# Yeast Gaa1p Is Required for Attachment of a Completed GPI Anchor onto Proteins

**Dirk Hamburger, Mark Egerton, and Howard Riezman**

Biozentrum of the University of Basel, CH-4056 Basel, Switzerland

*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, *GAA1*, encoding an essential ER protein required for GPI anchoring. *gaa1* mutant cells synthesize the complete GPI anchor precursor at nonpermissive temperatures, but do not attach it to proteins. Overexpression of *GAA1* improves the ability of cells to attach anchors to a GPI-anchored protein with a mutant anchor attachment site. Therefore, Gaa1p is required for a terminal step of GPI anchor attachment and could be part of the putative GPI:protein transamidase.

ANCHORING of cell surface proteins with various functions to membranes by covalent attachment of glycosylphosphatidylinositol (GPI)[1] 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 circumsporozoite 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).

Address correspondence to Howard Riezman, Biozentrum of the University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland. Tel.: (41) 61 267 2160. Fax: (41) 61 267 2148. E-mail Riezman@ubaclu.unibas.ch.

The current address of D. Hamburger is MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824.

The current address of M. Egerton is ICI Pharmaceuticals, Mereside, Alderly Park, Macclesfield, Cheshire SK10 4TG, UK.

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.

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). 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).

GPI-anchored proteins are synthesized as precursors with a classic, cleavable signal sequence at the $NH_2$ 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; Gerber et al., 1992; Kodukula et al., 1993; Nuoffer 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 (*MATa gaal-1 leu2 ura3 barl-1*) was obtained after four outcrosses of the original mutant, EMRH557 (Chvatchko et al., 1986), to isogenic wild-types RH144-3A (*MATa his4 leu2 ura3 barl-1*) or RH144-3D (*MATα his4 leu2 ura3 barl-1*). RH172-1D was used for the experiments shown in Figs. 1, 2, and 3 and for cloning of *GAAl*. RH2837 (*MATα gaal-1 his4 leu2 ura3 barl-1*) was obtained after another backcross to RH144-3A. RH2837 was crossed with C4 (*MATa pmi40 leu2 ura3*) to introduce the phosphomannose isomerase conditional mutation. Strains RH401-7A (*MATa pmi40 ura3 leu2*), RH401-7B (*MATa pmi40 gaal-1 ura3 leu2*), RH401-7C (*MATα gaal-1 ura3 leu2*), and RH401-7D (*MATα ura3 leu3 his4*) were generated and used for analyzing GPI synthesis. RH392-3A (*MATa ura3 leu2 trp1 lys2 gas1Δ::LEU2*) was used for analyzing the effects of overexpression of *GAAl* on maturation of mutant Gas1 proteins.

### Cloning and Characterization of the GAA1 Gene

The *GAAl* gene was cloned by complementation of the temperature-sensitive phenotype of the original mutation using strain RH172-1D. After transformation (Ito et al., 1983) with a plasmid DNA library constructed in plasmid pFL1 (Chevalier et al., 1980) and growth at 24°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 GAA1 clone and transferred into various vectors (Gietz and Sugino, 1988). A 4.1-kb fragment comprising this HindIII fragment was subcloned into the integration vector YIplac211 (URA3 based; Gietz and Sugino, 1988) to give plasmid pDH16, which was integrated into the genome of the diploid strain DH3 (*Mata gaal-1 ura3 leu2 barl-1/Matα ura3 leu2 his4 barl-1*) after restriction digest of the single MluI site contained in the DNA insert. The resulting diploid strains were sporulated, and tetrads were analyzed for segregation of the temperature-sensitive phenotype of *gaal* and Ura prototrophy, marking the integrated DNA. Two types of segregation were found in the different diploids. In the first type, 2:2 segregation of the temperature-sensitive phenotype and 2:2 segregation of Ura prototrophy were found, with the temperature-sensitive spores always being Ura auxotrophs. In the second type, 2:2 segregation of Ura prototrophy was seen, but no temperature-sensitive spores were found. This is consistent only with integration at the *GAAl* locus in the diploid, with integration at the wild-type locus giving the first segregation pattern and integration at the mutant locus giving the second segregation pattern. Therefore, the cloned DNA maps to the *GAAl* locus.

Deletion disruption alleles were constructed by replacing the NcoI fragment within the coding region of *GAAl* (Fig. 4) with either the *URA3* or *LYS2* genes. HindIII fragments carrying the deletion disruption construct were integrated into the genome of strain RH1201 (*Mata/αhis4/his4 ura3/ura3 leu2/leu2 lys2/lys2 barl-1/barl-1*). The transformants 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 diploids heterozygous for the disruption received plasmid pME43 (2.8-kb HindIII fragment with *GAAl* in YCplac211), colonies from spores carrying this plasmid and the disrupted allele of *gaal* could be obtained, but these cells could not lose the plasmid, showing that *GAAl* is essential for cell viability.

*GAAl* was sequenced (Sanger et al., 1977) using the Sequenase 2.0 kit from United States Biochemical Corp. (Lucerna Chem, Lucerne, Switzerland). Site-directed mutagenesis was performed by standard PCR protocols (Ho et al., 1989). The entire fragments amplified in this manner were sequenced to ensure that no mutations other than the ones wanted were introduced.

### Characterization of Gaalp

Antibodies were raised in rabbits against the peptides $NH_2$-MALLEK-LHRRIV-CCOOH and $NH_2$-CSVVRSKEKQS-COOH 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 (Towbin et al., 1979). The filters were blocked in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Tween 20 and incubated for 1-3 h 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 instruction. 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 3,500 *g* for 5 min to form pellet 1 (P1) and supernatant 1 (S1). S1 was spun at 10,000 *g* for 15 min to form P2 and S2, and S2 was spun at 100,000 *g* for 1 h to form S3 and P3. After no treatment or carbonate treatment, >95% of Gaalp was found in P1 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, directly fixed with 3.7% formaldehyde, and further processed as described (Hall et al., 1990) by attaching the fixed cells to poly-L-lysine-treated slides. Incubation with affinity-purified antibodies and localization of DNA by incubation with 10 μg/ml 4',6-diamidino-2-phenylindole (DAPI) were performed as previously described (Zanolari and Riezman, 1991).

To assay for N-linked glycosylation, cell extracts were prepared from the 1 $OD_{600}$ unit of cells by the glass bead extraction method (Horvath and Riezman, 1994) and treated with 35 μU of endoglycosidase H (Boehringer Mannheim, Switzerland) for 2 h. A control was performed without enzyme. The extracts were then denatured in SDS and prepared for SDS-PAGE and Western blotting.

### 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 (Dulić et al., 1991). 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.

Pulse-chase studies at 37°C on spheroplasts to study invertase maturation and secretion were performed as described, except that the spheroplasts were preincubated for 30 min at 37°C before labeling (Kübler et al., 1994). After separation and SDS denaturation of medium and spheroplasts, invertase was immunoprecipitated and analyzed by SDS-PAGE and fluorography

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 *gas1* alleles have been described (Nuoffer et al., 1993) and were transformed into strain RH392-3A. These strains were transformed with the control vector YEplac195 (2μ/TRP1 based; Gietz and Sugino, 1988), or with the same vector with insertion of a 2.8-kb HindIII fragment carrying the *GAA1* gene (pDH17). These cells were grown for ~20 h in SD medium (Dulić et al., 1991) supplemented with 40 μg/ml lysine to $OD_{600}$ 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.

## [$^3$H]Mannose and [$^3$H]Inositol Labeling

Strains RH144-3D and RH172-1D with and without plasmid pDH15 (YEplac195 with the 2.8-kb *GAA1*-containing HindIII restriction fragment) were grown overnight in semisynthetic medium, washed in SD medium lacking inositol, and immediately labeled for 1 h with [2-$^3$H]*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-$^3$H]*myo*-inositol or [2-$^3$H]mannose (DuPont-NEN) was performed after growing cells for 20 h to log phase (<4 × $10^7$ cells per ml) in SDCU medium (Sipos et al., 1994). For mannose labeling, 8 × $10^7$ cells per labeling reaction were washed twice and resuspended in 0.25 ml of SPCU medium (Sipos et al., 1994). The cells were then placed into a 37°C shaking water bath and incubated for 20 min to inactivate the temperature-sensitive phosphomannose isomerase and deplete internal mannose pools. 25 μCi of [2-$^3$H]mannose was added, and incubation was continued for an additional 30 min. For labeling with inositol, a similar procedure was followed, except that the cells were washed with and incubated in 0.5 ml of SD lacking inositol. 14 μCi of [2-$^3$H]*myo*-inositol was added after 3 min in the 37°C shaking water bath to allow the cells to reach 37°C, and the incubation was continued for 37 min. The labeling reaction was stopped by transferring the cells to a 1.5-ml Eppendorf tube on ice containing 250 μl of 20 mM $NaN_3$, 20 mM NaF, centrifuging, and washing two times with $H_2O$. The cell pellet was extracted by shaking for 30 min in 0.5 ml of CMW ($CHCl_3/CH_3OH/H_2O$; 10:10:3) containing 0.5 g of glass beads (0.45 mm). After centrifugation, the supernatant was removed to a fresh tube, and the pellet was reextracted with 0.4 ml of CMW by shaking for 20 min. The combined supernatants were dried in the Speed-vac (Savant, Switzerland) and dissolved in 150 μl of $H_2O$-saturated *n*-butanol. The butanol was extracted with 150 μl of 5 mM Tris-HCl, pH 7.5, 0.1 mM EDTA. The aqueous phase was back-extracted with 75 μl of butanol. The combined butanol phases were finally extracted with 150 μl of *n*-butanol-saturated $H_2O$ and dried in the Speed-vac. The lipids were dissolved in CMW for TLC (Horvath et al., 1994) on Kieselgel 60 plates (20 × 20; Merck, Darmstadt, Germany) using CMW as a solvent system or dried down again for chemical or enzymatic treatments. After chromatography the plates were sprayed with En$^3$Hance (DuPont-NEN) and exposed to film at −70°C for 1–4 d.

## Chemical and Enzymatic Treatments of Lipids

For treatment with α-mannosidase, mannose-labeled glycolipids were dissolved in 25 μl of 0.1 M sodium acetate, pH 5.0, 1 mM $ZnCl_2$, 0.1% taurodeoxycholate with or without 0.9 U of jack bean α-mannosidase (JBAM; Oxford Glycosystems, Switzerland) and incubated for 8 h at 37°C (Güther et al., 1994).

For nitrous acid deamination, mannose-labeled glycolipids were resuspended in 10 μl of 0.1 M sodium acetate, pH 4.0, 0.01% Zwittergent 3-16 (Calbiochem Corp., La Jolla, CA), and 10 μl of freshly prepared 0.5 M $NaNO_2$ or 0.5 M NaCl (control) was added followed by sonication in a water bath for 2 min. After a 1-h incubation at 60°C, this procedure was repeated two more times without sonication (Güther et al., 1994).

For phosphoinositide phospholipase C (PI-PLC) digestion, inositol-labeled lipids were resuspended in 50 μl of 20 mM Tris-HCl, pH 7.5, 20% propanol, 1 mM EDTA and digested with or without 0.05 U of PI-specific PLC (Boehringer Mannheim) for 2 h at 30°C (Puoti and Conzelmann, 1992).

For GPI-PLD (phospholipase D) treatment, inositol-labeled lipids were dissolved in 20 μl of 50 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM $CaCl_2$, 0.1% NP-40 and digested with 1.6 U of partially purified (sp act, 1,700 U/mg protein), GPI-specific PLD from bovine serum (kind gift from U. Brodbeck, University of Bern, Switzerland) for 2 h at 37°C. Two controls were performed: one without enzyme and one with enzyme, but with 2 mM *o*-phenanthroline substituted for $CaCl_2$.

After chemical or enzymatic treatments, the solutions were extracted twice with 100 μl of $H_2O$-saturated butanol. The combined butanol phases were extracted twice with 100 μl of butanol-saturated $H_2O$. After drying, the lipids were dissolved in CMW and analyzed by TLC as previously described.

# Results

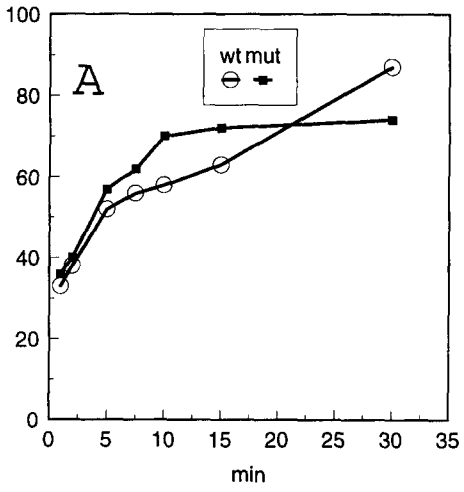
Previously, we reported the isolation of a yeast mutant, *end2*, that is defective for accumulation of an endocytic marker, Lucifer yellow, in the vacuole (Chvatchko et al., 1986). To determine where in the endocytic pathway the mutant was affected, we analyzed the ability of mutant cells to internalize and degrade α-factor, a marker of receptor-mediated endocytosis (Dulić et al., 1991). After 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 next 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 [$^{35}$S]cysteine and [$^{35}$S]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 ($t_{1/2}$ = 35 min) than in wild-type cells ($t_{1/2}$ = 10 min). At 37°C, the defect in Gaslp maturation in mutant cells was almost complete (Fig. 2 *B*). 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. 2 *B*). 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.



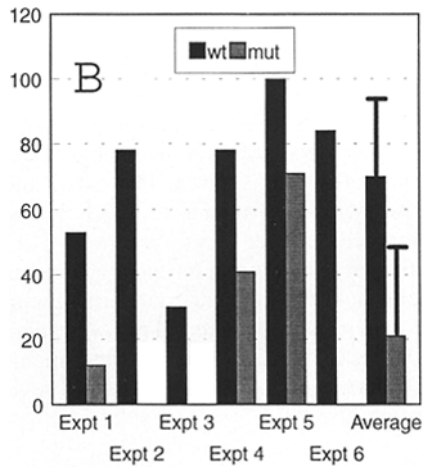




*Figure 1.* Mutant cells are defective in α-factor degradation. Wild-type (RH144-3D) and mutant (RH172-1D) cells were grown overnight at 24°C and tested for α-factor internalization and degradation after a 30-min preincubation at 37°C. (*A*) The percentage of bound α-factor that was not removed by washing with pH 1.1 buffer was expressed as the percent internalized. (*B*) 60 min after the initiation of internalization at 37°C, the radioactivity was extracted from the cells after washing at pH 1.1, and separated by TLC. After fluorography, the percentage of the maximum amount of internalized intact α-factor that remained at 60 min was determined by densitometry.

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 [$^3$H]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 *GAA1*, for GPI anchor attachment.

### *Gaalp 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 allele. After generation of a heterozygous diploid by integrative transformation, sporulation, and tetrad dissection, it was found that the *gaa1* disrupted cells did not grow. The *gaa1*::*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 5 *A*). We raised rabbit antibodies against peptides corresponding to the $NH_2$ 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 5 *C*; 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 $NH_2$ and COOH termini in the cytoplasmic compartment. If this were the case, then only one of the two potential

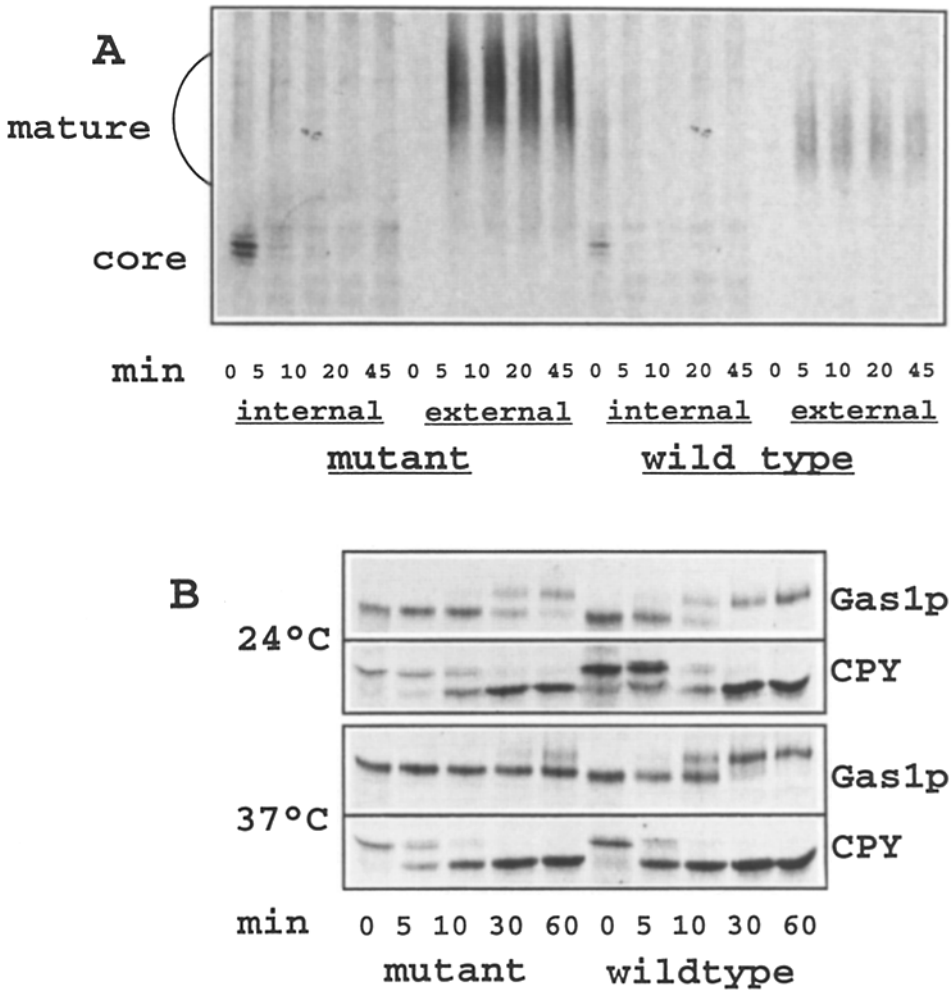



*Figure 2.* Gaslp biogenesis is defective in mutant cells. (*A*) Spheroplasts were prepared from mutant (RH172-1D) and wild-type (RH144-3D) cells. These spheroplasts were incubated at 37°C for 30 min, pulse labeled with [$^{35}$S]methionine and [$^{35}$S]cysteine for 4 min, and chased for the indicated times. Supernatant (*external*) and cell pellets (*internal*) were prepared, and invertase was immunoprecipitated from these for analysis by SDS-PAGE and fluorography. (*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 fluorography. The lower band in the Gaslp immunoprecipitates is the 105-kD form; the upper band is the 125-kD form.

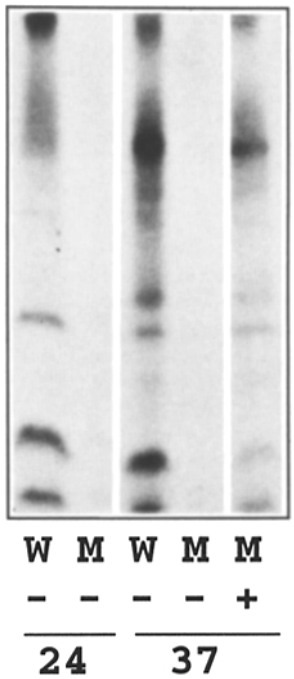



*Figure 3.* Mutant cells do not incorporate inositol into proteins. Mutant (RH172-1D) cells with or without plasmid pDH15 and wild-type (RH144-3D) cells were resuspended in prewarmed medium at 24 or 37°C and immediately labeled with [$^{3}$-H]*myo*-inositol for 1 h. Total protein extracts were prepared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

N-linked glycosylation sites ($N^{87}$ and $N^{383}$; Fig. 4) could be used.

To examine whether Gaalp is a glycoprotein, protein extracts were treated with endoglycosidase H and separated by SDS-PAGE, and Gaalp was detected by Western blotting. Upon endoglycosidase H treatment, Gaalp shifted in apparent molecular mass from 70 to 68 kD (Fig. 5 *C*), indicating that it contains N-linked carbohydrate. Therefore, Gaalp is likely to be inserted into the ER membrane. To determine whether the predicted glycosylation site, $N^{87}$, was used, we introduced a mutation ($A^{87}$) destroying this site. The mutant Gaalp 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 $A^{87}$ mutant was due to gross misfolding or mistargeting of the mutant protein because the mutant allele was able to complement the *gaal* mutant. Therefore, Gaalp is a membrane glycoprotein with an apparently large (~306 amino acids) luminal domain.

At the extreme COOH terminus of Gaalp there is a KXKXX sequence 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 Gaalp is localized to the ER, the protein was overproduced and local-

```
-792  tctagaccttcacattctacggaaattttacagggaagctttgctttttgccagaagaa
-732  gacttgttattctctatcaattctgcttttctgcaatttaaaagccagtatttccatgtt
-672  aaactaccctctaataccgaaattgtataatctttgtgttactcggaaaggttttaaaa
-612  tgtattcgaccacctagtaaggagacacgaagtgtctcaaacatttatcttgattttcgc
-552  tcgtttccatagtagccattctaatataaaacttacaaggtatgcatagattcttcccat
-492  gtagaatatcattgtcaaacatactactaccgtgagaatccaatctactaaaaagaccca
-432  gctaaagtccttttggtgtgtcaatggaacaccacgtaattgtgaaatagcttccatgct
-372  tgctaacttattaccgtcaatagaagacttgagtcaggtggctttaatgagtactattct
-312  tttttttttccaaagagcactatgttgataataccgcagtaatttttttgagtatcct
-252  gtagcctagaaaggttgaagcttataaaaccgtgccaacagctttatagtggggagtttg
-192  gcttccctctatttgtatattgatcgccatccctacgaagttattgggaacgcatcgtga
-132  acctctcactttaatgccagcggtagaaaaaaaagtcataaacaatacacgccggctaca
 -72  cttagaaagaaatgacatttgctgtctttataaaaggacttgacagaccaaagacgcgtat
 -12  aatacaccaagaatggccttattggagaagttgcatcgaaggattgttgatatggggctt
                M  A  L  L  E  K  L  H  R  R  I  V  D  M  G  L      16
  49  gtcccgcgtataatcgccttattaccagttatttccatgctatgcgctctatttgggttt
      V  P  R  I  I  A  L  L  P  V  I  S  M  L  C  A  L  F  G  F      36
 109  atttctatagctattctgcctatggatggacagtacagaagaacatacatttctgagaat
      I  S  I  A  I  L  P  M  D  G  Q  Y  R  R  T  Y  I  S  E  N      56
 169  gcattgatgccttcacaagcgtatagttactttagagaatctgaatggaacattttgagg
      A  L  M  P  S  Q  A  Y  S  Y  F  R  E  S  E  W  N  I  L  R      76
 229  ggctatcgatctcaaattaaagaaatggtaaacatgacttctatggaaagaaacaatttg
      G  Y  R  S  Q  I  K  E  M  V  N  M  T  S  M  E  R  N  N  L      96
 289  atgggttcttggttacaagaatttggtactaagactgctatttacgaaaatgaacaatat
      M  G  S  W  L  Q  E  F  G  T  K  T  A  I  Y  E  N  E  Q  Y     116
 349  ggagaaacattgtacggtgtaatgcacgctcctaggggtgatggaacagaagcgatggtg
      G  E  T  L  Y  G  V  M  H  A  P  R  G  D  G  T  E  A  M  V     136
 409  cttgccgtt***ccatgg***tttaattcagatgatgaattcaatattggcggcgcagctttgggt
      L  A  V  P  W  F  N  S  D  D  E  F  N  I  G  G  A  A  L  G     156
 469  gtatctttagcaagatttttctcacgttggccagtatggtccaagaatataattgttgtc
      V  S  L  A  R  F  F  S  R  W  P  V  W  S  K  N  I  I  V  V     176
 529  ttcagcgaaaatcctcgtgcagcattaagatcatgggttgaggcataccatacttcctta
      F  S  E  N  P  R  A  A  L  R  S  W  V  E  A  Y  H  T  S  L     196
 589  gatttgactggtggttccattgaagctgctgttgtgttggattattcgagtacggaagat
      D  L  T  G  G  S  I  E  A  A  V  V  L  D  Y  S  S  T  E  D     216
 649  ttcttcgagtatgtagaaatctcatacgacggtctgaatggtgagctgcccaatttggat
      F  F  E  Y  V  E  I  S  Y  D  G  L  N  G  E  L  P  N  L  D     236
 709  cttgtcaacatcgctatatccattacggaacatgaaggtatgaaagtttctttgcacggt
      L  V  N  I  A  I  S  I  T  E  H  E  G  M  K  V  S  L  H  G     256
 769  ctacccagtgatcagttaactaataataatttctggtcaagattaaaaatattatgcctg
      L  P  S  D  Q  L  T  N  N  N  F  W  S  R  L  K  I  L  C  L     276
 829  ggaataagggattgggcgttgtccggtgttaaaaagcc***ccatgg***taacgaggcatttagc
      G  I  R  D  W  A  L  S  G  V  K  K  P  H  G  N  E  A  F  S     296
 889  ggctggaggattcaatctgtaacattgaaagcacatggaaacagtggtcatgatattact
      G  W  R  I  Q  S  V  T  L  K  A  H  G  N  S  G  H  D  I  T     316
 949  acatttggacgtatacccgaagcaatgtttcgctctattaataacctttggaaaaattt
      T  F  G  R  I  P  E  A  M  F  R  S  I  N  N  L  L  E  K  F     336
1009  caccaatcgttcttctttattgttattagcaccacgtcagttcgtatccattagtagt
      H  Q  S  F  F  F  Y  L  L  L  A  P  R  Q  F  V  S  I  S  S     356
1069  tatttgccaagcgctgtggctttatctatagcattcgccataagttcattaaatgcattt
      Y  L  P  S  A  V  A  L  S  I  A  F  A  I  S  S  L  N  A  F     376
1129  ataaacaatgcttatgcaaatatatccttattttccgagtataatttggtagcgttgttg
      I  N  N  A  Y  A  N  I  S  L  F  S  E  Y  N  L  V  A  L  L     396
1189  gtttggttcgtgtcattggtgatatcatttgttgtttcacaagcgtttcttctaatacct
      V  W  F  V  S  L  V  I  S  F  V  V  S  Q  A  F  L  L  I  P     416
1249  tcatcgggattattgatgacaattagcatggcatcttgtttttttacctttgatactttcc
      S  S  G  L  L  M  T  I  S  M  A  S  C  F  L  P  L  I  L  S     436
1309  agaaaaattcacatctcagaaccactatcatacaggttgaaaaatgttgctttttatat
      R  K  I  H  I  S  E  P  L  S  Y  R  L  K  N  V  A  F  L  Y     456
1369  ttcagtttggtttcaacatctttgctaatgataaactttgcaatggctttactgatcggc
      F  S  L  V  S  T  S  L  L  M  I  N  F  A  M  A  L  L  I  G     476
1429  acattggcatttcctatgacatttgtgaagaccattgttgaaagttctagcgaacatgag
      T  L  A  F  P  M  T  F  V  K  T  I  V  E  S  S  S  E  H  E     496
1489  gtgacaactcaatcctctaacccaataaaaactgagccgaaagatgagatagagctcgtc
      V  T  T  Q  S  S  N  P  I  K  T  E  P  K  D  E  I  E  L  V     516
1549  gagaatcacatggatacaacgccagcaacccccaacaacagaaacaaaaactaaaaaat
      E  N  H  M  D  T  T  P  A  T  P  Q  Q  Q  K  Q  K  L  K  N     536
1609  ttagtactattaattttgacaaatccatttatttcaataaccttattcggactatttttt
      L  V  L  L  I  L  T  N  P  F  I  S  I  T  L  F  G  L  F  F     556
1669  gatgatgaatttcatggatttgatataataaacaaactggtttcagcatggttggatttg
      D  D  E  F  H  G  F  D  I  I  N  K  L  V  S  A  W  L  D  L     576
1729  aaatgttggagttggtttgtactttgtataggttggcttccatgttggctattgatatta
      K  C  W  S  W  F  V  L  C  I  G  W  L  P  C  W  L  L  I  L     596
1789  gcgtcatcgtttgaatctaaatctgtcgtagtaaggtcgaaagaaaagcaaagttaggtg
      A  S  S  F  E  S  K  S  V  V  V  R  S  K  E  K  Q  S  *       614
1848  ccattggtaagaagtaaaggattaacgggtgttcgaactacgcaattgattttatacgca
1909  caaacattacaaatcgaagcaaaagaagatgaaatgatatgatatactgatacaaatatg
1969  tattaaataaaacataagtattgatggaaacatggcatagagaagcgaagatgcgataat
2029  atcaaataactatatgtgagttgtggaagaaagaaacgatcacattattataataacta
2089  gctatttaaatgtttattgaagactgttctgccccctttattcagtcacggtattggtc
2149  aaaaaattctttatggaaactttcccatttcttaatccattccagaccaggtgccaaaaa
2209  tgttgttcttaaatggtaagtaccaggttcttgaccaaacccagaaccgggaagagtgca
2269  aatgccagtagattctaacaacttcttacaataaaattcatccggagttaactctaagtg
2329  gcgagcttcttgaactgccttgaaaggtaagtctatcttagggaataaatacatggcacc
2389  ttgaggcttttgacattcaatgccttctaaagagttaaatgtctcatacagtgtcattgc
2449  tcttgtaattaacttttcatggatggagttacgttctgcttggtccgactcgaatgattc
2509  ctccccttccactggtggacgaaccatcaaatcaaccaaagctt
```

*Figure 4.* Sequence analysis of *GAA1*. The DNA sequence of the *GAA1* gene is shown with nucleotide positions indicated in the left margin. The deduced protein sequence of Gaa1p is shown in single-letter code, with the amino acid numbers in the right margin. Uncharged and/or hydrophobic regions of Gaa1p are in boldface. N-linked glycosylation consensus sequences are underlined. The putative ER localization sequence is double underlined. The NcoI sites used to construct the deletion disruption mutant are in italics and boldface. The GenBank accession number is X79409.

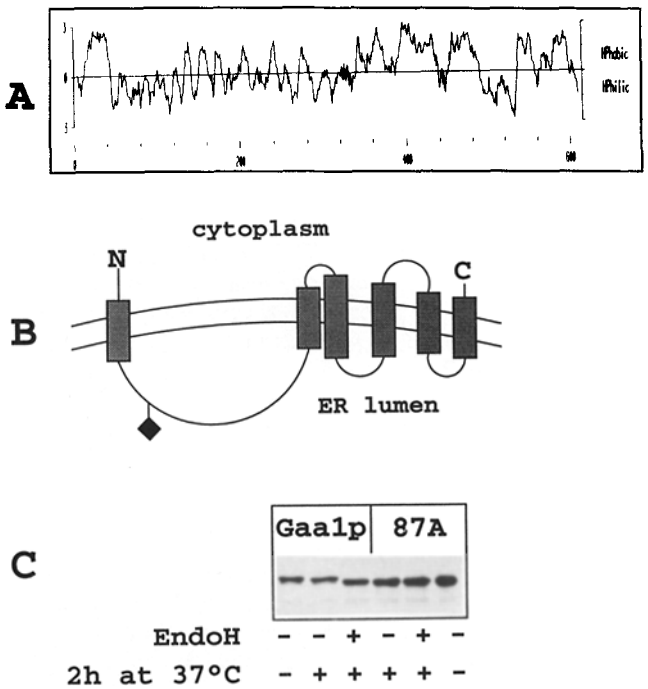



*Figure 5.* Gaalp is glycosylated on $N^{87}$. (*A*) Kite-Doolittle hydrophobicity blot of Gaalp. (*B*) Model of the transmembrane orientation of Gaalp. The diamond is the N-linked glycosylation site. The hydrophobic stretches are in shaded boxes. (*C*) Extracts from cells overexpressing wild-type *GAA1* or *gaal-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.

ized by 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 Wbplp, a part of the oligosaccharyl transferase that is known to be localized to the ER in yeast (te Heesen et al., 1991, 1993). Without overproduction of Gaalp, no fluorescence signal was seen, illustrating the specificity of the antibody and staining protocol (data not shown). Attempts to use immunochemical amplification techniques did not allow detection of Gaalp expressed at normal levels. In addition, when the predicted cytoplasmic tail of Gaalp (amino acids 603–614) was transferred onto a plasma membrane protein, the α-factor receptor (Ste2p), the latter was localized to the ER (Hamburger, 1994), showing that the COOH terminus is sufficient for ER localization. Therefore, Gaalp can be localized to the ER, where the process of GPI anchoring occurs.

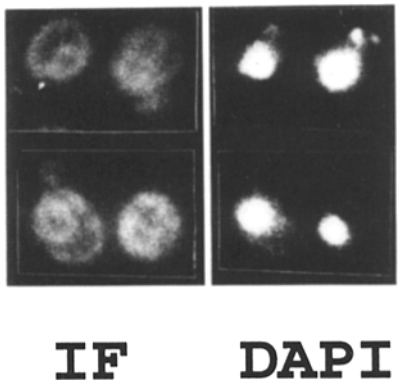



*Figure 6.* Immunofluorescence localization of overexpressed Gaalp. RH144-3D carrying plasmid pDH15 were fixed and processed for immunofluorescence using affinity-purified antibody raised against a COOH-terminal peptide of Gaalp. On the left side is the immunofluorescence due to Gaalp; on the right side is DAPI staining to localize the nucleus. Virtually no immunofluorescence was seen when strain RH144-3D did not carry pDH15.

### *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 [$^3$H]mannose or [$^3$H]inositol. Since the complete GPI anchor precursors (CP1 and CP2) are very difficult to detect in wild-type cells by [$^3$H]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. [$^3$H]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

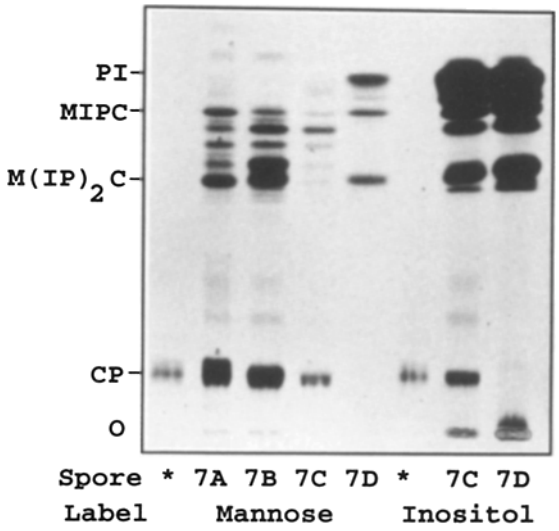



*Figure 7.* Mannose and inositol labeling of *gaal*, *pmi40* segregants. Strains RH401-7A (*pmi40*), -7B (*pmi40,gaal*), -7C (*gaal*), and -7D were grown overnight and labeled with [$^3$H]mannose or [$^3$H]*myo*-inositol at 37°C as described in Materials and Methods. Lipids were extracted and separated 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.

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 mannosylinositoldiphosphoceramide. 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 $M(IP)_2C$ 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 inositolphosphoceramides 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*

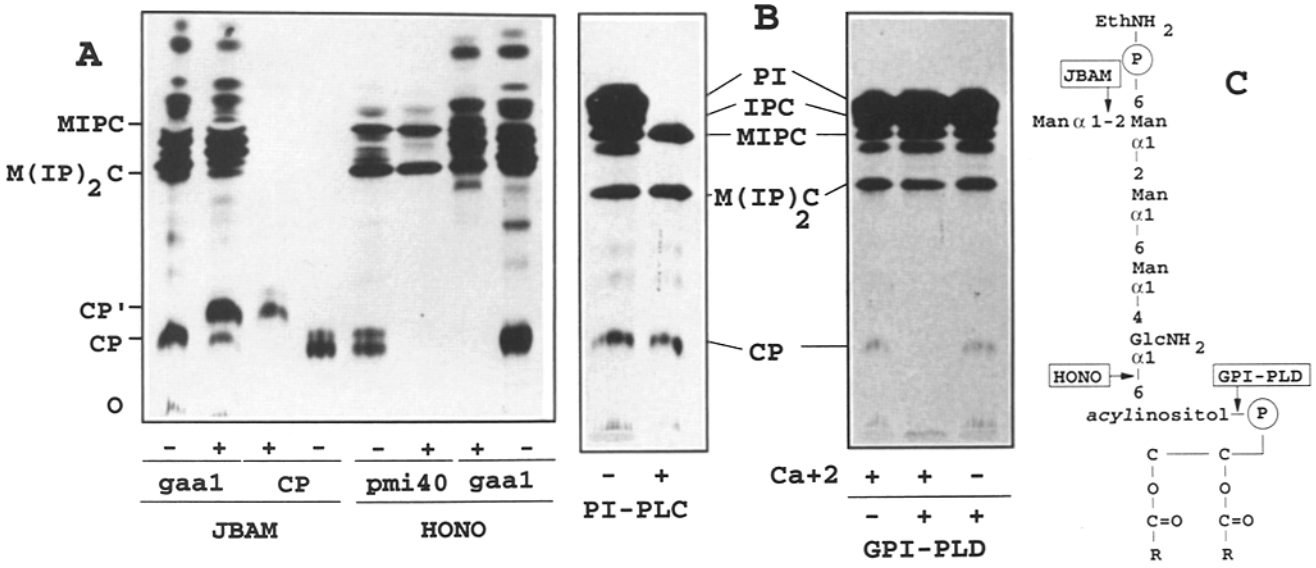



*Figure 8.* Enzymatic and chemical treatments of lipid extracts. (*A*) Mannose-labeled lipids were prepared as in Fig. 7 from strains RH401-7A (pmi40) and RH401-7B (gaal) and subjected to nitrous acid deamination (*HONO*), or lipids from RH401-7B were treated along with the complete GPI precursor standard (*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*, mannosylinositolphosphoceramide, *M(IP)$_2$C*, mannosyldiinositolphosphoceramide. −, 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 inositolphosphoceramide (*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 *gaal* 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 ($N^{506}$; 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 ($-T^{509}N^{510}$) and three anchor attachment site mutants, $D^{506}$, $C^{506}$, and $Q^{506}$. Cells expressing the mutant Gasl 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 $D^{506}$ mutation (data not shown), and a statistically significant difference was found with the $C^{506}$ mutation (Fig. 9). Without Gaalp overproduction, 27 ± 6% of the $C^{506}$-Gaslp was matured and thus GPI anchored, whereas with Gaalp overproduction, 43 ± 6% of the $C^{506}$-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.

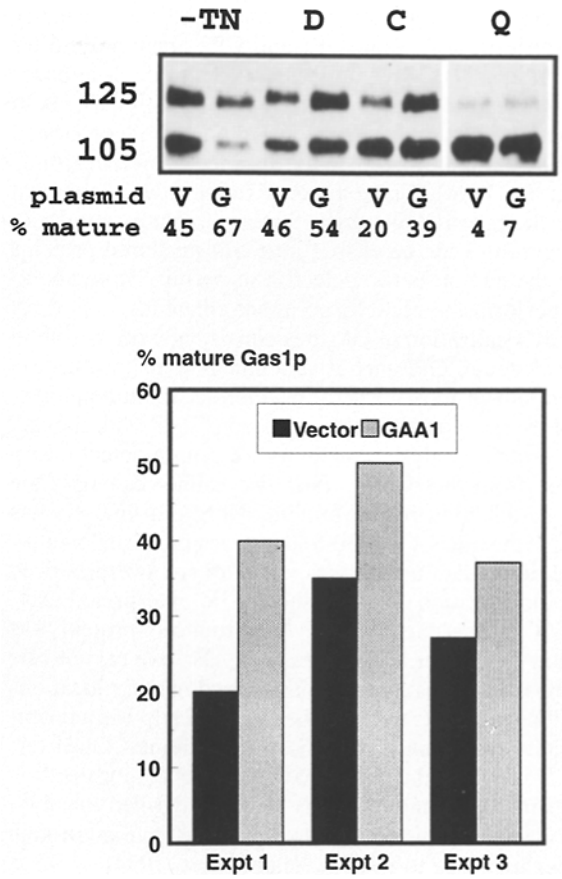



*Figure 9. GAA1* overexpression partially suppresses Gaslp anchor attachment site mutants. Strain RH392-3A (*gasl*Δ::*LEU2*) carrying plasmids encoding Gaslp anchor attachment site or spacer mutants were transformed with plasmid YEplac195 as control (*V*) or plasmid pDH17 (*G*) to cause *GAA1* overexpression. Total protein extracts were prepared from log phase cells and analyzed by Western blotting using antibodies against Gaslp, secondary antibody coupled to peroxidase, and enhanced chemiluminescence. Some variation in the total amount of Gaslp found by Western blotting could be due to the fact that the mutant proteins were expressed from plasmids. The signals were quantified by scanning of the x-ray films. Mature, GPI-anchored Gaslp, 125, immature, unanchored Gaslp, 105. (*Top panel*) Analysis of Gaslp mutants with residues $T^{509}N^{510}$ in the spacer region deleted (−TN), attachment site mutants $D^{506}$ (*D*), $C^{506}$ (*C*), and $Q^{506}$ (*Q*). (*Bottom panel*) Quantitation of three separate experiments analyzing the $C^{506}$ *gasl* mutant.

## *Discussion*

In this study we have shown that the *gaal* 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 *gaal* mutant synthesized only the complete precursor CP2 and not CP1. This could be explained by the fact that CP2 synthesis is predominant when cells are under stress (Sipos et al., 1994). As the *gaal* mutant is already at least partially defective for GPI anchor-

ing at permissive temperature, the cells could be constantly under stress. In any event, CP1 and CP2 apparently differ only in their fatty acyl chains, as no detectable differences in the complex head groups were found. Additionally, both CP1 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 (KEKQS) 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 defective 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 Gaslp 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.

We would like to thank F. Hamburger-Crausaz and T. Aust for excellent technical assistance, A. Conzelmann (University of Fribourg, Switzerland) for the *pmi40* mutant strain and complete GPI standard, A. Conzelmann and M. Ferguson for their advice on glycolipid analysis, and C. Sütterlin for critical reading of the manuscript.

This work was funded by the Canton of Basel-Stadt, a grant from the Swiss National Science Foundation (H. Riezman), and a European Molecular Biology Organization postdoctoral fellowship (M. Egerton).

Received for publication 13 December 1994 and in revised form 25 January 1995.

### *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. Hillmen, T. Miyata, N. Yamada, J. Takeda, L. Luzzatto, 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 glycophosphoinositol-anchored membrane proteins of *Saccharomyces cerevisiae. EMBO (Eur. Mol. Biol. Organ.) J.* 11:457–466.

Dulić, V., M. Egerton, I. Elguindi, S. Raths, B. Singer, and H. Riezman. 1991. Yeast endocytosis assays. *Methods Enzymol.* 194:697–710.

Englund, P. T. 1993. The structure and biosynthesis of glycosyl phosphatidylinositol protein anchors. *Annu. Rev. Biochem.* 62:121–138.

Fankhauser, C., and A. Conzelmann. 1991. Purification, biosynthesis and cellular localization of a major 125-k Da glycophosphatidylinositol-anchored membrane glycoprotein of Saccharomyces cerevisiae. *Eur. J. Biochem.* 195:439–448.

Fankhauser, C., S. M. Homans, J. E. Thomas-Oates, M. J. McConville, C. Desponds, A. Conzelmann, and M. A. J. Ferguson. 1993. Structures of glycosylphosphatidylinositol membrane anchors from *Saccharomyces cerevisiae*. *J. Biol. Chem.* 268:26365–26374.

Gaynor, E. C., S. te Heesen, T. R. Graham, M. Aebi, and S. D. Emr. 1994. Signal-mediated retrieval of a membrane protein from the Golgi to the ER in yeast. *J. Cell Biol.* 127:653–665.

Gerber, L. D., K. Kodukula, and S. Udenfriend. 1992. Phosphatidylinositol glycan (PI-G) anchored membrane proteins. Amino acid requirements adjacent to the site of cleavage and PI-G attachment in the COOH-terminal signal peptide. *J. Biol. Chem.* 267:12168–12173.

Gietz, R. D., and A. Sugino. 1988. New yeast–*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair recognition sites. *Gene.* 74:527–534.

Güther, M. L., W. J. Masterson, and M. A. Ferguson. 1994. The effects of phenylmethylsulfonyl fluoride on inositol-acylation and fatty acid remodeling in African trypanosomes. *J. Biol. Chem.* 269:18694–18701.

Hall, M. N., C. Craik, and Y. Hiraoaka. 1990. Homeodomain of yeast repressor $\alpha$2 contains a nuclear localization signal. *Proc. Natl. Acad. Sci. USA.* 87:6954–6958.

Hamburger, D. 1994. Characterization of the End2 protein which is required for endocytosis in *Saccharomyces cerevisiae*. Ph.D. thesis. University of Basel, Basel, Switzerland. 117 pp.

Harlow, E., and D. Lane. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Hirose, S., R. P. Mohney, S. C. Mutka, L. Ravi, D. R. Singleton, G. Perry, A. M. Tartakoff, and M. E. Medof. 1992. Derivation and characterization of glycoinositol-phospholipid anchor-defective human K562 cell clones. *J. Biol. Chem.* 267:5272–5278.

Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene.* 7:51–59.

Horvath, A., and H. Riezman. 1994. Rapid protein extraction from *Saccharomyces cerevisiae*. *Yeast.* 10:1305–1310.

Horvath, A., C. Sütterlin, U. Manning-Krieg, N.-R. Movva, and H. Riezman. 1994. Ceramide biosynthesis enhances transport of GPI-anchored proteins to the Golgi apparatus in yeast. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:3687–3695.

Hyman, R. 1988. Somatic genetic analysis of the expression of cell surface molecules. *Trends Genet.* 4:5–8.

Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* 153:163–168.

Jackson, M. R., T. Nilsson, and P. A. Peterson. 1990. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. *EMBO (Eur. Mol. Mol. Organ.) J.* 9:3153–3162.

Kodukula, K., D. Cines, R. Amthauer, L. Gerber, and S. Udenfriend. 1992. Biosynthesis of phosphatidylinositol-glycan (PI-G)-anchored membrane proteins in cell-free systems: cleavage of the nascent protein and addition of the PI-G moiety depend on the size of the COOH-terminal signal peptide. *Proc. Natl. Acad. Sci. USA.* 89:1350–1353.

Kodukula, K., L. D. Gerber, R. Amthauer, L. Brink, and S. Udenfriend. 1993. Biosynthesis of glycosylphosphatidylinositol (GPI)-anchored membrane proteins in intact cells: specific amino acid requirements adjacent to the site of cleavage and GPI attachment. *J. Cell Biol.* 120:657–664.

Kübler, E., F. Schimmöller, and H. Riezman. 1994. Calcium-independent calmodulin requirement for endocytosis in yeast. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:5539–5546.

Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Leidich, S. D., D. A. Drapp, and P. Orlean. 1994. A conditionally lethal yeast mutant blocked at the first step in glycosyl phosphatidylinositol anchor synthesis. *J. Biol. Chem.* 269:10193–10196.

McConville, M. J., and M. A. J. Ferguson. 1993. The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. *Biochem. J.* 294:305–324.

Menon, A. K., R. T. Schwarz, S. Mayor, and G. A. Cross. 1990. Cell-free synthesis of glycosyl-phosphatidylinositol precursors for the glycolipid membrane anchor of Trypanosoma brucei variant surface glycoproteins. Structural characterization of putative biosynthetic intermediates. *J. Biol. Chem.* 265:9033–9042.

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

Moran, P., and I. W. Caras. 1991. Fusion of sequence elements from non-anchored proteins to generate a fully functional signal for glycophosphatidylinositol membrane anchor attachment. *J. Cell Biol.* 115:1595–1600.

Moran, P., and I. W. Caras. 1992. Proteins containing an uncleaved signal for glycophosphatidylinositol membrane anchor attachment are retained in a post-ER compartment. *J. Cell Biol.* 119:763–772.

Moran, P., and I. W. Caras. 1994. Requirements for glycosylphosphatidylinositol attachment are similar but not identical in mammalian cells and parasitic protozoa. *J. Cell Biol.* 125:333–343.

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

Munn, A., and H. Riezman. 1994. Endocytosis is required for the growth of vacuolar $H^+$-ATPase-defective yeast: identification of six new *END* genes. *J. Cell Biol.* 127:373–386.

Nuoffer, C., P. Jeno, A. Conzelmann, and H. Riezman. 1991. Determinants for glycophospholipid anchoring of the Saccharomyces cerevisiae GAS1 protein to the plasma membrane. *Mol. Cell. Biol.* 11:27–37.

Nuoffer, C., A. Horvath, and H. Riezman. 1993. Analysis of the sequence requirements for glycosylphosphatidylinositol anchoring of Saccharomyces cerevisiae Gas1 protein. *J. Biol. Chem.* 268:10558–10563.

Puoti, A., and A. Conzelmann. 1992. Structural characterization of free glycolipids which are potential precursors for glycophosphatidylinositol anchors in mouse thymoma cell lines. *J. Biol. Chem.* 267:22673–22680.

Sanger, F., S. Nicklen, and S. R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463–5471.

Schönbächler, M., A. Horvath, J. Fassler, and H. Riezman. 1995. The yeast *SPT14* gene is homologous to the human PIG-A gene and is required for GPI synthesis. *EMBO (Eur. Mol. Biol. Organ.) J.* In press.

Simons, K., and G. van Meer. 1988. Lipid sorting in epithelial cells. *Biochemistry.* 27:6197–6202.

Singer, B., and H. Riezman. 1990. Detection of an intermediate compartment involved in transport of alpha-factor from the plasma membrane to the vacuole in yeast. *J. Cell Biol.* 110:1911–1922.

Singer Krüger, B., R. Frank, F. Crausaz, and H. Riezman. 1993. Partial purification and characterization of early and late endosomes from yeast. Identification of four novel proteins. *J. Biol. Chem.* 268:14376–14386.

Sipos, G., A. Puoti, and A. Conzelmann. 1994. Glycosylphosphatidylinositol membrane anchors in Saccharomyces cerevisiae: absence of ceramides from complete precursor glycolipids. *EMBO (Eur. Mol. Biol. Organ.) J.* 13: 2789–2796.

Stevens, T., B. Esmon, and R. Schekman. 1982. Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. *Cell.* 30:439–448.

Stevens, V. L. 1993. Regulation of glycosylphosphatidylinositol biosynthesis by GTP. Stimulation of N-acetylglucosamine-phosphatidylinositol deacylation. *J. Biol. Chem.* 268:9718–9724.

Takeda, J., T. Miyata, K. Kawagoe, Y. Iida, Y. Endo, T. Fujita, M. Takahashi, T. Kitani, and T. Kinoshita. 1993. Deficiency of the GPI anchor caused by a somatic mutation of the PIG-A gene in paroxysmal nocturnal hemoglobinuria. *Cell.* 73:703–711.

te Heesen, S., R. Rauhut, R. Aebersold, J. Abelson, M. Aebi, and M. W. Clark. 1991. An essential 45 kD yeast transmembrane protein reacts with anti-nuclear pore antibodies: purification of the protein, immunolocalization and cloning of the gene. *Eur. J. Cell Biol.* 56:8–18.

te Heesen, S., R. Knauer, L. Lehle, and M. Aebi. 1993. Yeast Wbp1p and Swp1p form a protein complex essential for oligosaccharyl transferase activity. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:279–284.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

Vidugiriene, J., and A. K. Menon. 1993. Early lipid intermediates in glycosylphosphatidylinositol anchor assembly are synthesized in the ER and located in the cytoplasmic leaflet of the ER membrane bilayer. *J. Cell Biol.* 121:987–996.

Vidugirene, J., and A. K. Menon. 1994. The GPI anchor of cell-surface proteins is synthesized on the cytoplasmic face of the endoplasmic reticulum. *J. Cell Biol.* 127:333–341.

Zanolari, B., and H. Riezman. 1991. Quantitation of alpha-factor internalization and response during the Saccharomyces cerevisiae cell cycle. *Mol. Cell. Biol.* 11:5251–5258.