Analysis of the Sequence Requirements for Glycosylphosphatidylinositol Anchoring of *Saccharomyces cerevisiae* Gas1 Protein*

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The *Saccharomyces cerevisiae* Gas1 protein is synthesized as a precursor with a hydrophobic extension at the carboxyl terminus which is removed and replaced with an inositol containing glycolipid that anchors the protein to the plasma membrane. We performed saturation mutagenesis on the anchor attachment site (Asn<sup>508</sup>) and showed that only a subset of amino acids with small side chains could act as substrates for peptide cleavage and glycolipid addition. After Asn, which is the most efficient anchor attachment site, Ser, Gly, Ala, Asp, and Cys function with decreasing effectiveness. Mutational analysis also revealed that the 2 adjacent amino acids to the carboxyl side of the anchor attachment site are important for efficient anchoring. These two amino acids should have relatively short side chains with the second position being more critical. Analysis of the region between the anchor attachment site and the carboxyl-terminal hydrophobic region indicated that this region may not simply perform a spacer function.

A large number of eukaryotic proteins are anchored to the membrane via a covalently attached glycosylphosphatidylinositol (GPI)<sup>1</sup> moiety. The process of GPI anchoring is highly conserved, occurring in *protozoa*, *Dictyostelium*, *Saccharomyces*, and various animal cells (see Cross (1990), for review). In fact, the analysis of the structure of GPI anchors from *protozoa* (Ferguson et al., 1988) and animal cells (Homans et al., 1988) has shown that they all contain a common core structure consisting of lipid-linked inositol, glucosamine, three mannoses, and ethanolamine phosphate in an amide linkage to the COOH terminus of the protein. All GPI anchored proteins described thus far contain 2 hydrophobic amino acid stretches, one near the NH<sub>2</sub>-terminal side and another one at the COOH terminus. Both hydrophobic regions are removed rapidly from the newly synthesized protein (Bangs et al., 1985; Conzelmann et al., 1987) and a preformed GPI is attached to the newly exposed COOH terminus (Masterson et al., 1989; Menon et al., 1990). GPI anchoring is known to occur in the ER because of the rapidity of the process and because anchoring still occurs at nonpermissive temperature in the yeast sec18 mutant (Conzelmann et al., 1988) which is blocked in protein transport from the ER to the Golgi (Novick et al., 1980). *In vitro* studies using a recombinant placental alkaline phosphatase miniprotein suggested that preformed GPI is an obligatory co-substrate for COOH-terminal processing (Kodukula et al., 1992). Analysis of the structural requirements for GPI anchoring have shown that a COOH-terminal hydrophobic region is necessary (Caras et al., 1987; Caras et al., 1989). It is apparently the hydrophobic character of the region and not a specific sequence that is required for GPI anchoring because other unrelated hydrophobic regions can substitute (Caras and Weddell, 1989) and because introduction of a single charge into the middle of the hydrophobic domain blocks GPI anchoring (Nuoffer et al., 1991). Comparison of known cleavage/attachment sites (called ω sites by Gerber et al. (1992)) and mutagenesis studies on two mammalian proteins (Micanovic et al., 1990; Moran et al., 1991) have shown that only 5 or 6 amino acids with small side chains can act efficiently as ω sites. Two other studies (Moran and Caras, 1991; Gerber et al., 1992) showed that the amino acids at positions ω + 1 and ω + 2 are important determinants and should be amino acids with small side chains. In addition, Moran and Caras (1991) suggested that the ω site must be to the NH<sub>2</sub>-terminal side and placed 10-12 amino acids from the hydrophobic domain. Gerber et al. (1992) found that amino acids with large side chains are tolerated less well at the ω + 2 than the ω + 1 position. The detailed studies of the sequence requirements for GPI anchoring were all performed on two mammalian proteins. It is necessary to examine these requirements on a protein from an unrelated organism. First, clearly more examples are needed before rules for GPI anchoring can be established. Second, if small differences are found between organisms these differences could possibly be exploited to develop specific inhibitors of GPI anchoring to be used as antimicrobials.

In this study we examined the sequence requirements at the ω + 1, and ω + 2 sites on the *S. cerevisiae* Gas1 protein by site-directed mutagenesis and began to address the question of spacing between the ω site and the COOH-terminal hydrophobic domain. Consistent with findings in animal cells, only a small subset of amino acids can work at the ω site even though we found larger differences between the efficiency of GPI anchoring with acceptable amino acids. In agreement with Gerber et al. (1992), we also find that amino acids with...
large side chains are tolerated less well at the ω + 2 than at the ω + 1 site. Our data suggest that the region between the ω site and the COOH-terminal hydrophobic domain is not simply a spacer, because an internal deletion of 1 amino acid in this region produces a substrate that is less efficiently GPI-anchored than when 2 amino acids are deleted.

**EXPERIMENTAL PROCEDURES**

**Construction of Mutant gas1 Alleles—**A series of gas1 ω site variants were constructed using a cassette mutagenesis approach (Wells et al., 1985). A SalI-HindIII fragment containing both a BglII site at position 1975 and a BamHI site at position 1996 was constructed as described (Nuoffer et al., 1991). In addition to these two sites, the resulting sequence comprises at position 1972 a new XbaI site that is sensitive to dam methylation. The SalI-HindIII fragment was then inserted into pBR322 and was propagated in the Escherichia coli dam strain B237 (from S. Kvit, Stockholm) to permit XbaI restriction.

The XbaI-BamHI fragment was excised and a mutagenic DNA cassette with a degerganger codon/anticodon for position 506 of Gaslp was inserted into the gap. This cassette was assembled by annealing two complementary oligonucleotides, which were designed to form a duplex with single-stranded overlaps compatible for ligation into the XbaI and BamHI ends of the vector. The A at position 1977 was replaced with a C to eliminate the XbaI and BglII sites, and to restore the wild-type Ser residue at position 506 of Gaslp. Mutant clones were identified by DNA sequencing, and the recombinant HindIII- or SalI-HindIII fragments were cloned back into the CEN vector carrying the GAS1 gene, pCNCG (Nuoffer et al., 1991). The same techniques were used to create the ω site deletion mutant (Aω^66) by using a cassette lacking nucleotides encoding position 506 of Gaslp.

All other mutants were constructed using the polymerase chain reaction. The reactions were performed using T35 DNA polymerase according to the instructions of the supplier (Perkin-Elmer Cetus). For some mutations a SacI site was introduced at position 1981 which results only in silent mutations. The polymerase chain reactions were performed with a mutagenic sense oligonucleotide encompassing either the BamHI site in the SacI site and the desired mutation and an antisense oligonucleotide complementary to a vector sequence located 5' to the HindIII site. The polymerase chain reaction products were purified by agarose gel electrophoresis and ligated into the EcoRV site of pBlueScript II (Stratagene). The inserts were sequenced and appropriate fragments (SacI by HindIII or BarnHI by HindIII) were cloned back into pCNCG templates. Information on the precise codon changes made can be obtained upon request.

**Analysis of Mutant gas1 Alleles—**Mutant gas1 alleles constructed in plasmid pCNCG (CBN-ARS, URA3) were introduced into the strain RH273-1A (MATa, urd-1, ura3, leu2, bar-1, gas1::LEU2) and precultures were grown in selective medium (Dulic et al., 1991). Precultures were used to inoculate overnight cultures in YPD medium with 10% trichloroacetic acid, washed with cold acetone, and exposed to x-ray film (Kodak X-AR5). Alternatively, the protein pattern on the nitrocellulose was revealed using Ponceau S and the sheet was cut into two halves. The filters were treated with TBS (bovine serum albumin 50 mM Tris-HCl, 150 mM NaCl, 30 mg/ml bovine serum albumin) for at least 30 min at room temperature and then washed with TBS. One filter was treated with 80 units/ml phospholipase C from Bacillus cereus (Sigma) diluted into TBS and the other half was mock treated under the same conditions for 90 min at 37 °C. The filter was then washed with TBS and treated again with TBS/bovine serum albumin. The filter was then decorated following a standard Western blotting protocol with rabbit anti-CRD, followed by goat anti-rabbit IgG coupled to a peroxidase. The peroxidase was revealed using chemiluminescence with ECL. The soluble and membrane forms of variant surface glycoprotein from trypanosomes and the anti-CRD were kindly provided by M. Lucido de Almeida (Sao Paolo, Brazil).

**RESULTS**

The S. cerevisiae Gas1 protein (Gaslp) is a typical GPI-anchored protein with a cleavable hydrophobic signal sequence for import into the ER and a COOH-terminal hydrophobic stretch. The latter hydrophobic region is part of the 31 amino acids that are removed form the protein when GPI is added to the asparagine at residue 506 (Fig. 1) (Nuoffer et al., 1991). To facilitate the construction of mutant alleles of gas1, a BamHI site was introduced by site-directed mutagenesis converting the codons for A507 and A508 to codons for G507 and S508. These changes did not effect GPI anchoring when the ω site was asparagine (Nuoffer et al., 1991). Nω^66 was then mutated to encode each of the 19 other amino acids. To determine which amino acid residues can act as a cleavage/attachment site (ω site), the mutant gas1 alleles were subcloned into a centromere containing vector and expressed from their natural promotor in the strain RH273-1A (gas1::LEU2) that carries a deletion in the GAS1 gene and therefore has no endogenous Gaslp expression from its chromosomal GAS1 gene (Nuoffer et al., 1991). The cells were grown overnight in YPD medium, harvested, and total proteins were extracted and analyzed by Western blotting using antibodies raised against Gaslp. When the ω site is asparagine (wild-type), one detect almost exclusively the 125-kDa fully glycosylated Gaslp (Fig. 2). There is a very slight amount of a 105-kDa species of the protein that comigrates with the core glycosylated protein when it is in the ER (Pankhauser and Conzelmann, 1991). When the gas1-I507 and gas1-S508 alleles are expressed, the pattern is similar with a small increase in the amount of the 105-kDa band. The gas1-A507 allele leads to the expression of about equal amounts of 105- and 125-kDa Gaslp, whereas only a small fraction of the Gaslp encoded by the D506 and C506 alleles is in the 125-kDa form. All of the other mutant gas1 alleles lead to almost exclusive synthesis of a 105-kDa species. This would suggest that only 6 amino acids at the ω site, N, S, G, A, D, and C, lead to

![Fig. 1. COOH-terminal sequence of the Gas1p precursor.](https://example.com/fig1.png)

The COOH-terminal sequence of the Gas1p precursor. The presence of the COOH-terminus of the Gas1p precursor is shown starting with amino acid 498 (Nuoffer et al., 1991). The cleavage/attachment site (ω site), Nω^66, is shown as a large character. The COOH-terminal hydrophobic stretch of amino acids is underlined.
substantial maturation of Gaslp with N, S, and G being more efficient than the others.

The lack of maturation of Gaslp could be due to a defect in GPI anchoring and a consequent lack of transport from the ER to the Golgi (site of carbohydrate chain elongation) or simply a lack in transport. Therefore, we tested whether the mutant Gaslp proteins were GPI-anchored. GPI-anchored Gaslp partitions into the detergent phase after Triton X-114 extraction (Nuoffer et al., 1991). Δgasl::LEU2 cells carrying mutant Gaslp alleles on a plasmid were grown overnight, the 105-kDa species detected in Triton X-114 was not efficiently revealed by this treatment. The filter was treated with PI-PLC, whereas the mutant Gaslp that was detected by this treatment corresponded in mobility to the 125-kDa species detected in Triton X-114 extraction. Gaslp was detected by Western blotting. As expected, the wild-type Gaslp (N506) partitioned into the detergent phase after Triton X-114 extraction (Fig. 3). Mutant Gaslp1 proteins that showed poor maturation (Q506) showed a different behavior. The 105-kDa species partitions approximately equally between detergent and aqueous phases. In addition, a small amount of Gaslp1 can be found secreted into the medium. Gaslp that showed partial maturation (D506) shows a complex fractionation. Most of the matured Gaslp1 is found in the detergent phase, whereas the immature protein partitions about equally between the detergent and aqueous phases. The analysis of all of the other mutant Gaslp mutants gave similar results to those presented with the above mutants (data not shown). Although these results suggest that the extent of Gaslp maturation reflects the extent of GPI anchoring the data remain open to alternative interpretations.

To obtain a more definitive answer whether the 105-kDa species contains a GPI-anchor, we used an antibody that reacts specifically with GPI-anchors. Upon lysis of trypanosomes, a variant surface glycoprotein (VSG) is converted from a membrane form (mfVSG) to a soluble form (sfVSG) by the action of an endogenous phospholipase (Cardoso de Almeida and Turner, 1983). Different VSGs all react with a common antibody that is directed against a CRD whose overlapping epitopes involve carbohydrates of the anchor and the inositol 1,2-cyclic phosphate formed by the action of phospholipase C (Zamze et al., 1988). Gaslp reacts with this antibody when the protein was treated with phospholipase C (Conzelmann et al., 1988). Total protein extracts from cells expressing wild-type and Q506 Gaslp were separated by SDS-PAGE and transferred to a nitrocellulose membrane in duplicate. As controls, sfVSG and mfVSG were run alongside Gaslp. The membrane was then treated with PI-PLC, incubated with anti-CRD, followed by goat anti-rabbit IgG coupled to peroxidase, and CRD was detected by subsequent chemiluminescence. As expected, sfVSG reacted with anti-CRD independent of PI-PLC treatment, whereas mfVSG was only recognized after PI-PLC treatment (Fig. 4). As shown previously, wild-type Gaslp1 (N506) reacted strongly with anti-CRD only when the filter was treated with PI-PLC, whereas the mutant Gaslp1 (Q506) was not efficiently revealed by this treatment. The small amount of mutant Gaslp1 that was detected by this procedure corresponded in mobility to the 125-kDa species and probably represents a small percentage of molecules that were anchored despite the mutation. These results show that the 105-kDa species detected in gasl mutants is not GPI-anchored, even though it does comigrate in SDS-PAGE with the GPI-anchored ER-localized biosynthetic intermediate (Fig. 2) (Fankhauser and Conzelmann, 1991). This allows us to quantify the efficiency of anchoring in gasl mutants by determining the percentage of the protein that is in the fully glycosylated (125 kDa) form.

In addition to the missense mutations that were introduced at the codon for N506, this codon was deleted. Mutant Gaslp1 (Δ506) was expressed in Δgas1::LEU2 cells, the cells were extracted, and Gaslp was analyzed by Western blotting. The mutant Gaslp1 (Δ506) was only slightly matured and therefore,
there is some other inhibitory effect of the $\Delta^{506}$ mutation on GPI anchoring of Gaslp, perhaps due to the alteration of the distance between the $\omega$ site and the COOH-terminal hydrophobic amino acid stretch.

To test the effect of changes in the distance between the $\omega$ site and the COOH-terminal hydrophobic domain on the GPI anchoring of Gaslp, five additional mutants were created starting from the $G^{506}$ allele. We used this allele rather than the $N^{506}$ allele because mutations created downstream of the $\omega$ show stronger phenotypes when the $\omega$ site is G than when it is N (see below). A T or TN was inserted at position $\omega + 3$ (+T$^{+3}$, +TN$^{+3}$), the T at $\omega + 3$ was removed (−T$^{-3}$), the N at position $\omega + 4$ was removed (−N$^{-4}$), or the both of the latter 2 amino acids were removed (−TN$^{-3,4}$). All of these mutations caused defects in Gaslp maturation, but to varying degrees (Fig. 6). Addition of 1 amino acid (−T$^{+3}$) inhibited GPI anchoring of Gaslp significantly (36% maturation) while adding 2 amino acids (+TN$^{+3}$) inhibited this process more strongly (7% maturation). Removal of 1 amino acid, be it the T or N at positions $\omega + 3$ or $\omega + 4$, respectively, caused a strong defect in GPI anchoring of Gaslp (14 and 7% maturation, respectively). Surprisingly, when both of these amino acids were removed, GPI anchoring of Gaslp was not inhibited as strongly (54% maturation) as when either of the single amino acids were removed. These latter results suggest that it is not simply the number of amino acids that are contained between the $\omega$ site and the COOH-terminal hydrophobic domain that is determinant for the function of this region.

To exclude that the above results were due to the presence of a mutant $\omega$ site we also created the +T$^{+3}$ and −T$^{-3}$ mutations where S or N is the $\omega$ site amino acid. When S is the $\omega$ site, the +T$^{+3}$ and −T$^{-3}$ mutations showed 83 and 53% maturation, respectively. When N, the wild-type residue, is the $\omega$ site the +T$^{+3}$ and −T$^{-3}$ mutations showed 99 and 65% maturation, respectively. With all 3 $\omega$ site amino acids the tendency is the same. The −T$^{-3}$ mutation gives a more severe phenotype than the +T$^{+3}$ mutation, suggesting that the phenotype obtained by insertion or removal of a T at position +3 is independent of the $\omega$ site amino acid.

To explore whether there are strict primary structural requirements in the region between the $\omega$ site and the COOH-terminal hydrophobic region we constructed mutations in this region and analyzed the behavior of the mutant proteins as above (see Table I). N$^{+10}$ was changed to K or T and K$^{+2}$ was changed to E or A. Neither mutation of N$^{+10}$ had a substantial effect on Gaslp maturation, including the introduction of a charged lysine residue. Changing K$^{+2}$ to a residue of opposite charge (E) or to a neutral amino acid had the same effect, resulting in a partial defect in GPI anchoring of Gaslp. These results suggest that the function of this region does not depend heavily on its primary structure.

In order to test the importance of the $\omega + 1$ and $\omega + 2$ sites...
TABLE I

Mutations in the "spacer" region

The mutations described below were introduced into the GAS1 gene, which was expressed in a gas1 mutant strain (RH273-1A). The percentage of Gaslp that was matured was determined by Western blotting as described under "Experimental Procedures." The results from the indicated number of experiments were averaged. Sometimes only two experiments were averaged if the results were very similar.

| Sequence starting at 506 | Maturation | No. of determinations |
|--------------------------|------------|-----------------------|
| GGSTNVK...              | 89%        | 8                     |
| GGSTTKV...              | 90%        | 2                     |
| GGSTTVK...              | 91%        | 2                     |
| GGSTNVE...              | 61%        | 4                     |
| GGSTNVA...              | 65%        | 4                     |

TABLE II

Mutations at the ω + 1 and ω + 2 sites

The mutations described below were introduced into the GAS1 gene, which was expressed in a gas1 mutant strain (RH273-1A). The percentage of Gaslp that was matured was determined by Western blotting as described under "Experimental Procedures." The results from the indicated number of experiments were averaged. Sometimes only two experiments were averaged if the results were very similar. NAA is the wild-type sequence.

| Sequence starting at 506 | Maturation | No. of determinations |
|--------------------------|------------|-----------------------|
| NAA                      | 99%        | 4                     |
| NGG                      | 99%        | 2                     |
| NAG                      | 99%        | 2                     |
| NGA                      | 99%        | 2                     |
| NAS                      | 99%        | 2                     |
| NSA                      | 99%        | 2                     |
| NTA                      | 99%        | 2                     |
| NTC                      | 99%        | 2                     |
| NTS                      | 99%        | 2                     |
| NST                      | 97%        | 2                     |
| NGS                      | 99%        | 2                     |
| NGS                      | 99%        | 5                     |
| NAT                      | 97%        | 3                     |
| NAT                      | 97%        | 2                     |
| NAL                      | 69%        | 2                     |
| NLA                      | 96%        | 2                     |
| NAV                      | 87%        | 2                     |
| NVA                      | 94%        | 2                     |
| GAA                      | 95%        | 2                     |
| GAS                      | 77%        | 4                     |
| GSA                      | 94%        | 2                     |
| GAT                      | 24%        | 3                     |
| GTA                      | 95%        | 2                     |
| GST                      | 22%        | 6                     |
| GTS                      | 70%        | 3                     |
| GGA                      | 97%        | 3                     |
| GGS                      | 89%        | 8                     |
| GGT                      | 77%        | 4                     |

several mutations were introduced at these positions into the N<sup>506</sup> and G<sup>506</sup> alleles. After expression in the Δgas1::LEU2 strain the percentage of mature Gaslp was determined by Western blotting. The Western blots were quantified by densitometry and the results are shown (Table II). Analysis of these mutants leads to several conclusions. N<sup>506</sup> is a better ω site amino acid in Gaslp than is G<sup>506</sup> with any possible equivalent variations at the ω + 1 and ω + 2 positions. Amino acids with small side chains are preferred at positions ω + 1 and ω + 2 (compare GAA, GAS, GAT; GAS, GTS) even though the requirement is less strict for the ω + 1 position (compare GTA, GAT; GTS, GST; NLA, NAL). The combination of amino acids at the ω + 1 and ω + 2 positions may also to be crucial. Introduction of a relatively large amino acid at ω + 2 can be tolerated if the amino acid at ω + 1 is very small (compare GAT, GGT, and GST). The opposite is probably also true (compare GTA, GTS; GAS, GGS).

DISCUSSION

In this paper we have investigated the primary structural requirements for GPI anchoring at and near the cleavage/attachment site (ω site) of the S. cerevisiae Gas1 protein. Our measurement of anchoring efficiency relied on the demonstration that, when unanchored, Gaslp remains in a 105-kDa form. This 105-kDa species comigrates with the ER form of the protein (Fankhauser and Conzelmann, 1991), but almost certainly still retains its hydrophobic COOH-terminal peptide sequence. This conclusion can be drawn from the fact that the protein that did not receive a GPI-anchor was not efficiently secreted. Removal of the hydrophobic COOH-terminal region by site-directed mutagenesis led to secretion of Gaslp (Nuoffer et al., 1991).

The size of the unanchored form of Gaslp suggests that transport of the protein is blocked in the ER. While we have no direct evidence for this, it would be consistent with the results found with decay accelerating factor (DAF) and placental alkaline phosphatase (Micanovic et al., 1990). Two separate hypotheses could explain this block in transport. The GPI anchor could be necessary for transport of this class of proteins from the ER to the Golgi or the COOH-terminal hydrophobic sequence when not removed may retain the protein in the ER. If the latter hypothesis is correct this could reflect the natural function of this domain. It could function to retain the protein in the ER until it can be recognized and processed by the GPI anchoring machinery.

Using saturation mutagenesis of the ω site of Gaslp we showed that only amino acids with small side chains (N, S, G, A, D, and C) could act as cleavage/attachment sites. N, the wild-type ω site, was clearly the best while S and G were approximately equal and clearly more effective ω site amino acids than A, D, and then C. This is similar to but not identical with the findings on two human cell proteins where a systematic analysis was performed. For DAF (Moran et al., 1991), 5 amino acids (S, A, D, N, and G) gave relatively high levels of GPI-anchored DAF, while 2 amino acids (V and E) gave low, but detectable levels of GPI-anchored DAF. From their data it is not possible to determine whether there are quantitative differences between the efficiencies of the 5 amino acids that gave high levels of GPI-anchored DAF. For placental alkaline phosphatase (Micanovic et al., 1990), 6 amino acids were also suitable ω sites for GPI anchoring (D, G, A, C, S, and N) with N and S being approximately equal and the best, followed by D, G, and A, which also showed about equal activity, followed by C. From the analysis of the ω sites from human and yeast cells we can conclude that only amino acids with small side chains can act as suitable cleavage/attachment sites. The preference for a particular amino acid may depend on the protein being investigated and not the cell type, because GPI anchoring of DAF and placental alkaline phosphatase were investigated in the same cell type (Micanovic et al., 1990; Moran et al., 1991). It is not clear what structural features of the protein determine the ω site preference. It is unlikely to be the amino acids at the ω + 1 or ω + 2 sites because all the variants we tried at these positions still showed a preference for N as ω site amino acid. It will be necessary to compare the analyses of more ω sites before we can determine the structural information that confers ω site specificity. In any event, the high degree of conservation of ω site specificity suggests that the active site of the putative transamidase has
been highly conserved between yeast and humans.

Analysis of the sequences downstream from the Gas1p ω site suggest that the ω + 1 and ω + 2 positions are also important. The ω + 1 position of Gas1p can tolerate fairly large amino acids with only a small reduction of GPI anchoring efficiency. This is entirely consistent with the results found from the analysis of the same sites on placental alkaline phosphatase (Gerber et al., 1992). Even though small amino acids are preferred at the ω + 1 site, they are not essential. Indeed, in the case of human Thy-1 an E residue is found at this position naturally (Seki et al., 1985). At the ω + 2 position of Gas1p, large amino acids are less well tolerated than at the ω + 1 position. When T was placed at position ω + 2 of the gas1-G706 allele a reduction in the efficiency of GPI anchoring was seen. However, with the optimal ω site amino acid (N508) a T at the ω + 2 position was well tolerated. This is consistent with the finding that T occupies the natural ω + 2 position in DAF (Moran et al., 1991). It seems that both the ω site and the ω + 2 site amino acids influence the efficiency of GPI anchoring and that the effects are cumulative. A less than optimal amino acid in one of these positions can be compensated for by an optimal amino acid in the other position to yield an efficiently processed site. The same concept seems to hold true for the ω + 1 and ω + 2 positions. Large amino acids at one of these positions can be compensated for by very small amino acids in the other position. A comparison of our results on Gas1p with results on placental alkaline phosphatase may also help to illustrate this point. Gas1p has the optimal amino acid at its ω site, whereas placental alkaline phosphatase does not (Micanovic et al., 1990), and Gas1p tolerates larger amino acids at the ω + 1 and ω + 2 positions than does placental alkaline phosphatase. This concept suggests that suboptimal amino acids in one of these three critical positions can be tolerated if the other positions are optimal for high level processing. This way we can understand the fact that suboptimal amino acids are found at these positions in natural processing sites (Gerber et al., 1992).

Moran and Caras (1991) claimed that the only requirements for GPI anchoring are two small residues positioned 10–12 residues NH2-terminal to a hydrophobic domain. We began to address the question of a distance requirement for Gas1p. While a certain minimal and maximum distance may exist it is not necessarily the number of residues in this region that is important. Addition of 1 or 2 amino acids in the region between the hydrophobic domain and the ω + 2 position show equal and strong effects on GPI anchoring of Gas1p. One of the possible explanations for this would be that the ω site is naturally placed at its maximal distance (12 amino acids) from the start of the hydrophobic domain. This would be consistent with the above hypothesis. Removal of 2 amino acids between the hydrophobic domain and the ω + 2 position showed a less pronounced effect on GPI anchoring than the removal of only 1 amino acid. This is not consistent with a simple distance requirement. In addition, several known anchor attachment sites (compiled by Gerber et al. (1992)) do not fit this rule. If the region between the ω site and the hydrophobic domain is not simply a spacer, then what role could it play? For the moment this is not clear, but a comparison of this region in the different mutants we constructed suggests that no one particular amino acid residue is essential. The changes that caused the largest defect in GPI anchoring from this region (K532 to E or A532) had some effect on GPI anchoring, but still approximately 60% (compared to 89% for the corresponding wild-type) of the Gas1p was anchored. Changes in the polar N510 residue to a charged residue or to a T had no effect. Among the natural examples, there is also a large sequence diversity in this region. One possibility is that the COOH-terminal hydrophobic domain and the ω and ω + 2 sites bind to the processing enzyme (putative transamidase). The region in between these two elements would have to be able to fold appropriately to allow these binding steps to occur. The lack of sequence conservation in this region would simply reflect the large number of possible ways to fold into an acceptable structure and could help to explain why the number of residues between the ω site and the COOH-terminal hydrophobic stretch is not tightly conserved.

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