sim 1\) kD larger.

**Immunoprecipitation Analysis**

The cDNAs encoding these proteins were transiently expressed in COS cells under control of the cytomegalovirus promoter. The cells were labeled with \([\text{35S}]\)methionine and hGH was immunoprecipitated from both cell extracts and culture media using a purified goat anti-hGH antibody. All four fusion proteins were localized exclusively in the cell lysates (Fig. 2); no protein was detected in the culture media (not shown). We previously reported that the processed and unprocessed forms of these fusion proteins can be separated on 15% polyacrylamide gels, the uncleaved forms migrating more slowly than the GPI-linked forms (Moran et al., 1991). As shown in Fig. 2 (lanes 1 and 2), the previously characterized GPI-anchored fusion proteins hGH-DAF37 (containing the complete GPI signal of DAF) (Caras et al., 1989) and HLD.S\(^{3\text{rd}G}\) (containing the 17-residue COOH-terminal hydrophobic domain of DAF) (Moran and Caras, 1991), both migrate as doublets, indicating that processing occurs but is incomplete. Pulse-chase experiments indicate that the uncleaved species permanently escapes processing and is not a newly synthesized precursor to the cleaved form (our unpublished observations). HLPsigl produced a single \(\sim 22\)-kD species (lane 3) that migrates with the GPI-linked form of HLD.S\(^{3\text{rd}G}\), suggesting that this protein is processed to completion. HLHsigl produced a slightly larger \(\sim 22.5\)-kD species (lane 4), as well as a minor \(\sim 22\)-kD species. The major HLPsig2 and HLHsig2 polypeptides (lanes 5 and 6) migrate with an apparent molecular mass of \(\sim 25\) kD, consistent with that of the uncleaved form of the protein (\(\sim 1\) kD larger than uncleaved HLD.S\(^{3\text{rd}G}\)).

**Immunofluorescence Microscopy**

To determine whether the fusion proteins on the cell surface are GPI anchored, transfected COS cells were incubated with phosphatidylinositol-specific PIPLC from *Bacillus thuringiensis* and the released hGH was measured by an ELISA. HLPsigl produced large amounts of PIPLC-releasable protein, comparable to those observed with the previously characterized GPI-anchored proteins, HGH-DAF37 and HLD.S\(^{3\text{rd}G}\) (Table I), suggesting that HLPsigl is efficiently processed to a GPI-linked form. PIPLC released a small but detectable amount of hGH from cells expressing HLHsigl, suggesting that this protein also becomes GPI anchored, although \(\sim 18\)-fold less efficiently than HLPsigl. The remaining fusion proteins, HLPsig2 and HLHsig2, containing the complete prolactin or hGH signal peptide at their COOH termini, also showed evidence of PIPLC-releasable protein, suggesting that they are GPI anchored. However, this conclusion is complicated by the observation of significant release of protein in the absence of PIPLC, possibly due to proteolysis.

**[\text{3H}]Ethanolamine Labeling Confirms the Presence of a GPI Anchor**

To verify the presence of a GPI anchor, transfected cells were labeled metabolically with [\text{3H}]ethanolamine, a specific component of the GPI anchor, and analyzed by immunoprecipitation. As a negative control we included the fusion protein, HLD, which lacks a cleavage/attachment site and fails to become GPI anchored. Whereas HLD showed no [\text{3H}]ethanolamine-labeled bands (Fig. 4, lanes 1 and 7), all four fusion proteins containing a synthetic GPI signal incorporated [\text{3H}]ethanolamine, indicating conclusively that they are GPI anchored (Fig. 4). However, the degree of GPI anchoring varied. HLPsigl (lane 3) is processed very efficiently, producing as much GPI-linked protein as HLD.S\(^{3\text{rd}G}\), containing the DAF hydrophobic domain (lane 2). HLHsigl (lane 4) is processed poorly, producing only small amounts of [\text{3H}]ethanolamine-labeled protein. This result is consistent with the results obtained using PIPLC (Table I). All three proteins, HLPsigl, HLHsigl and HLD.S\(^{3\text{rd}G}\), produced a similar \(\sim 21.8\)-kD [\text{3H}]ethanolamine-labeled GPI-linked species, suggesting that all are cleaved at the same processing site, presumably the Ser-Gly sequence. As previously observed, this [\text{3H}]ethanolamine-labeled species comigrated with the lower molecular weight of the two [\text{35S}]methionine-labeled species of HLD.S\(^{3\text{rd}G}\) (not shown).

Both HLPsig2 and HLHsig2, containing the complete prolactin or hGH signal peptide at their COOH termini, showed a more complex pattern of four [\text{3H}]ethanolamine-labeled bands (lanes 5 and 6 or 9 and 10). The smallest of these (\(\sim 23\) kD for HLHsig2; 23.3 kD for HLPsig2) is \(\sim 1.2\) kD (HLHsig2) or \(\sim 1.5\) kD (HLPsig2) larger than the \(\sim 21.8\)-kD GPI-linked form of HLPsigl, HLHsigl, and HLD.S\(^{3\text{rd}G}\).

\[\text{Figure 2. Immunoprecipitation of fusion proteins from [\text{35S}]methionine-labeled, transfected COS cells. COS cells were labeled with [\text{35S}]methionine for 6 h, 24 h after transfection with DNAs encoding the indicated fusion proteins. The proteins were immunoprecipitated from cell lysates using a purified goat antibody against hGH.}\]
indicating cleavage COOH terminal to the Ser-Gly sequence. The molecular sizes suggest an addition of approximately 11 amino acids to the processed form of HLHsig2 consistent with cleavage at or near the Ala-Ser sequence (Fig. 1). GPI-linked HLPsig2 appears to be slightly larger, containing ~14 additional residues. This suggests cleavage at an even more distal site, possibly the Asp-Ser sequence within the prolactin signal peptide (Fig. 1). These assignments of the cleavage sites, deduced from the electrophoretic mobility of the labeled proteins, place the processing sites in both cases at a distance of 10 residues NH2 terminal to the hydrophobic domain. Although these assignments are tentative rather than proven, they are in good agreement with previous results suggesting that 10 to 12 residues is the optimal distance for GPI attachment (Moran and Caras, 1991). The largest of the four labeled species migrate with apparent molecular masses of ~27 and 30 kD (HLHsig2) and ~27.5 and 30.5 kD (HLPsig2). These species are larger than the uncleaved polypeptides (~25 kD), indicating that they are post-translationally modified (presumably glycosylated) forms of a GPI-linked molecule rather than products of processing at alternative cleavage sites. Since the LDLR sequence present in these proteins is part of a Ser/Thr-rich domain known to be the site of O-linked glycosylation in the LDLR (Cummings et al., 1983; Russell et al., 1984), this is not unlikely. The fourth labeled species (~24 kD for HLHsig2; ~24.3 kD for HLPsig2) could represent either a partially glycosylated species, or GPI addition at an alternative site. Considering the latter possibility, a molecular mass increase of ~2.2-2.5 kD over that of the ~21.8-kD GPI-linked form of HLHsig1 and HLPsig1 suggests the presence of 20-23 additional residues, thereby placing the putative alternative cleavage site al-
Table 1. ELISA of hGH in Supernatants from Transfected COS Cells with or without PIPLC

| Fusion protein       | hGH ng/ml -PIPLC | hGH ng/ml +PIPLC |
|----------------------|-------------------|------------------|
| hGHDAF-37            | 2.8               | 43.3             |
| HLD.S12G             | 1.7               | 30.2             |
| HLPsig1              | 2.0               | 29.0             |
| HLDHsig1             | <1.0              | 6.2*             |
| HLPsig2              | 9.6               | 12.8             |
| HLHsig2              | 9.6               | 14.2             |

* Cell aliquots contained ~4 × 10^6 cells in 100 μl (determinations using 1 × 10^6 cells/100 μl did not produce reliable signals after background subtraction).

Transfected COS cells grown in 60-mm dishes were removed with 7 mm EDTA in PBS and resuspended in 10% FCS in 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 removed by centrifugation and hGH in the supernatants was measured by an ELISA as described in Materials and Methods. Shown is a representative of three experiments.

Discussion

Previous reports, based on an analysis of the DAF GPI signal, have suggested that a hydrophobic domain combined with an appropriately positioned cleavage/attachment site consisting of a pair of small residues, are all that is needed for signaling GPI attachment (Caras et al., 1989; Moran and Caras, 1991). In the present work we tested these rules by showing that a series of synthetic signals, meeting these requirements but assembled entirely from sequence elements not normally involved in GPI anchoring, are able to direct hGH, a secreted protein, to the plasma membrane via a GPI anchor. PIPLC-dependent release as well as metabolic labeling with [3H]ethanolamine were used as criteria for the presence of a GPI anchor, and immunofluorescence analysis of intact cells clearly indicated that hGH was on the cell surface.

Our data illustrate the generality of the above rules in a number of ways. (a) Two different COOH-terminal hydrophobic domains, derived from either the hGH or prolactin signal peptide, were able to signal GPI attachment, supporting and extending earlier conclusions that the only important structural feature of this domain is its hydrophobicity (Caras and Weddell, 1989). (b) Different paired combinations of small residues were apparently able to serve as cleavage/attachment sites. Cleavage and GPI anchoring of the fusion protein hGH.S12G, containing the DAF hydrophobic domain, has previously been shown to depend on the presence of a correctly positioned Ser-Gly sequence, indicating that this sequence provides the necessary processing site (Moran and Caras, 1991). The GPI-linked forms of HLPsig1 and HLHsig1 were electrophoretically indistinguishable from that of hGH.S12G, strongly suggesting that they are also cleaved at the Ser-Gly sequence. In contrast, the GPI-linked forms of HLPsig2 and HPHsig2 were larger, indicating cleavage at a site COOH terminal to the Ser-Gly sequence. The molecular sizes suggest that HLHsig2 is cleaved at or near the Ala-Ser sequence at the junction of the LDLR sequence and the signal peptide, whereas HLPsig2 is cleaved at or near the Asp-Ser sequence within the prolactin signal peptide. We suggest that only a single cleavage site is used for each fusion protein, the observed heterogeneity in the band pattern being entirely due to O-linked glycosylation. This conclusion is consistent with the observations that (i) the larger GPI-linked species are too large to represent alternatively processed products; and (ii) while the four labeled HLPsig2 polypeptides migrate slightly faster than the corresponding HLPsig2 proteins, in both cases we observe the same pattern of stepwise size increments. Furthermore, the LDLR sequence remaining in these fusion proteins (but cleaved from the HLPsig1 and HLHsig1 proteins) is derived from a Ser/Thr-rich domain known to be the site of O-linked glycosylation in the LDLR (Cummings et al., 1983; Russell et al., 1984).

The above assignments of the processing sites place the points of cleavage at positions either 10 residues (HLHsig2 and HLPsig2) or 12 residues (HLPsig1 and HLPsig1) NH2 terminal to the hydrophobic domain. Previous data has indicated that these are the optimal positions for GPI attachment (Moran and Caras, 1991), and examination of natural GPI-linked proteins where the cleavage site is known suggests that these are the positions used in nature. As predicted, our data indicate that although the Ser-Gly sequence functions as a processing site in HLHsig1 and HLPsig1, this sequence is not used in HLPsig2 or HPHsig2, presumably because of its suboptimal position, 21 or 24 residues distal to the hydrophobic domain. Furthermore, the Ala-Ser sequence of HLPsig2, positioned 13 residues NH2 terminal to the hydrophobic domain, is apparently bypassed in favor of the Asp-Ser sequence, positioned at a distance of 10 residues. These
membrane proteins, and if found, why they are not recognized. The cleavage/attachment site is a critical feature of these observations strengthen our earlier conclusions that the position of the cleavage/attachment site is a critical feature of the GPI-signal.

(c) Different nonspecific hydrophilic sequences are able to serve as spacer sequences between the processing site and the hydrophobic domain. We previously showed that the hydrophilic sequences NH$_2$ terminal to the DAF hydrophobic domain can be replaced with nonspecific sequences from the LDLR (Moran and Caras, 1991). Our results with HLHsиг2 and HLPSиг2 now indicate that alternative hydrophilic sequences derived from the charged, NH$_2$-terminal regions of either the hGH or prolactin signal peptide are similarly able to serve as spacers between the cleavage site and hydrophobic domain, confirming that this region does not contain specific structures necessary for anchor attachment.

While our present results verify the minimal requirements of the GPI signal and suggest that potentially any hydrophobic domain combined with a pair of small residues could trigger anchor attachment, they also reveal that not all sequence combinations are recognized with equal efficiency. Subtle conformational differences in either the hydrophobic domain itself or the precise spatial arrangement of the cleavage site relative to the hydrophobic domain, determined by the spacer sequences, may influence the interaction with the GPI attachment machinery. For example, HLPsig2 is processed more efficiently than HLPSig2, although in both cases GPI attachment is triggered by the prolactin signal peptide.

The generality of the GPI signal, it is of interest to ask how frequently potential GPI signals are found in transmembrane proteins, and if found, why they are not recognized. The absence of a cytoplasmic tail is not an obligatory feature of the GPI signal; internal signals, although less efficient than COOH-terminal signals, are functional in anchor attachment (Caras, 1991). In a survey of 41 transmembrane proteins two were found to contain a potential cleavage/attachment site positioned 10 or 12 residues NH$_2$ terminal to the hydrophobic domain, suggesting that selection against potential processing sites might be one mechanism for preventing GPI attachment. In transmembrane proteins where such sites do occur, factors such as the presence of a cytoplasmic domain combined with a stop-transfer sequence might reduce the efficiency of the signal to a point that it is no longer recognized.

We conclude that a fully functional GPI signal can be assembled from sequence elements totally unrelated to GPI anchoring, confirming that the minimal requirements for GPI attachment are a pair of small amino acids, serving as a cleavage/attachment site, positioned 10-12 residues NH$_2$ terminal to a hydrophobic domain.

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