Requirements for Glycosylphosphatidylinositol Attachment Are Similar but Not Identical in Mammalian Cells and Parasitic Protozoa

Paul Moran and Ingrid W. Caras
Department of Neurobiology, Genentech, Inc., South San Francisco, California 94080

Abstract. The general features of the glycosylphosphatidylinositol (GPI) signal have been conserved in evolution. To test whether the requirements for GPI attachment are indeed the same in mammalian cells and parasitic protozoa, we expressed the prototype GPI-linked protein of *Trypanosoma brucei*, the variant surface glycoprotein (VSG), in COS cells. Although large amounts of VSG were produced, only a small fraction became GPI linked. This impaired processing is not caused by the VSG ectodomain, since replacement of the VSG GPI signal with that of decay accelerating factor (DAF) produced GPI-linked VSG. Furthermore, whereas fusion of the DAF GPI signal to the COOH terminus of human growth hormone (hGH) produces GPI-linked hGH, an analogous hGH fusion using the VSG GPI signal does not, indicating that the VSG GPI signal functions poorly in mammalian cells. By constructing chimeric VSG-DAF GPI signals and fusing them to the COOH terminus of hGH, we show that of the two critical elements that comprise the GPI-signal—the cleavage/attachment site and the COOH terminal hydrophobic domain—the former is responsible for the impaired activity of the VSG GPI signal in COS cells. To confirm this, we show that the VSG GPI signal can be converted to a viable signal for mammalian cells by altering the amino acid configuration at the cleavage/attachment site. We also show that when fused to the COOH terminus of hGH, the putative GPI signal from the malaria circumsporozoite (CS) protein produces low levels of GPI-anchored hGH, suggesting that the CS protein is indeed GPI linked, but that the CS protein GPI signal, like the VSG signal, functions poorly in COS cells. The finding that the requirements for GPI attachment are similar but not identical in parasitic protozoa and mammalian cells may allow for the development of selective inhibitors of GPI-anchoring that might prove useful as antiparasite therapeutics.

In eukaryotic cells, a diverse group of cell surface proteins are anchored to the cell membrane by a glycosylphosphatidylinositol (GPI) glycolipid, covalently attached to the COOH terminus of the protein (reviewed by Englund, 1993; Cross, 1990). The GPI membrane anchor contains phosphatidylinositol, carbohydrate, and ethanolamine, and it is preassembled before being added to proteins. Attachment to protein is directed by a COOH-terminal signal, and involves a coupled reaction in which the signal itself is proteolytically removed and replaced with the GPI lipid. This reaction requires translocation of the nascent chain across the ER membrane (Caras, 1991), and it is catalyzed by an as yet unidentified enzyme, possibly a membrane-associated transamidase.

Although there is no sequence homology between the COOH-terminal sequences of GPI-anchored proteins, the general features of the GPI signal have been elucidated. Using the GPI-anchored protein decay accelerating factor (DAF) as a model system, we showed that the last 29 amino acids contain the information for GPI attachment, and when fused to the COOH-terminus of a normally secreted protein, human growth hormone (hGH), will target hGH to the cell surface as a GPI-anchored protein (Moran et al., 1991). This 29-residue DAF sequence contains two critical elements for GPI-attachment—a 17-residue COOH-terminal hydrophobic domain and a cleavage/attachment site for anchor addition—separated by a short hydrophobic spacer sequence (Caras et al., 1989). The distance between these two elements is important, 10–12 residues being optimal (Moran and Caras, 1991a). Two to three residues form the cleavage/attachment site: the residue on the NH2 terminal side of the cleavage point (also referred to as the $\omega$ position), to which the GPI lipid is added, must be a small side chain amino acid; e.g.,
Ser, Gly, Ala, Asp, Asn, or possibly Cys (Moran et al., 1991; Micanovic et al., 1990); the residue on the COOH-terminal side of the cleavage point (the ω+1 position) is generally also small. Experiments using alkaline phosphatase suggest that only certain amino acids are tolerated in the position adjacent to the ω+1 residue (the ω+2 position), suggesting that this residue also plays a role in forming the cleavage/attachment site (Kodokula et al., 1993). However, in the context of the DAF GPI signal, this position appears to be less restricted (Moran and Caras, 1991a; Moran and Caras, 1991b).

The core structure of the GPI lipid and many features of the biosynthesis have been conserved in evolution (for review, see Englund, 1993; Cross, 1990). The GPI signal also appears to have been conserved, the same general features being present in GPI-linked proteins from organisms as distantly related as mammals and parasitic protozoa. However, the assumption that the requirements for GPI attachment are the same in mammals and parasitic protozoa has never been tested.

The goals of this study were twofold: (a) to express the prototype GPI-linked protein of the African parasite, Trypanosoma brucei (Ferguson et al., 1986, 1988) in mammalian cells and ask if it becomes GPI anchored, and (b) to express in mammalian cells the immunodominant surface antigen of the malaria sporozoite, the circumsporozoite (CS) protein (Nussenzweig and Nussenzweig, 1985) (suspected to be GPI-linked but hitherto untested) and test the prediction that it is GPI linked.

**Materials and Methods**

**Antibodies and Reagents**

A rabbit antisera against variant surface glycoprotein (VSG) variant 117 was from Drs. M. Field and G. Cross at The Rockefeller University (New York); an antibody against the CS protein of Plasmodium berghei was provided by Dr. D. J. Eichinger of New York University (New York). Affinity-purified antibodies against hGH were supplied by the Medicinal and Analytical Chemistry Department at Genentech; fluorescent anti-mouse or anti-rabbit IgG was from Cappel Laboratories (Cochrane, PA). Phosphatidylinositol-specific phospholipase C (PIPLC) purified from Bacillus thuringiensis was kindly provided by Dr. Martin G. Low of Columbia University (New York).

**Recombinant Plasmids and Fusion Proteins**

A cDNA encoding a full-length VSG, variant 117, isolated from T. brucei was kindly made available by Dr. Hon Ip of Case Western Reserve University (Cleveland, Ohio). The gene encoding the CS protein of the malaria parasite P. berghei was provided by Drs. D. J. Eichinger and V. Nussenzweig at New York University and Dr. S. M. Beverley at Harvard Medical School (Boston, MA). These DNAs were cloned into a mammalian expression vector containing the cytomegalovirus enhancer/promoter and an SV-40 polyadenylation sequence (Eaton et al., 1986). Expression plasmids encoding DAF or an hGH-DAF fusion protein (hGHDAF) have been previously described (Caras et al., 1987; Moran et al., 1991). New fusion proteins were constructed using the PCR essentially as described by Yon and Fried (1989) using synthetic 15-20mers as "outer oligos" and 40mers as "linking oligos." Fragments of <800 bp generated by the polymerase chain reaction and containing the precise gene fusion were used to reconstruct the complete fusion protein by standard cloning procedures. In all cases, the regions generated by PCR were verified by sequencing.

**Transfections, Metabolic Labeling, and Immunoprecipitation**

COS cells were transfected using the DEAE-dextran method as described by Seldon (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 or [3H]ethanolamine and analysis of proteins by immunoprecipitation was as previously described (Caras et al., 1989).

**Immunofluorescent Labeling**

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

**hGH ELISA**

hGH levels were measured by an ELISA as previously described (Moran and Caras, 1991a).

**Results**

**GPI Anchoring of VSG in Mammalian Cells**

To test whether the GPI signal of T. brucei is recognized in mammalian cells, a cDNA encoding the prototype GPI-linked protein of T. brucei, the VSG (Ferguson et al., 1986, 1988), was cloned into a mammalian expression vector and transiently expressed in COS cells. Analysis of both the cell lysates and culture media by immunoprecipitation with an anti-VSG antibody revealed the presence of a cell-associated, ~50 kD VSG polypeptide in transfected cultures (Fig. 1, lane 1), but not in nontransfected control cells (not shown). Immunofluorescence microscopy suggested that some of this VSG was present on the cell surface, although the signal was low (see Fig. 4 below). To determine whether this cell surface VSG is GPI linked, metabolically labeled cells were incubated with PIPLC from B. thuringiensis and the incubation supernatants were analyzed by immunoprecipitation and SDS-PAGE. Although we detected VSG protein in these supernatants, the release was not PIPLC dependent (Fig. 1, lanes 2 and 3). In contrast, the release of two control proteins that are known to be GPI linked, DAF (Davitz et al., 1987) and a hGHDAF fusion protein, containing the DAF GPI signal fused to the COOH terminus of hGH (Caras et al., 1989; Moran et al., 1991), was completely PIPLC dependent (Fig. 1, lanes 4-7). (The small difference in the intensity of the VSG band in lane 3 vs lane 2 might indicate a small amount of PIPLC-releasable protein; nevertheless, the behavior of VSG is clearly different from that of DAF and hGHDAF.)

This result, while unexpected, suggests that the VSG protein might not be processed to a GPI-linked protein in mammalian cells. To confirm this, transiently transfected cells expressing either VSG or DAF were labeled with [3H]ethanolamine, a specific component of the GPI anchor, and the labeled proteins were analyzed by immunoprecipitation. We observed 10- to 20-fold less [3H]ethanolamine-labeled VSG compared to DAF (Fig. 2), although a parallel immunoprecipitation from [35S]methionine-labeled cells indicated that both proteins were expressed at similar levels (not shown). We concluded that the bulk of the expressed VSG does not become GPI linked in mammalian cells.

**The Failure to Become GPI Linked Can Be Attributed to the VSG GPI Signal**

The failure of VSG to be correctly processed and expressed
on the surface of COS cells as a GPI-linked protein could be a consequence of the heterologous system rather than a problem with the GPI signal; i.e., it is possible that the parasite protein does not fold correctly in the mammalian cell environment, thereby blocking posttranslational processing and transport to the cell surface. To test if this is the case, we used DNA manipulation to construct two chimeric proteins: VSGDAF, containing the VSG ectodomain fused to the DAF GPI signal in place of the VSG GPI signal; and hGHVSG, containing the VSG GPI signal fused to the COOH terminus of hGH (Fig. 3). The latter fusion protein is analogous to the hGHDAF fusion protein that we have previously described (Caras et al., 1989; Moran et al., 1991). These fusion proteins were transiently expressed in COS cells together with authentic VSG and hGHDAF as controls, and produced cell-associated proteins of the predicted molecular masses (~50 kD for VSGDAF and ~22 kD for hGHVSG) (data not shown). To determine the cellular localization of these proteins, we analyzed the cells by immunofluorescence micro-

**Figure 1.** Expression of VSG in COS cells and the effect of PIPLC on its release. COS cells were transfected with expression plasmids encoding VSG, DAF, or hGHDAF as indicated. 24 h after transfection, the cells were labeled with [35S]methionine and incubated with or without PIPLC as indicated, for 2 h at 37°C. The cell lysates (shown for VSG only, lane 1) and incubation supernatants (lanes 2–7) were analyzed by immunoprecipitation using appropriate antibodies, followed by SDS-PAGE.

- VSG
- DAF
- hGHDAF
- PIPLC

~50 kD

**Figure 2.** [3H]Ethanalamine labeling and immunoprecipitation of VSG and DAF expressed in COS cells. 24 h after transfection, COS cells were labeled with [3H]ethanolamine (200 μCi/35-mm dish) for 16 h, and the cell lysates were then analyzed by immunoprecipitation with antibodies to VSG (lanes 1 and 2) or DAF (lanes 3 and 4); lane 1, VSG-transfected cells; lanes 2 and 3, nontransfected control cells; lane 4, DAF-transfected cells.

**Figure 3.** Schematic diagram showing the COOH-terminal sequences of authentic VSG and various fusion proteins as indicated. The source of each block of sequence is indicated above, at the start of the sequence. Small print, hGH COOH terminus; boxed sequence, COOH-terminal hydrophobic domain. The anchor-addition site is underlined, and the three additional residues in the spacer sequence of hGHVSGsp are shown in bold. Note that the hGHDAF fusion shown is hGHDAF15, containing the last 30 amino acids of DAF (Moran et al., 1991), whereas hGHDAF15 was constructed from hGHDAF37 (Moran et al., 1991) and contains some additional DAF sequence NH2-terminal to the cleavage site; we have previously shown that this sequence plays no role in GPI addition (Moran et al., 1991).

- VSG
- DAF
- hGHDAF
- hGHVSG
- hGHDAF.VH
- hGHVSG.DH
- hGHVSG_sp

Defective GPI Attachment is Due to the Cleavage/Attachment Site

GPI signal domains are comprised of two functional elements—a COOH-terminal hydrophobic domain and a cleavage/attachment site for anchor addition—connected by a
short spacer sequence (Caras et al., 1989). To determine whether one or both of these elements in the VSG signal is functionally impaired in COS cells, we constructed two hybrid GPI signals and fused them to the COOH terminus of hGH (Fig. 3): hGHDAF.V organic signal combined with the COOH terminal hydrophobic domain from the VSG GPI signal; hGHVSG.DH contains the reverse arrangement; i.e., the cleavage/attachment site and spacer sequences are from VSG and the hydrophobic domain.

Figure 4. Immunofluorescent labeling of transfected COS cells expressing the proteins indicated. Left, Fixed, nonpermeabilized cells labeled as described in Materials and Methods, showing cell surface protein; right, permeabilized cells.
is from DAF. In addition, we questioned whether the position of the cleavage site relative to the hydrophobic domain might be suboptimal in the VSG GPI signal; i.e., most mammalian GPI signals contain spacer sequences of 9-11 residues, whereas the VSG spacer contains only 8 residues. Therefore, we reasoned that this might result in misalignment of the processing site in the VSG GPI signal with the active site of the mammalian GPI transamidase. To test if this is the case, we lengthened the VSG spacer by inserting an additional three hydrophilic residues (Fig. 3), and fused this altered VSG signal to the COOH terminus of hGH to make hGHVSGsp.

After expression in COS cells, the chimeric proteins were localized by immunofluorescence microscopy using an anti-hGH antibody (Fig. 6). Although permeabilization of the cells demonstrated good expression of all of the chimeras, only hGHDAF and hGHDAFVn showed strong cell surface expression; hGHVSGDn and hGHVSGsp showed staining patterns indicative of intracellular retention. To test for the presence of GPI-anchored hGH, we incubated the cells with PIPLC and measured the released hGH by an ELISA (Table 1). We observed significant PIPLC-dependent release of hGHDAF and hGHDAFVn, indicating that these proteins are GPI anchored on the cell surface; no release was observed with cells expressing hGHVSGDn or hGHVSGsp, suggesting that these proteins are not GPI linked. To confirm this, we immunoprecipitated the proteins from cells labeled with [3H]ethanolamine (Fig. 7). Of the fusion proteins containing a hybrid GPI signal, only hGHDAFVn was strongly labeled with [3H]ethanolamine, verifying the presence of a GPI anchor. The [3H]ethanolamine-labeled band corresponding to hGHDAFVn was similar in intensity to that of hGHDAF, indicating that the COOH terminal hydrophobic domains from DAF and VSG are completely interchangeable. In contrast, hGHVSGDn failed to become GPI linked, suggesting that the parasite cleavage/attachment site is not functional in mammalian cells. Since hGHVSGsp also failed to become GPI linked, we conclude that the defect is caused by an intrinsic property of the cleavage site itself rather than to a spatial misalignment of the cleavage site relative to the hydrophobic domain.

**Conversion of the VSG GPI Signal to a Mammalian GPI Signal by Substitution Mutagenesis**

The above results suggest that the VSG cleavage/attachment site is not compatible with the putative mammalian GPI transamidase. To test this conclusion and to better understand how the mammalian and parasite signals differ, we asked whether specific amino acid substitutions in the VSG GPI signal could produce a viable GPI signal for mammalian cells. The three residues immediately surrounding the cleavage point, termed the ω, ω+1, and ω+2 residues (see Fig. 8 A), are thought to be important for correct processing and GPI attachment (Kodukula et al., 1993). Using hGHVSG as the reference protein, we replaced these three residues in the VSG GPI signal (Asp-Ser-Ser) with the corresponding three residues from the DAF GPI signal (Ser-Gly-Thr), and termed the mutated fusion protein, hGHVSGDn (Fig. 8 A). (Note that the three-letter subscript denotes the residues in the ω, ω+1, and ω+2 positions. For reference, hGHVSG corresponds to hGHVSGDn).

We also tested the effect of replacing only the ω and ω+1 residues of VSG (Asp-Ser) with the corresponding DAF sequence (Ser-Gly), while keeping serine in the ω+2 position, and termed this protein hGHVSGn (Fig. 8 A). Studies using alkaline phosphatase have suggested that a Ser residue in the ω+2 position, as occurs in the VSG sequence, can be detrimental for GPI addition in COS cells. To test if this might be the cause of the impaired activity of the VSG signal, we substituted Ala in place of Ser in the ω+2 position, while keeping the ω and ω+1 residues unaltered, and termed this protein hGHVSGan (Fig. 8 A).

In contrast to the reference protein hGHVSG (hGHVSGDn), all three of the mutated fusion proteins were efficiently targeted to the cell surface after transfection in COS cells, as evidenced by strong surface staining with an anti-hGH antibody (Fig. 8 B). Treatment of the cells with PIPLC resulted in significant release of hGH in all three cases, indicating that these fusion proteins are GPI anchored (Table 1). In addition, all three mutated proteins were strongly labeled with [3H]ethanolamine (Fig. 9), confirming the presence of a GPI anchor. We conclude from these results that the VSG GPI signal can be converted to a viable signal for mammalian cells by altering the amino acid configuration at the cleavage/attachment site.

**Malaria Circumporozoite Protein and GPI Anchoring**

One of the goals of this study was to test whether the CS antigen, found on the surface of malaria parasite sporozoites (Nussenzweig and Nussenzweig, 1985), is GPI anchored. Inspection of the sequence suggests that this is the case, although direct evidence is lacking. To potentially provide such evidence, as well as to test whether mammalian cells can process the GPI signal from a parasite other than trypanosomes, a cDNA encoding the CS protein of the rodent parasite *P. berghei* (Eichinger et al., 1986) was cloned into a mammalian expression vector and transiently ex-
pressed in COS cells. As has been observed by other groups 
(Adams et al., 1992), the expressed CS protein, which is 
normally transported to the cell surface in micronemes (in-
vasion organelles), remained trapped in internal organelles 
(data not shown). Therefore, to avoid trafficking difficulties 
caused by the protein ectodomain, we constructed an 
hGHCS fusion protein in which the last 28 residues of the 
CS protein, containing the putative GPI signal, were fused 
to the COOH terminus of hGH (Fig. 8 A). After expression 
in COS cells, the immunofluorescent staining pattern sug-
gested that most of the hGHCS is retained intracellularly, al-
though we detected low levels on the surface of transfected 
cells (Fig. 10). Although our ELISA was not sensitive 
enough to measure release of hGHCS after treatment of the
Table I. PIPLC-sensitivity of hGH Fusion Proteins, Determined by ELISA of hGH in Supernatants from Transfected COS Cells Incubated With or Without PIPLC

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Fusion protein</th>
<th>-PIPLC</th>
<th>+PIPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>hGHDAF</td>
<td>2.4</td>
<td>20.7</td>
</tr>
<tr>
<td></td>
<td>hGHVSG</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td></td>
<td>hGHVSG.Dh</td>
<td>2.2</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td></td>
<td>hGHDAF.Vh</td>
<td>1.0</td>
<td>17.3</td>
</tr>
<tr>
<td></td>
<td>hGHVSGsp</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>2</td>
<td>hGHDAF</td>
<td>3.7</td>
<td>46.3</td>
</tr>
<tr>
<td></td>
<td>hGHVSG</td>
<td>&lt;1.0</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>hGHVSG.DO</td>
<td>&lt;1.0</td>
<td>14.6</td>
</tr>
<tr>
<td></td>
<td>hGHVSG.OD</td>
<td>2.2</td>
<td>23.6</td>
</tr>
<tr>
<td></td>
<td>hGHVSGDAS</td>
<td>&lt;1.0</td>
<td>18.1</td>
</tr>
<tr>
<td></td>
<td>hGHVSCS</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>

Transfected COS cells grown on 60-mm dishes were removed with 7 mM EDTA in PBS and resuspended in 200 μl 10% fetal calf serum in PBS. All aliquots of 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.

Discussion

Based on our analysis of the DAF GPI signal, we previously 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 to signal GPI attachment in mammalian cells. A similar analysis using placental alkaline phosphatase arrived at similar conclusions, except that the cleavage/attachment site was found to be comprised of a domain of three small residues, including two residues on the COOH-terminal side of the anchor addition site rather than just one (Kodukula et al., 1993). Inspection of the COOH-terminal sequences of various VSGs from the African trypanosomes, T. brucei or Trypanosoma congolense (Cross, 1990), suggests that the parasite GPI signals obey similar rules, leading to the prediction that VSG would be processed to a GPI-linked protein in mammalian cells. In the present study, we tested this prediction by expressing a VSG of T. brucei in COS cells. Cell surface expression, PIPLC-mediated release, and metabolic labeling with [3H]ethanolamine were used as criteria of GPI anchoring. Surprisingly, our data show that VSG is not efficiently processed to a GPI-linked protein in COS cells, suggesting that the VSG GPI signal functions poorly in mammalian cells. To verify this and rule out potential difficulties caused by the VSG ectodomain, we showed that (a) replacement of the VSG GPI signal with the DAF GPI signal produces high levels of GPI-anchored VSG, and (b) an hGHVSG fusion protein containing the VSG GPI signal is processed poorly compared to hGHDAF, which produces high levels of GPI-anchored hGH. These results confirm that the VSG GPI signal is indeed poorly recognized in mammalian cells.

The VSG Cleavage/Attachment Site is Incompatible with the Mammalian Machinery for GPI Attachment

Our data show that a chimeric GPI signal composed of the COOH-terminal hydrophobic domain from VSG combined with the cleavage/attachment site and spacer sequences from DAF, is as efficient as the DAF GPI signal in targeting hGH to the cell surface via a GPI anchor. We conclude from this result that the VSG and DAF hydrophobic domains are completely interchangeable. In contrast, a chimeric GPI signal containing the DAF hydrophobic domain combined with the cleavage/attachment site and spacer sequences from VSG, failed to produce significant amounts of GPI-anchored hGH when fused to the COOH terminus of hGH. To rule out the possibility that this is caused by the difference in length between the VSG and DAF spacer sequences—potentially resulting in misalignment of the processing site in the GPI signal with the active site of the putative GPI transamidase—we added three hydrophobic residues to the VSG spacer sequence, thereby equalizing the distance between the processing site and hydrophobic domain in the VSG and DAF GPI signals. This altered VSG signal was no better than the authentic VSG signal in directing GPI attachment. There-
Figure 8. (A) Schematic diagram showing the COOH-terminal sequences of hGHVSG fusion proteins containing amino acid substitutions at the cleavage/attachment site. The COOH terminus of the hGHCS fusion protein is also shown. Small print, hGH COOH terminus; boxed sequence, COOH-terminal hydrophobic domain; the \( \omega \), \( \omega+1 \), and \( \omega+2 \) residues are indicated in bold print and the anchor-addition site (\( \omega \)) is underlined. In the case of hGHCS, the anchor-addition site is not known; therefore, the underline indicates a probable addition site. (B) Immunofluorescent labeling of transfected COS cells expressing hGHVSG fusion proteins containing substitutions at the cleavage/attachment site as indicated. Shown are intact, nonpermeabilized cells, labeled as described in Methods. Staining of permeabilized cells (not shown) indicated that all four proteins were strongly expressed.
Figure 9. [3H]Ethanolamine labeling and immunoprecipitation of hGHVSG fusion proteins with substitutions at the cleavage/attachment site as indicated, and of the hGHCS fusion protein. Transfected COS cells were labeled as described in the Fig. 2 legend. The proteins were immunoprecipitated from cell lysates using a purified goat antibody to hGH.

Therefore, we concluded that the failure to direct GPI attachment in COS cells can be attributed to the VSG cleavage/attachment site.

Amino Acid Requirements of the Cleavage Site

The confirm the above conclusion, we replaced either a three-residue (Asp-Ser-Ser) or a two-residue (Asp-Ser) sequence at the cleavage/attachment site of the VSG signal (i.e., the \(\omega, \omega+1\) and \(\omega+2\) positions, or the \(\omega\) and \(\omega+1\) positions only) with the corresponding sequences from the DAF GPI signal (Ser-Gly-Thr or Ser-Gly) (Fig. 8 A). Both of these changes converted the inactive VSG GPI signal to a viable signal for mammalian cells. In addition, a single substitution replacing Ser with Ala at the \(\omega+2\) position overcame the defect and produced a viable GPI signal. Superficially, these results are contradictory; i.e., if only the \(\omega\) and possibly \(\omega+1\) positions need be replaced, Ser in the \(\omega+2\) position is by inference not detrimental; yet, the latter result suggests that the defective processing in COS cells is caused by the Ser residue in the \(\omega+2\) position. These results can be reconciled by assuming that there is cooperativity between the residues surrounding the processing site. For example, when Ser is in the \(\omega\) position as in the DAF sequence, \(\omega+2\) is relatively promiscuous, tolerating the presence of Ser or Thr in addition to Ala or Gly, the most common residues found at this position. When Asp is in the \(\omega\) position as in the VSG sequence, \(\omega+2\) is more restricted, tolerating Ala but not Ser (see Fig. 11). This view also reconciles our earlier results using DAF with those of Kodukula et al. 1993, using alkaline phosphatase (PLAP). The latter study showed that the sequences Asp-Ala-Ala and Asp-Ala-Gly at the cleavage site (Fig. 11) yield GPI-anchored PLAP, whereas Asp-Ala-Ser and Asp-Ala-Thr do not. Although a detailed understanding of these results will have to await isolation of the transamidase and elucidation of its structure, a simplistic interpretation is that it's a matter of size. It's not unreasonable to speculate that the binding pocket of the mammalian transamidase is large enough to accommodate two Ser residues or a Ser plus Thr in the \(\omega\) and \(\omega+2\) positions, respectively; however, if the \(\omega\) position is occupied by the slightly larger Asp, then to compensate, the \(\omega+2\) residue must be smaller, allowing only Ala or Gly. If this hypothesis turns out to be correct, it is logical to infer that the binding pocket of the trypanosome transamidase is slightly larger than its mammalian counterpart, being able to accommodate Ser in the \(\omega+2\) position when the \(\omega\) position is occupied by Asp, which the mammalian enzyme cannot do (see Fig. 11). This small but critical difference may present an opportunity for the design of small molecule inhibitors that might effectively block GPI attachment in trypanosomes and hence be lethal to this organism, but are too large to enter the binding pocket of the mammalian enzyme, and hence, leave mammalian GPI attachment intact.

The Malaria Circumsporozoite Protein

The sequence of the malaria CS protein predicts that it is GPI
linked. However, it has not hitherto been possible to test this because of practical difficulties in working with malaria parasites. After expression in COS cells, the full-length CS protein was blocked in transport to the cell surface, remaining instead in internal cell organelles. This transport block could be caused by the ectodomain of the CS protein, which normally traffics to the cell surface in micronemes (Adams et al., 1992). Thus, to circumvent difficulties caused by the ectodomain, we used only that portion of the CS protein encoding the putative GPI signal, fused to the COOH terminus of a normally secreted protein, hGH. We observed low cell surface expression and weak $[^3H]$ethanolamine labeling of the hGHCS fusion after expression in COS cells. However, since we do not detect any immunoprecipitable $[^3H]$ethanolamine-labeled bands in mock-transfected cells or in cells expressing secreted hGH, we interpret the latter result as being significant, suggesting that the COOH terminus of the CS protein does indeed contain a GPI signal, albeit one that is processed poorly in mammalian cells. This conclusion is supported by the observation that rather than being secreted, most of the hGHCS was retained in the cells. Previous reports using hGHDAF fusion proteins suggest that an uncleaved GPI signal acts as a retention signal in mammalian cells (Moran and Caras, 1992); retention of the unprocessed hGHCS protein is thus consistent with the presence of an uncleaved GPI signal (uncleaved because it is not recognized in mammalian cells). The CS GPI signal contains a possible cleavage/attachment site comprised of Cys-Ser-Ser (Fig. 8A). Our results are, therefore, consistent with the “size exclusion hypothesis” outlined above, which predicts that this hypothesis is purely speculative at present, one might speculate further that the malaria GPI transamidase is more like the trypanosome enzyme than its mammalian counterpart, having a slightly larger binding pocket. Thus selective inhibitors of the trypanosome GPI transamidase might also be effective in blocking the malaria transamidase. We considered attempting to confirm the existence of a GPI signal in the CS protein by introducing mutations in the region of the cleavage site and asking if, by analogy with VSG, this produces a viable GPI signal in COS cells. However, since we have previously shown that irrelevant sequences from nonanchored proteins can produce a viable GPI signal by these means (Moran and Caras, 1991b), a positive result cannot be taken as proof that the unaltered CS sequence normally functions in GPI anchoring. Further verification of the CS GPI signal will have to await the development of suitable transfection systems for parasitic protozoa.

Received for publication 3 December 1993 and in revised form 31 January 1994.

References


