Yeast Gaalp Is Required for Attachment of a Completed GPI Anchor onto Proteins

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Abstract. Anchoring of proteins to membranes by glycosylphosphatidylinositols (GPIs) is ubiquitous among all eukaryotes and heavily used by parasitic protozoa. GPI is synthesized and transferred en bloc to form GPI-anchored proteins. The key enzyme in this process is a putative GPI:protein transamidase that would cleave a peptide bond near the COOH terminus of the protein and attach the GPI by an amide linkage. We have identified a gene, GAAL, encoding an essential ER protein required for GPI anchoring. gaal mutant cells synthesize the complete GPI anchor precursor at nonpermissive temperatures, but do not attach it to proteins. Overexpression of GAAL improves the ability of cells to attach anchors to a GPI-anchored protein with a mutant anchor attachment site. Therefore, Gaalp is required for a terminal step of GPI anchor attachment and could be part of the putative GPI:protein transamidase.

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1. Abbreviations used in this paper: DAPI, 4,6-diamidino-2-phenylindole; GPI, glycosylphosphatidylinositol; JBAM, jack bean α-mannosidase; PI-PLC, phosphoinositide phospholipase C; PLD, phospholipase D.

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ANCHORING of cell surface proteins with various functions to membranes by covalent attachment of glycosylphosphatidylinositols (GPIs) is used by all eukaryotes examined thus far. Even though all eukaryotes have GPI-anchored proteins, the use of GPI anchoring is of particular importance in protozoal pathogens. In Trypanosoma brucei, the variable surface glycoprotein that forms part of the protective coat around the bloodstream form of the parasite is GPI anchored. The malarial circumporozoite protein is also believed to have a GPI anchor (Englund, 1993; McConville and Ferguson, 1993). For this reason, GPI anchoring has been considered as a possible target pathway for intervention in diseases caused by protozoa and possibly fungi. In yeast cells, GPI synthesis and/or anchoring are essential for viability (Leidich et al., 1994), whereas in certain mouse and human cell lines, GPI anchoring is not required for growth and division (Hyman, 1988; Hirose et al., 1992). Despite the apparent ability of some human cells to survive without GPI anchoring, a human somatic cell disease, paroxysmal nocturnal hemoglobinuria, has been found to be due to a defect in this process (Takeda et al., 1993; Bessler et al., 1994).

GPI-anchored proteins are synthesized as precursors with a classic, cleavable signal sequence at the NH₂ terminus and an additional hydrophobic region at the COOH terminus of the protein, which acts as a signal to direct GPI anchoring. After or during import into the ER, the COOH-terminal hydrophobic region is removed and replaced with a preformed, complete GPI anchor (McConville and Ferguson, 1993; Englund, 1993). Initial experiments defining GPI anchoring signals showed an apparent similarity between different organisms. The signal was proposed to comprise a COOH-terminal hydrophobic region, a short "spacer" between it, and the cleavage/attachment site (ω site), which must be an amino acid with a small side chain. The 2 amino acids after the ω site should also have small side chains, but this requirement seems to depend upon the context of the signal (Micanovic et al., 1990; Moran and Caras, 1991; Moran et al., 1991; Conzelmann et al., 1992; Fankhauser et al., 1993). Despite the similarity in core structures among all organisms, the mode of GPI biosynthesis is not completely conserved. In trypanosomes, the mannose residues of the core structure are added onto GlcNH-PI (Menon et al., 1990), whereas in mammalian cells and yeast, they are added onto GlcNH-PI with an acylated inositol ring (Stevens, 1993; Leidich et al., 1994).

The structures of GPI anchors from several organisms have been determined, and there is a core structure that is highly conserved among all characterized GPI anchors. This core includes an inositol, glucosamine, three mannoses, and a phosphoethanolamine linked in an amide linkage to the COOH terminus of the protein. Apart from the core structure, there is a wide variety of side chain modifications and variation in glycerolipid and ceramide structures that are attached to the inositol of GPI anchors (McConville and Ferguson, 1993; Englund, 1993; Conzelmann et al., 1992; Fankhauser et al., 1993).
et al., 1991, 1993). Despite this apparent consensus, recent experiments have shown that the signal requirements are not identical among protozoa and animal cells. Protozoan GPI-anchoring signals do not function in animal cells (Moran and Caras, 1994). Therefore, the protein recognizing this signal, presumably the GPI-protein transamidase, is a potential target for chemotherapy.

In this study we have characterized a yeast mutant, gaal, that is able to synthesize the entire GPI precursor but cannot transfer it to proteins. The corresponding essential protein, Gaalp, is a multi-membrane-spanning protein of the ER with a relatively large luminal domain. Overproduction of Gaalp can partially suppress the GPI anchoring defect found in a protein with a mutated anchor attachment site. These data show that Gaalp is required for a terminal step of GPI anchor attachment and suggest that it may be part of the putative GPI-protein transamidase.

Materials and Methods

Yeast Strains

Strain RH172-1D (MATα gaal-1 leu2 ura3 bar1-1) was obtained after four outcrosses of the original mutant, EMRH557 (Chvaichko et al., 1986), to inbreed with EMRH260 (MATα his4 leu2 ura3 bar1-1) or RH261-3 (MATα his4 leu2 ura3 bar1-1). RH172-1D was used for the experiments shown in Figs. 1, 2, and 3 and for cloning of Gaalp. RH2837 (MATa gaal-1 his4 leu2 ura3 bar1-1) was obtained after another backcross to RH44-3A. RH2837 was crossed with C4 (MATα pmr40 leu2 ura3) to introduce the phosphomannose isomerase conditional mutation. Strains RH460-7A (MATa pmr40 ura3 leu2), RH401-7B (MATα pmr40 gaal-1 ura3 leu2), RH401-7C (MATα gaal-1 ura3 leu2), and RH401-7D (MATα ura3 leu2 his4) were generated and used for analyzing Gaalp synthesis. RH392-3A (MATα gaal-1 leu2 trpl lys2 gaalΔ:LEU2) was used for analyzing the effects of overexpression of Gaalp on maturation of mutant Gaa1 proteins.

Cloning and Characterization of the Gaalp Gene

The Gaalp gene was cloned by complementation of the temperature-sensitive phenotype of the original mutation using strain RH172-1D. After transformation (Io et al., 1983) with a plasmid DNA library constructed in pBlom (Chevalier et al., 1980) and growth at 30°C, over 8,000 colonies were scraped from the plates, diluted, and spread onto selective plates at 37°C. 16 colonies grew under these conditions and showed plasmid-dependent growth at 37°C. A total of two different plasmids (pDH1 and pDH2) that had overlapping inserts, as judged by their DNA restriction patterns, were isolated from these clones. The complementing activity was mapped to a 2.8-kb HindIII fragment. This fragment was used as a full-length Gaalp clone and transferred into various vectors (Gietz and Sngino, 1991). A 1.4-kb fragment comprising this HindIII fragment was subcloned into the integration vector Yplac211 (URA3 based) and used for cloning Gaalp. The resulting diploid strains were sporulated, and tetrads were dissected. A 2:2 segregation of growth/non-growth was found, with the colonies formed always either Ura or Lys auxotrophs, depending on the integrative DNA used. The spores that did not form colonies usually germinated and divided from one to a few times. If the spores were heterozygous for the disruption event, the disrupted allele of gaal could be obtained, but these cells could not lose the plasmid, showing that Gaalp is essential for cell viability.

Characterization of Gaalp

Antibodies were raised in rabbits against the peptides NH2-MALLEKHRRIV-COOH and NH2-CSVVRSEKQCOOH following the strategy described (Harlow and Lane, 1988) using the terminal cysteine residues (not naturally found in Gaalp) for coupling to the carrier protein or to affinity resins. Peptide-specific antibodies were bound to peptide columns and eluted with 0.1 M glycine, pH 2.3, and immediately neutralized. For detection of Gaalp, protein samples were prepared using the glass bead lysis method (Horvath and Riezman, 1994), separated by 7.5% SDS-PAGE (Laemmli, 1970), and transferred to nitrocellulose (Tobin et al., 1979). The filters were blocked in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Tween 20. After incubation with affinity-purified antibodies (diluted 1:100) in blocking buffer. After washing and incubation in blocking buffer containing 3% nonfat dry milk, the filters were incubated with goat anti-rabbit IgG coupled to peroxidase, which was detected by enhanced chemiluminescence (Amersham Buchler GmbH, FRG) according to the manufacturer's instructions. Gaalp could be detected using this protocol only when the gene was present in the cells in multiple copies.

To determine whether Gaalp is membrane associated, cells overproducing Gaalp were lysed with DEAE-dextran (Singer and Riezman, 1990), and the extracts were brought to either 0.1 M sodium carbonate, pH 11, or 0.5% Triton X-100, incubated for 30 min on ice, and centrifuged at 100,000 g for 1 h to form S3. After no treatment or carbonate treatment, >95% of Gaalp was found in S1 by Western blotting. After Triton X-100 treatment, >95% of Gaalp was found in S3. These results confirm that Gaalp is tightly associated with a rapidly sedimenting membrane.

For immunofluorescence, cells were grown at 30°C to 1-2×10^7 cells per ml in YPUAD medium. For α-factor internalization and degradation measurements, the continuous presence protocol was used with a 0- or 30-min preincubation at 37°C in YPUAD medium. The assay was also performed at 24 and 30°C. α-Factor degradation was quantified by scanning densitometry of the x-ray films obtained after fluorography of the TLC plates. The disappearance of intact α-factor was quantified, as not all of the radioactivity resulting from α-factor degradation was recovered.

Endocytosis Assays and Protein Biogenesis

Lucifer yellow CH accumulation, α-factor internalization, and degradation were measured as described after overnight growth to 2×10^7 cells per ml in YPUAD medium. For α-factor internalization and degradation measurements, the continuous presence protocol was used with a 0- or 30-min preincubation at 37°C in YPUAD medium. The assay was also performed at 24 and 30°C. α-Factor degradation was quantified by scanning densitometry of the x-ray films obtained after fluorography of the TLC plates. The disappearance of intact α-factor was quantified, as not all of the radioactivity resulting from α-factor degradation was recovered.
as described (Horvath et al., 1994). Pulse-chase studies to follow Gaslp and CPY biogenesis were performed on whole cells. After glass bead lysis of the cells, the proteins were denatured using SDS and subjected to immunoprecipitation, SDS-PAGE, and fluorography as described (Horvath et al., 1994).

Plasmids (URA3/CEN-ARS-based) for expressing mutant gas/l alleles have been described (Nussler et al., 1993) and were transformed into strain RH392-3A. These strains were transformed with the control vector YEplac195 (2μ/TrPI-based; Gietz and Sugino, 1988), or with the same vector with insertion of a 2.8 kb HindIII fragment carrying the GAAI gene (gDH17). These cells were grown for 20 h in SD medium (Dulic et al., 1991) supplemented with 40 μM lysine to OD600 0.1-1, and total proteins were extracted by the glass bead lysis method, separated by 7.5% SDS-PAGE, transferred to nitrocellulose sheets, and detected by using enhanced chemiluminescence with rabbit serum against Gaslp and goat anti-rabbit IgG coupled to peroxidase (Horvath and Riezman, 1994). The enhanced chemiluminescence signals were quantified by scanning densitometry (Molecular Dynamics Inc., Sunnyvale, CA) of the x-ray films from different exposures to ensure linearity of the response.

**[PH]Mannose and [PH]Inositol Labeling**

Strains RH144-3D and RH172-4D with and without plasmid pDH15 (YEplac195 with the 2.8 kb Gasl-containing HindIII restriction fragment) were grown overnight in semisynthetic medium, washed in SD medium lacking inositol, and immediately labeled for 1 h with [2-3H]myo-inositol (DuPont-NEN, Boston, MA) in SD medium lacking inositol that was prewarmed to the indicated temperature (Horvath et al., 1994). The cells were lysed with glass beads, and proteins were precipitated with 10% TCA, the pellet was washed with acetone, dried, and dissolved in SDS gel sample buffer containing 2% 2-mercaptoethanol. The proteins were separated on 10% polyacrylamide gels containing SDS and prepared for fluorography in 1 M sodium salicylate.

Glycolipid labeling (Sipos et al., 1994) with [2-3H]myo-inositol or [2-3H]mannose (DuPont-NEN) was performed after growing cells for 20 h to log phase (<4 × 10^7 cells/ml) in SD medium, supplemented with 40 μM lysine to OD600 0.3-1, and total proteins were extracted by the glass bead lysis method, separated by 7.5% SDS-PAGE, transferred to nitrocellulose sheets, and detected by using enhanced chemiluminescence with rabbit serum against Gaslp and goat anti-rabbit IgG coupled to peroxidase (Horvath and Riezman, 1994). The enhanced chemiluminescence signals were quantified by scanning densitometry (Molecular Dynamics Inc., Sunnyvale, CA) of the x-ray films from different exposures to ensure linearity of the response.

**Results**

Previously, we reported the isolation of a yeast mutant, end2, that is defective for accumulation of an endocytic marker, Lucifer yellow, in the vacuole (Chvatchko et al., 1986). To determine where in the endocytic pathway the mutant was affected, we analyzed the ability of mutant cells to internalize and degrade α-factor, a marker of receptor-mediated endocytosis (Dulic et al., 1991). A 30-min incubation at restrictive temperature (37°C), α-factor was added, and its internalization was measured. No defect in α-factor internalization was found (Fig. 1). After internalization, α-factor is delivered, via two intermediate compartments, to the vacuole, where it is degraded (Singer and Riezman, 1990; Singer-Krüger et al., 1993). We examined the ability of wild-type and mutant cells to degrade the internalized pheromone after a 30-min preincubation at restrictive temperature. The results of several experiments are shown in Fig. 1. In each experiment, the mutant degraded less α-factor than the wild-type cells, but the results were highly variable. Since mutant cells exhibit a growth defect already at permissive temperature, we also measured α-factor degradation at 24 and 30°C. No defect in α-factor degradation was found. In addition, mutant cells that were not preincubated at 37°C before adding α-factor showed no detectable defect in pheromone degradation (data not shown). These data and data presented in the following report led us to conclude that the effect of the mutation on the endocytic pathway is indirect. The indirect effect of the mutation on endocytosis was specific for this gene, because mutant cells transformed with plasmid carrying the wild-type gene (see below) were normal for endocytosis. We then examined whether other membrane trafficking events were also disturbed after the relatively long preincubation at 37°C.

**GAA1 Is Required for GPI Anchoring**

The secretion of invertase was followed as a marker for the secretory pathway. Yeast cells were converted to spheroplasts, preincubated for 30 min at 37°C, pulse labeled with [35S]cysteine and [35S]methionine, and chased for the indicated times. The spheroplasts were collected by centrifugation, and the secreted invertase was recovered in the supernatant. After the pulse labeling, core glycosylated invertase was found in both mutant and wild-type spheroplasts (Fig. 2 A). After 5 min of chase, all of the invertase was secreted by both mutant and wild-type spheroplasts. The secreted in-
Vertase migrated at an apparently larger molecular weight in mutant cells, suggesting that additional outer chain glycosylation could have occurred in these spheroplasts.

Next we tested for the ability of cells to mature a GPI-anchored protein, Gaslp. Gaslp is synthesized as a 105-kD glycoprotein in the ER, where it obtains a GPI anchor. After and depending upon anchor attachment, the protein can move to the Golgi apparatus, where its carbohydrate chains are elongated, leading to an increase in its apparent molecular mass to 125 kD (Nuoffer et al., 1991, 1993; Fankhauser and Conzelmann, 1991). Preliminary experiments performed after a 30-min preincubation at 37°C showed that Gaslp maturation was completely blocked. Therefore, we also examined Gaslp biosynthesis without preincubation at 24 and 37°C. At 24°C, Gaslp maturation was significantly slower in mutant cells (t1/2 = 35 min) than in wild-type cells (t1/2 = 10 min). At 37°C, the defect in Gaslp maturation in mutant cells was almost complete (Fig. 2B). Under the same conditions, biogenesis of the vacuolar enzyme, carboxypeptidase Y (Stevens et al., 1982), was virtually identical in mutant and wild-type cells (Fig. 2B). The 67-kD ER form of the enzyme was rapidly converted to the 69-kD Golgi form, which in turn was matured in the vacuole to the 61-kD form.

The defect in maturation of Gaslp could be the result of a defect in GPI anchoring of the protein, causing a transport defect, or it could be due directly to a transport defect (see Horvath et al., 1994, for a discussion of this problem). To test for a defect in GPI anchoring, we analyzed the incorporation of [3H]inositol into proteins by pulse labeling without preincubation at 24 and 37°C, followed by total protein extraction and SDS-PAGE. Under these conditions, all detectable protein-bound inositol incorporation in S. cerevisiae is into GPI-anchored proteins (Conzelmann et al., 1990). Inositol incorporation into proteins was greatly reduced at both temperatures, and the defect was reversed by introduction of a plasmid carrying the wild-type gene (Fig. 3). Inositol was efficiently incorporated into lipids under similar conditions (see Fig. 7). Therefore, we concluded that the primary defect in the mutant cells was not in endocytosis, but in GPI anchoring, and we renamed the gene GAA/, for GPI anchor attachment.

**Galp Is an Essential ER Protein with a Large Luminal Domain**

The sequence of the GAA1 gene is shown in Fig. 4. The single, large open reading frame encodes a protein of ~68 kD that shows no strong homologies with other known proteins upon searching the available databases using the TFASTA or BLAST programs of the UWGCG package. To create a disruption of the GAA1 gene, a selectable marker, URA3 or LYS2, was substituted for the NcoI restriction fragment (Fig. 4), generating a disruption deletion fragment (Fig. 4), generating a disruption deletion allele. After generation of a heterozygous diploid by integrative transformation, sporulation, and tetrad dissection, it was found that the gaal disrupted cells did not grow. The gaal::LYS2 mutant cells could be rescued if they carried a wild-type allele of GAA1 on a URA3-based plasmid, but these cells were unable to lose this plasmid and retain viability. These results show that the GAA1 gene is essential.

Gaalp contains six regions of hydrophobic and/or uncharged amino acids that would be of sufficient length and hydrophobicity to span a lipid bilayer (Figs. 4 and 5A). We raised rabbit antibodies against peptides corresponding to the NH2 and COOH termini of Gaalp (see Materials and Methods), and both were able to recognize a protein of ~70 kD, but only when multiple copies of the GAA1 gene were present (Fig 5C; Hamburger, 1994). This suggests that there are no major processing events at the termini, such as removal of a signal sequence. Gaalp is indeed a membrane protein, because it was not solubilized by treatment with 0.1 M carbonate, pH 11, but was completely solubilized by 0.5% Triton X-100 (see Materials and Methods for details). We predicted that the protein spans the membrane six times with its NH2 and COOH termini in the cytoplasmic compartment. If this were the case, then only one of the two potential...
Figure 2. Gaslp biogenesis is defective in mutant cells. (A) Spheroplasts were prepared from mutant (RH172-1D) and wild-type (RH144-3D) cells. These spheroplasts were incubated at 37°C for 30 min, pulse labeled with [35S]methionine and [35S]cysteine for 4 min, and chased for the indicated times. Supernatant (external) and cell pellets (internal) were prepared, and invertase was immunoprecipitated from these for analysis by SDS-PAGE and autoradiography. (B) The same yeast strains were grown at 24°C, washed, resuspended in prewarmed labeling medium at the indicated temperature, and immediately pulse-chase labeled as previously described. At the indicated times of chase, extracts were made from the cells, and Gaslp and CPY were sequentially immunoprecipitated and analyzed by SDS-PAGE and autoradiography. The lower band in the Gaslp immunoprecipitates is the 105-kD form; the upper band is the 125-kD form.

Figure 3. Mutant cells do not incorporate inositol into proteins. Mutant (RH172-1D) cells with or without plasmid pDH15 and wild-type (RH144-3D) cells were resuspended in prewarmed medium at 24 or 37°C and immediately labeled with [3H]myo-inositol for 1 h. Total protein extracts were prepared and separated by SDS-PAGE, and inositol incorporation was revealed by autoradiography. W, wild type; M, mutant; -, without plasmid; +, with plasmid pDH15.

Figure 4. N-linked glycosylation sites (N\textsuperscript{7}7 and N\textsuperscript{3}3; Fig. 4) could be used.

To examine whether Gaslp is a glycoprotein, protein extracts were treated with endoglycosidase H and separated by SDS-PAGE, and Gaslp was detected by Western blotting. Upon endoglycosidase H treatment, Gaslp shifted in apparent molecular mass from 70 to 68 kD (Fig. 5 C), indicating that it contains N-linked carbohydrate. Therefore, Gaslp is likely to be inserted into the ER membrane. To determine whether the predicted glycosylation site, N\textsuperscript{7}7, was used, we introduced a mutation (A\textsuperscript{7}77) destroying this site. The mutant Gaslp was not affected by endoglycosidase H treatment (Fig. 5 C), indicating that it was no longer modified by N-linked glycosylation. It is unlikely that the absence of glycosylation in the A\textsuperscript{7}7 mutant was due to gross misfolding or mistargeting of the mutant protein because the mutant allele was able to complement the gaal mutant. Therefore, Gaslp is a membrane glycoprotein with an apparently large (~306 amino acids) luminal domain.

At the extreme COOH terminus of Gaslp there is a KXXXsequence that has been characterized as a signal for retrieval of membrane proteins to the ER (Jackson et al., 1990; Gaynor et al., 1994). To determine whether Gaslp is localized to the ER, the protein was overproduced and local-
A protein Gaalp is glycosylated on N^87. (A) Kite-Doolittle hydrophobicity blot of Gaalp. (B) Model of the transmembrane orientation of Gaalp. The diamond is the N-linked glycosylation site. The hydrophobic stretches are in shaded boxes. (C) Extracts from cells overexpressing wild-type GAA1 or gaa1-87A were subjected to digestion with endoglycosidase H (EndoH) for 2 h at 37°C. Controls were performed without enzyme. Gaalp was revealed by Western blotting. + indicates addition of Endo H or incubation at 37°C for 2 h. - indicates no Endo H or no incubation.

The gaal Mutant Synthesizes the Complete GPI Precursor

To define where the defect in GPI anchor attachment of the gaal mutant lies, we examined the biosynthesis of the GPI anchor after labeling with [3H]mannose or [3H]inositol. Since the complete GPI anchor precursors (CP1 and CP2) are very difficult to detect in wild-type cells by [3H]mannose labeling (Sipos et al., 1994), we introduced a mutation in phosphomannose isomerase, pmi40, which blocks production of mannose, thus improving the sensitivity of GPI detection by metabolic labeling (Sipos et al., 1994). The gaal mutant was crossed with the pmi40 mutant, and we analyzed a tetrad that comprised all four combinations of the two mutations. The cells were grown overnight, harvested, and incubated in labeling medium for 20 min at 37°C to deplete the endogenous mannose. [3H]mannose was added, and after an additional 30-min incubation, total lipids were extracted, separated by TLC, and revealed by fluorography (Fig. 7). In the pmi40 mutant (spore 7A), a heavily labeled

Figure 5. Gaalp is glycosylated on N^87. (A) Kite-Doolittle hydrophobicity blot of Gaalp. (B) Model of the transmembrane orientation of Gaalp. The diamond is the N-linked glycosylation site. The hydrophobic stretches are in shaded boxes. (C) Extracts from cells overexpressing wild-type GAA1 or gaa1-87A were subjected to digestion with endoglycosidase H (EndoH) for 2 h at 37°C. Controls were performed without enzyme. Gaalp was revealed by Western blotting. + indicates addition of Endo H or incubation at 37°C for 2 h. - indicates no Endo H or no incubation.

Figure 6. Immunofluorescence localization of overexpressed Gaalp. RH144-3D carrying plasmid pDH15 were fixed and processed for immunofluorescence using antibodies raised against the COOH terminus. When Gaalp was overproduced, a distinctive ER pattern was seen (Fig. 6), typified by the ring around the nucleus (localized using DAPI staining) and staining just below the plasma membrane. This staining pattern resembles that found for Wbp1p, a part of the oligosaccharyl transferase that is known to be localized to the ER in

Figure 7. Mannose and inositol labeling of gaal, pmi40 segregants. Strains RH401-7A (pmi40), 7B (pmi40, gaal), 7C (gaal), and 7D were grown overnight and labeled with [3H]mannose or [3H]inositol at 37°C as described in Materials and Methods. Lipids were extracted and separated by TLC. The positions of migration of PI, mannosylinositolphosphoceramide (MIPC), mannosyldiinositolphosphoceramide (M(IP)2C), and the complete GPI precursor standard (CP; obtained from A. Conzelmann), as well as the origin (O) are noted on the left. * denotes lanes in which the complete GPI precursor standard was loaded.

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doublet that comigrated with the complete GPI anchor precursor standard was seen just above the origin. The upper band is CP1 and the lower is CP2 (Sipos et al., 1994). The gaal, pmi40 double mutant (spore 7B) also had a heavily labeled band that comigrated with the standard and the complete precursor, CP2, as well as an increase in the amount of another band running just above mannosylinositolphosphocheramide. The gaal mutant (spore 7C) also showed the band comigrating with CP2, whereas in wild-type cells (spore 7D), this band was not detectable. In addition, wild-type cells showed a labeled band that was not seen in either the pmi40 or gaal cells.

In wild-type cells, CP1 and CP2 were not detectable when cells were labeled with [\(^{3}H\)]inositol (Sipos et al., 1994; Fig. 7, spore 7D), but if the band that accumulated in gaal cells is indeed the complete GPI precursor, then it should also be possible to label it with inositol. Indeed, in the gaal mutant, an inositol-labeled species that comigrated with the authentic precursor was found (Fig. 7, spore 7C). We suggest that we could detect the precursor in gaal cells, but not in wild-type cells because in wild-type cells the complete precursor was rapidly incorporated into proteins, whereas in the mutant cells it accumulated owing to the block in GPI anchor attachment.

To confirm that the mannose- and inositol-containing bands, comigrating with the complete GPI precursor, were complete precursors, we analyzed them by several chemical and enzymatic tests. The structure of the complete GPI precursor (Sipos et al., 1994) is shown in Fig. 8 C. The complete precursor should be partially sensitive to JBAM, sensitive to nitrous acid deamination because of the glucosamine-inositol linkage, sensitive to GPI-specific PLD cleavage, and insensitive to PI-PLC cleavage because of the acylated inositol. Therefore, we labeled pmi40 and gaal, pmi40 cells with [\(^{3}H\)]mannose and extracted the lipids. These lipids and authentic standards were treated with JBAM, reextracted, separated by TLC, and revealed by fluorography. The presumptive complete precursor accumulating in the gaal mutant was almost entirely shifted to a slightly greater mobility after JBAM treatment. The authentic precursor behaved in the same manner (Fig. 8 A). Furthermore, the presumptive complete precursor was completely sensitive to nitrous acid deamination, whereas most of the other lipids were unaffected (Fig. 8 A). The band that accumulated in the gaal, pmi40 double mutant and migrated just above M(IP)\(_{2}\)C was also partially sensitive to JBAM and cleaved by nitrous acid (easily seen on lighter exposures; data not shown), suggesting that it may be an intermediate in GPI anchor synthesis. Its accumulation could be due to a backup of the pathway when GPI anchor attachment is blocked.

To examine the sensitivity to phospholipases, gaal mutant cells were labeled with [\(^{3}H\)]inositol and lipids were extracted. These lipids were treated with PI-PLC or GPI-PLD, reextracted, separated by TLC, and revealed by fluorography. The presumptive complete precursor was insensitive to PI-PLC under conditions in which all of the PI and inositolphosphocheramides were cleaved (Fig. 8 B). As expected, it was sensitive to GPI-PLD cleavage under conditions in which all of the other inositol-labeled lipids were uncleaved (Fig. 8 B). The GPI-PLD action was dependent upon divalent cations. The results presented show that the [\(^{3}H\)]inositol- and [\(^{3}H\)]mannose-labeled band accumulating in the gaal

![Figure 8](https://example.com/figure8.png)

Figure 8. Enzymatic and chemical treatments of lipid extracts. (A) Mannose-labeled lipids were prepared as in Fig. 7 from strains RH401-7A (pmi40) and RH401-7B (gaal) and subjected to nitrous acid deamination (HONO), or lipids from RH401-7B were treated along with the complete GPI precursor standard (CP) with JBAM (JBAM), after which the lipids were reextracted, and separated by TLC. O, origin, CP, complete precursor, CP', complete precursor after JBAM treatment, MIPC, mannosylinositolphosphocheramide, M(IP)\(_{2}\)C, mannosyl-diinositolphosphocheramide. --, no enzyme or NaCl added as control in nitrous acid experiment, +, enzyme or NaNO\(_{2}\) added. (B) Inositol-labeled lipids from strain RH401-7C (gaal) were treated with (+) or without (−) PI-PLC or GPI-PLD in the (+) presence or (−) absence of CaCl\(_{2}\), reextracted and separated by TLC. Migration positions are indicated as in Fig. 7, with inositolphosphocheramide (IPC) indicated in addition. (C) The structure of the complete GPI anchor precursor was taken from Sipos et al., 1994. The positions where JBAM (JBAM), nitrous acid (HONO) and GPI-PLD (GPI-PLD) should cleave are indicated.
mutant has all the properties of the complete GPI precursor. Therefore, the defect in GPI anchoring lies somewhere after the synthesis of the complete glycolipid anchor.

**GAA1 Overexpression Partially Suppresses Gaslp Anchor Attachment Mutants**

Since the gaa1 mutant can synthesize the entire GPI precursor but does not attach it to proteins, Gaalp could be part of the putative GPI:protein transamidase. If this were the case, it is possible that overexpression of GAA1 could have effects on the rate of GPI anchoring. Under normal conditions, there is no evidence that GPI anchoring is a rate-limiting step in secretion of GPI-anchored proteins. However, when modifications are made at or near the anchor attachment site, GPI anchoring becomes rate limiting for transport of the normally GPI-anchored proteins to the Golgi apparatus (Moran and Caras, 1992; Nuoffer et al., 1993; Gerber et al., 1992). Previously, we have characterized various mutants (Nuoffer et al., 1993) at or near the cleavage attachment site (N°6; Nuoffer et al., 1991) that affect anchoring of the GPI-anchored protein Gaslp. Here we have tested the effects of overexpression of GAA1 on the biogenesis of one mutation in the putative “spacer” region of Gaslp (−T°N5°) and three anchor attachment site mutants, D°6, C°6, and Q°6. Cells expressing the mutant Gaslp proteins and harbouring either vector without insert or vector with GAA1 were grown overnight, proteins were extracted, and Gaslp was revealed by Western blotting. Under these conditions, the percentage of Gaslp found in the 125-kD form represents the percentage of protein that is GPI anchored (Nuoffer et al., 1993). Gaalp overproduction was verified by Western blotting (data not shown). The percentage of Gaslp that was GPI anchored was increased when Gaalp was overproduced in the cells expressing the −TN Gaslp mutant and in cells expressing Gaslp with mutant (D or C) attachment sites (Fig. 9). A small difference in anchoring efficiency with and without Gaalp overproduction was always seen with the D°6 mutation (data not shown), and a statistically significant difference was found with the C°6 mutation (Fig. 9). Without Gaalp overproduction, 27 ± 6% of the C°6-Gaslp was matured and thus GPI anchored, whereas with Gaalp overproduction, 43 ± 6% of the C°6-Gaslp was matured. When the anchor attachment site was mutated to Q, anchoring was virtually abolished. These data show that overexpression of GAA1 can partially suppress the defect in GPI anchoring of Gaslp mutants with defects at or near the anchor attachment site.

**Discussion**

In this study we have shown that the gaa1 mutant is defective for a terminal step in GPI anchor attachment. At restrictive temperature, the mutant cells were capable of synthesizing the entire GPI anchor precursor, but did not attach it to proteins. The authenticity of the precursor was established by metabolic labeling of the precursor, using both mannose and inositol, and comigration with the complete precursor, CP2 (Sipos et al., 1994). Several other structural characteristics of the putative precursor were confirmed. The presence of a mannose side chain and the terminal ethanolamine-P was inferred from the partial sensitivity to and change in chromatographic behavior after JBAM treatment. The glucosamine-inositol linkage was inferred from the sensitivity to nitrous acid deamination. The acylation of the inositol ring was shown by resistance to PI-PLC cleavage, whereas the GPI nature was confirmed by susceptibility to GPI-PLD cleavage. We can rule out the possibility that the band is an abnormal sphingolipid because its synthesis was not inhibited by myriocin, an inhibitor of the first step of ceramide biosynthesis (data not shown; Schönbächler et al., 1995).

It is interesting to note that the gaa1 mutant synthesized only the complete precursor CP2 and not CPI. This could be explained by the fact that CP2 synthesis is predominant when cells are under stress (Sipos et al., 1994). As the gaa1 mutant is already at least partially defective for GPI anchor-
ing at permissive temperature, the cells could be constantly under stress. In any event, CPI and CP2 apparently differ only in their fatty acyl chains, as no detectable differences in the complex head groups were found. Additionally, both CPI and CP2 could be chased and therefore incorporated into GPI-anchored proteins in the absence of cycloheximide (Sipos et al., 1994). Using the gaal mutant, we cannot test whether the putative precursor that accumulates at restrictive temperature can be chased into GPI-anchored proteins because the mutant is too defective at permissive temperature to perform the chase experiment efficiently.

The ER localization of Gaalp is consistent with its role in a terminal step of GPI anchor attachment. This localization has been shown most directly by indirect immunofluorescence of overproduced Gaalp and is consistent with the fact that the protein is glycosylated. As we cannot detect Gaalp expressed from the chromosome, we cannot calculate the fold overproduction of the protein. Northern analysis has shown that the mRNA is ∼50-fold overproduced under similar conditions (data not shown). As with any overproduced protein, localization studies should be interpreted cautiously. It is possible that the overproduced protein was trapped in the ER for various reasons. Also, we cannot rule out the possibility that the protein is found in other locations in addition to the ER. On the other hand, Gaalp has a potential ER retrieval signal (KEQKS) at its extreme COOH terminus. This part of the protein could confer ER localization to a plasma membrane protein, Ste2p, and when these lysines in Gaalp are mutated, the overproduced mutant protein no longer localizes to the ER (Hamburger, 1994).

Most of the steps of GPI biosynthesis have been shown to take place on the cytoplasmic side of the ER of trypanosomes. Only GPI molecules with acylated inositol are enriched on the luminal side of the membrane (Vidugiriene and Menon, 1993, 1994). Therefore, at some point in the pathway, the GPI precursor is likely to be transferred to the luminal side of the membrane, where the putative GPI-transamidase most likely acts posttranslationally on proteins that have been translocated into the ER lumen (Kodukula et al., 1992; Amthauer et al., 1993). The step in the GPI synthesis pathway at which the precursor crosses the ER membrane is not known in animal or yeast cells, but the inositol ring is acylated much earlier in the pathway. Gaalp is the first protein described in the GPI synthesis/anchoring pathway that presumably has a large hydrophilic part of its structure in the lumen of the ER, consistent with its role in the terminal stages of GPI anchor attachment.

When anchors are not attached to proteins that are normally GPI anchored, as is the case in the gaal mutant, these proteins are deficient for transport to the Golgi apparatus (Nuoffer et al., 1991; Moran and Caras, 1992; Micanovic et al., 1990). This could be the underlying reason for the indirect effect of the gaal mutation on endocytosis. Perhaps there is a GPI-anchored protein that is required, either directly or because of its enzymatic activity, for endocytosis. In this case, when GPI anchoring is completely blocked, this critical protein would be depleted from its normal location, and a delayed endocytic defect would ensue. Alternatively, it is possible that transport of GPI-anchored proteins, sphingolipids, and perhaps sterols, such as cholesterol in animal cells or ergosterol in yeast, are cotransported through the secretory pathway (Simons and van Meer, 1988; Horvath et al., 1994). In this case, it could be that depletion of a lipid or sterol causes the defect in endocytosis. One yeast mutant defective for endocytosis, end11 (Munn and Riezman, 1994) is defective for ergosterol biosynthesis (B. Stevenson, personal communication). Another indirect effect of the lack of GPI anchoring may be the apparent overglycosylation of invertase seen in Fig. 2. When GPI-anchored proteins, which could constitute a large pool of glycoprotein substrates for the outer chain glycosylation enzymes, are missing, the available GDP-mannose in the Golgi apparatus could be more efficiently used on the remaining glycoprotein substrates.

From the data presented here, we can conclude that Gaalp is required for GPI anchor attachment but not its synthesis. As previously mentioned, there are several functions that may be required for anchor attachment once the precursor is completed: translocation of the completed precursor into the lumen of the ER, recognition of the proteins to be anchored, cleavage at the anchor attachment site, and finally attachment of the GPI anchor. The latter two steps and perhaps all of the above steps could be coupled together and performed by single protein complex. The partial suppression of Gaalp anchor attachment site mutants would be consistent with Gaalp being part of the putative GPI:protein transamidase that interacts directly with proteins to be GPI anchored. However, it is also possible to reason that Gaalp acts to flip the complete GPI precursor to the lumen of the ER, thereby increasing the effective GPI concentration that may also improve anchoring efficiency. In any event, Gaalp is almost certainly involved in one of these important late functions. We hope that by using the gaal mutant and the powerful genetic techniques available in yeast that we can identify interacting genes and proteins and thereby define the other proteins required for addition of GPI anchors onto proteins.

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References

Amthauer, R., K. Kodukula, L. Gerber, and S. Udenfriend. 1993. Evidence that the putative COOH-terminal signal transamidase involved in glycosylphosphatidylinositol protein synthesis is present in the endoplasmic reticulum. Proc. Natl. Acad. Sci. USA. 90:3973-3977.

Bessler, M., P. J. Mason, P. Hillemen, T. Miyata, N. Yamada, J. Takeda, L. Lazzarotto, and T. Kinoshita. 1994. Paroxysmal nocturnal haemoglobinuria (PNH) is caused by somatic mutations in the PIG-A gene. EMBO (Eur. Mol. Biol. Organ.) J. 13:110-117.

Chevalier, M. R., J. C. Bloch, and F. Lacroute. 1980. Transcriptional and translational expression of a chimeric bacterial-yeast plasmid in yeasts. Gene. 11:11-19.

Chvatchko, Y., I. Howald, and H. Riezman. 1986. Two yeast mutants defective in endocytosis are defective in pheromone response. Cell. 46:355-364.

Conzelmann, A., C. Fankhauser, and C. Desponds. 1990. Myo-inositol gets incorporated into numerous membrane glycoproteins of Saccharomyces cerevisiae: incorporation is dependent on phosphomannomutase (SEC53). EMBO (Eur. Mol. Biol. Organ.) J. 9:653-661.

Conzelmann, A., A. Puoti, R. L. Lester, and C. Desponds. 1992. Two different types of lipid moieties are present in glycosphosphoinositol-anchored membrane proteins of Saccharomyces cerevisiae. EMBO (Eur. Mol. Biol. Organ.) J. 11:457-466.
Null,