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Structures of Glycosylphosphatidylinositol Membrane Anchors from *Saccharomyces cerevisiae**

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Metabolic labeling studies suggest that *Saccharomyces cerevisiae* contains many glycoproteins that are anchored in the lipid bilayer by glycosylphosphatidylinositol membrane anchors. Membrane anchors were purified from a crude yeast membrane protein fraction and analyzed by two-dimensional ^1H-^1H NMR, fast atom bombardment-mass spectrometry, compositional and methylation linkage analyses, as well as chemical and enzymatic modifications. The yeast glycosylphosphatidylinositol anchors consist of the following structures: ethanolamine-PO_4-6(R-2)Manα1–2Manα1–6Manα1–4Glc$NH_2\alpha$1–6*myo*-inositol-1-PO_4-lipid, where R is mainly Manα1- (80%) with some Manα1–2Manα1- (15%) and Manα1–3Manα1- (5%). The core region of the yeast anchors (ethanolamine-PO_4-6Manα1–2Manα1–6Manα1–4Glc$NH_2\alpha$1–6*myo*-inositol-1-PO_4) is identical to the conserved core region found in glycosylphosphatidylinositol anchors from protozoa and mammals. The lipid moieties of the total yeast glycosylphosphatidylinositol anchors are mainly ceramides, consisting mostly of C18:0 phytosphingosine and C26:0 fatty acid. However, the lipid moiety of the glycosylphosphatidylinositol anchor of the purified ggp125 protein is a *lyso*- or diacylglycerol, containing C26:0 fatty acids. This suggests that yeast adds different lipid components to the glycosylphosphatidylinositol anchors of different proteins.

The attachment of proteins to biological membranes by glycosylphosphatidylinositol (GPI)[1] anchors is a protein modification found in many different proteins from a wide range of eukaryotic organisms (for reviews see Ferguson and Williams (1988), Low (1989), Doering *et al.* (1990), Thomas *et al.* (1990), Cross (1990), and Ferguson (1991)). Analyses of the GPI anchors of the *Trypanosoma brucei* variant surface glycoprotein (VSG) (Ferguson *et al.*, 1988), rat brain Thy-1 (Homans *et al.*, 1988), the *Leishmania major* promastigote surface protease (Schneider *et al.*, 1990), and *Trypanosoma cruzi* 1G7 antigen (Güther *et al.*, 1992) revealed a common core structure that links the COOH terminus of the mature protein to the lipid moiety, *i.e.* ethanolamine-P-6Manα1–2Manα1–6Manα1–4Glc$NH_2\alpha$1–6*myo*-inositol-1-P. The partial structure of the human erythrocyte acetylcholinesterase GPI anchor is also consistent with this common core region (Roberts *et al.*, 1988b; Deeg *et al.* 1992). GPI anchors may contain species-specific side chains made of carbohydrate or ethanolamine (EtN) phosphate moieties (Ferguson, 1991). The lipid moieties of GPI anchors also are variable between different species and cell types and consist of *sn*-1,2-dimyristoylglycerol in *T. brucei* VSGs (Ferguson *et al.*, 1985a, 1985b), *sn*-1-stearoyl-2-*lyso*-glycerol in *T. brucei* procyclic acidic repetitive protein (Field *et al.*, 1991), *sn*-1-alkyl-2-acylglycerols in bovine and human erythrocyte acetylcholinesterase (Roberts *et al.*, 1987, 1988a, 1988b), human erythrocyte decay accelerating factor (Walter *et al.*, 1990), human folate-binding protein (Luhrs and Slomiany, 1989), *L. major* promastigote surface protease (Schneider *et al.*, 1990), *sn*-1,2-diacyl-glycerols in *Torpedo* acetylcholinesterase (Bütikofer *et al.*, 1990), and ceramide in *D. discoideum* contact site A protein (Stadler *et al.*, 1989) and in GPI anchors of yeast (Conzelmann *et al.*, 1992). In addition, an extra fatty acid (palmitate) is found in hydroxyester linkage to the inositol ring in some cases, such as human erythrocyte acetylcholinesterase (Roberts *et al.*, 1988a, 1988b), decay accelerating factor (Walter *et al.*, 1990) and *T. brucei* procyclic acidic repetitive protein (Field *et al.*, 1991; Ferguson, 1992a).

Metabolic labeling studies in the yeast *Saccharomyces cerevisiae* suggest the presence of numerous GPI-anchored membrane glycoproteins (Conzelmann *et al.*, 1988, 1990), only one of which has been purified (Vai *et al.*, 1990, 1991; Fankhauser and Conzelmann, 1991; Nuoffer *et al.*, 1991). Here we report on a method for the preparation of GPI anchors from a crude yeast membrane fraction without previous purification of individual proteins. The structures of GPI anchors in preparations obtained from wild type cells (X2180-1A) and from a strain lacking vacuolar hydrolases (82-2 *pep4-3*; Woolford *et al.* (1986)) and the lipid moiety of the purified GPI-anchored ggp125 protein are described. Our analyses thus elucidate not only the structure of the GPI anchor of a single protein but the whole spectrum of GPI anchors made by yeast cells.

EXPERIMENTAL PROCEDURES

Yeast Strains, Culture Conditions, and Materials

X2180-1A and 82-2 *pep4-3* yeast strains were maintained and grown

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[1] The abbreviations used are: GPI, glycosylphosphatidylinositol; AHM, anhydromannitol; COSY, correlated spectroscopy; Endo H, endoglycosidase H; EtN, ethanolamine; FAB, fast atom bombardment; GC-MS, gas chromatography-mass spectrometry; HF, hydrofluoric acid; HPLC, high pressure liquid chromatography; LAGP, lipid-containing anchor glycopeptide; NOESY, nuclear Overhauser effect spectroscopy; PI-PLC, phosphatidylinositol-specific phospholipase C; SAGP, soluble anchor glycopeptide; VSG, variant surface glycoprotein.

as recently described (Conzelmann *et al.*, 1990). Wickerham's minimal-medium (Wickerham, 1946) without inositol but with 2% glucose and 1% casein hydrolysate is referred to as SDC medium. Bio-Gel P10 and P4, AG3X4, AG50X12, and Chelex 100 were from Bio-Rad; GlcN and EtN standards were from Fluka (Buchs, Switzerland); the amino acid standards, 6 N HCl, and Teflon vials were from Pierce (Oud-Beijerlands, The Netherlands); Triton X-114 was from Serva (Heidelberg, Federal Republic of Germany (FRG)). Triton X-100, Pronase, QAE-Sephadex A25, the lipid standards C16:0, C17:0, C18:0, C20:0, C22:0, and C26:0 fatty acid methyl esters, as well as yeast phytosphingosine, D-sphingosine from bovine brain and DL-dihydrosphingosine were from Sigma. Jack bean α-mannosidase, PI-PLC from *Bacillus cereus*, and endoglycosidase H from *Streptomyces plicatus* (Endo H) were from Boehringer (Mannheim, FRG), triethylamine, D_2O (gold grade), and hydrazine were from Aldrich (Steinheim, FRG). Octyl-Sepharose, fast protein liquid chromatography system, and appropriate columns were from Pharmacia (Uppsala, Sweden). *Aspergillus phoenicis* α-mannosidase was from Oxford Glycosystems (Abingdon, United Kingdom), *scyllo*-inositol was from Calbiochem (San Diego, CA), BF_3-methanol and 0.5 N HCl in methanol were from Supelco (Gland, Switzerland), EN^{3}HANCE and [^{3}H]$NaBH_4$ (15 Ci mmol^{-1}) were obtained from DuPont NEN (Dreireich, FRG). Purified ggp125 was obtained from X2180-1A cells as described (Fankhauser and Conzelmann, 1991). β-Glucose oligomer standards were prepared by partial hydrolysis of dextran (Yamashita *et al.*, 1982). All other materials and chemicals were form sources described previously (Conzelmann *et al.*, 1990; Schneider *et al.*, 1990; Fankhauser and Conzelmann, 1991).

Preparation of Radiolabeled Tracer Proteins

Cells (1–2 × 10^8) from exponentially growing X2180-1A or 82-2 *pep4-3* cells were labeled in 1 ml of fresh SDC medium with 20–30 μCi of *myo*-[^{3}H]inositol for 60 min at 30 °C. After labeling for 1 h, approximately 99% of incorporated radioactivity is found in phospholipids and 1% in inositol-containing proteins (Conzelmann *et al.*, 1990). After the labeling the cells were broken with 100 μl of glass beads in 1.5-ml Eppendorf tubes in 100 μl of 3-fold concentrated "final sample buffer" (Laemmli, 1970) by vortexing (3 × 1 min). After boiling for 5 min and removal of insoluble material by centrifugation (8000 *g* × 5 min, 20 °C), solubilized proteins were precipitated by adding 100 μl of water, then 1 ml of solvent A (chloroform/methanol/water (10:10:3, v/v/v)) followed by vortexing for 30 s and centrifugation (8000 *g* × 5 min, 20 °C). The protein precipitate at the interphase was recovered and extracted four to six times with 1-ml aliquots of solvent A to remove detergent and free lipids. The delipidated proteins were dried under N_2 gas and resuspended in water by sonication in a bath sonicator (40 watts) and stored at −20 °C. When labeling 1–2 × 10^8 cells with 30 μCi of *myo*-[^{3}H]inositol, we typically recovered 130,000 cpm of delipidated protein. These tracer proteins were delipidated separately from the large amount of cold membrane proteins of the large scale preparation (see below) and were added to the latter before the Pronase digestion of the delipidated membrane proteins.

Isolation of Glycosylphosphatidylinositol Membrane Anchors

Preparation of Delipidated Membrane Proteins—The purification scheme is summarized in Fig. 1. X2180-1A or 82-2 *pep4-3* cells were grown aerobically at 30 °C in SDC medium in a 15-liter fermentor (Bioengineering) and harvested by centrifugation (6500 *g* × 5 min, 4 °C). Cells (100 g, wet weight) were resuspended in 300 ml of ice-cold buffer A (150 mM Na_2CO_3, pH 10.5) and broken for 2 min in a MSK glass bead homogenizer (Braun Melsungen) under refrigeration. Unbroken cells and large cell wall fragments (Fig. 1, *fraction I*) were removed by two successive centrifugations (1600 *g* × 5 min and 3000 *g* × 5 min, 4 °C, respectively). The cell wall pellet was washed with 300 ml of buffer A and centrifuged (3000 *g* × 5 min, 4 °C). The pooled 3000 × *g* supernatants were ultracentrifuged (30,000 *g* × 16 h, 4 °C) to sediment the membranes (Fig. 1, *fraction II*). The membrane pellet was extensively delipidated. For this, 10 g of membrane pellet (wet weight) were extracted with 45 ml of solvent A in a Dounce homogenizer (pestle B) with 20 strokes. The slurry was left on ice for 15 min and centrifuged (4000 *g* × 10 min, 4 °C). The protein precipitate at the interphase (Fig. 1, fraction III) was recovered and extracted six times with 45 ml of solvent A, whereby extensive Dounce homogenization after each centrifugation step achieved complete resuspension. The final protein pellet was dried, extracted with 30 ml of chloroform/methanol (1:1, v/v), and dried again (Fig. 1, *fraction IV*).

Preparation of Soluble Anchor Glycopeptides—Typically, 2 g of delipidated membrane proteins (dry weight) were digested with 100 mg of Pronase (Pronase treatment 1) in 20 ml of buffer B (100 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$, 0.02% NaN_3, 0.1 mg/ml gentamycin) for 48 h at 37 °C together with 10^6 cpm of *myo*-[^{3}H]inositol-labeled tracer proteins. The digest was boiled (15 min) to inactivate the Pronase, the suspension was diluted to 40 ml with 20 mM NaCl, and prepurified Triton X-114 (2% final) (Bordier, 1981) was added. After solubilization on ice for 15 min, the digest was centrifuged (4000 *g* × 5 min, 4 °C) to remove insoluble material.

The supernatant was incubated at 37 °C for 5 min to induce phase separation and was centrifuged (4000 *g* × 5 min, 25 °C) to sediment the Triton X-114 phase containing the hydropobic peptides (Fig. 1, *fraction V*) (Fankhauser and Conzelmann, 1991). The aqueous upper phase was discarded. The Triton X-114 phase was washed twice by addition of buffer C (150 mM disodium citrate, pH 5.5, 0.005% 2-mercaptoethanol, 0.02% NaN_3, 2 mM phenylmethylsulfonyl fluoride), resuspending at 0 °C, and precipitating at 37 °C. The washed detergent phase was finally diluted to 14 ml with buffer C containing 70 milliunits of Endo H. The reaction was incubated for 36 h at 37 °C with gentle shaking. After Endo H digestion the detergent phase was washed twice and then diluted to 20 ml with buffer B. After addition of 30 mg of Pronase, the peptides were incubated at 37 °C for 16 h (Pronase treatment 2). The protease was again inactivated by boiling, and the reaction was cooled down to 37 °C and centrifuged (4000 *g* × 3 min, 25 °C) to sediment the detergent phase (Fig. 1, *fraction VI*). The Triton X-114 phase was washed twice with 20 ml of buffer D (100 mM Tris, pH 7.4, 2 mM EDTA, 0.1 mM dithiothreitol, 0.02% NaN_3, 15 μg/ml leupeptin, 15 μg/ml pepstatin) and diluted to 25 ml with the same buffer, and 4.5 units of PI-PLC were added. After incubation at 37 °C for 24 h (gently shaking), phases were separated and the aqueous supernatant containing the soluble anchor glycopeptides (SAGP) was lyophilized. The dry material was dissolved in 2 ml of Triton X-100 (0.36%) and purified on a Bio-Gel P10 column (1.5 × 60 cm, 100-ml bed volume, equilibrated in 100 mM ammonium acetate, pH 7.0). Fractions containing ^{3}H were pooled and extracted with 1-butanol to remove detergent and stored at −20 °C (Fig. 1, *fraction VII*).

Purification of Lipid-containing Anchor Glycopeptides—To obtain complete GPI anchors, the peptides generated by Pronase treatment 1 were stored in 30% 1-propanol (propanol). After centrifugation (4000 *g* × 10 min, 20 °C) to remove insoluble material, soluble peptides were dried and resuspended in buffer E (5% propanol, 100 mM ammonium acetate) by heating to 100 °C, allowed to cool to 20 °C, and applied to an octyl-Sepharose column (2.5 × 40 cm, 110-ml bed volume, equilibrated in buffer E) connected to a fast protein liquid chromatography system.

The peptides were loaded onto the octyl-Sepharose column at a flow rate of 0.16 ml/min/cm^2. The column was washed with two column volumes of buffer E. Bound material was eluted at 20 °C with a linear gradient of 5–100% propanol over five column volumes at a flow rate of 0.2 ml/min/cm^2. Fractions (10 ml) were collected, and fractions containing the radioactive tracer were pooled and lyophilized. The propanol gradient was monitored by the refractive index. Eluted peptides (Fig. 1, *fraction VIII*) were further treated with Endo H and a second time with Pronase as described for SAGP and finally purified once more on octyl-Sepharose as described. Fractions containing radiolabel (Fig. 1, *fraction IX*) were kept at 4 °C for 16 h to precipitate a subclass of LAGP (Fig. 1, *fraction X*) containing short peptides only. The LAGP were resuspended in water and stored at −20 °C.

Amino Acid Analysis

The samples were hydrolyzed and derivatized with phenylisothiocyanate as described (Schneider *et al.*, 1990) and analyzed using a Pico Tag system (Waters, Milford, MA). Amino acid, EtN, and GlcN standards were analyzed before and after HCl treatment. Control experiments demonstrated a relatively high destruction of GlcN during acid hydrolysis, and all data were corrected correspondingly to obtain molar ratios. In order to separate the GlcN derivative from amino acid derivatives, it was necessary to add 600 μl of triethylamine to eluent A (see Pico Tag system operator's manuals 88140 and 07124) and, most importantly, to adjust the pH of eluent A to 5.10 with glacial acetic acid.

Mild Alkaline Hydrolysis

LAGP (820 cpm, 8 nmol) was solubilized in 240 μl of methanol/water (2:1, v/v) containing 100 mM NaOH, incubated for 30 min at 37 °C, and neutralized with 600 mM acetic acid in methanol. The neutralized reaction was dried and resuspended in water containing Triton X-114, and phases were separated as described (Fankhauser and Conzelmann, 1991). As control, an equal aliquot of the sample was incubated in parallel with a neutralized reaction mixture.

Published methods were used for the phenol sulfuric acid carbohy-

drate assay (Chaplin, 1986) and PI-PLC treatment of GPI anchors with enzyme from *B. cereus* (Fankhauser and Conzelmann, 1991).

Preparation of Tritiated Neutral Glycans

Twenty nmol of compound 2 (X2180-1A SAGP) or 14* (82-2 *pep4-3* SAGP) were deaminated with nitrous acid and reduced with [^{3}H]$NaBH_4$ and $NaBD_4$ as described (Ferguson, 1992b). Deaminated and reduced products were purified from impurities by descending paper chromatography on Whatman no. 3MM paper in 1-butanol/ethanol/water (4:1:1, v/v/v) and converted to compounds 4 and 15* by aqueous HF dephosphorylation (Ferguson, 1992b) or converted to compounds 11 and 19* by treatment with jack bean α-mannosidase prior to HF dephosphorylation. Tritium-labeled neutral glycans were desalted by passage through a column of 0.1 ml of AG50X12(H^+) layered over 0.2 ml of AG3X4(OH^-) over 0.1 ml of QAE-Sephadex(OH^-) and further purified by high voltage electrophoresis on Whatman no. 3MM paper in pyridine/acetic acid/water (3:1:387, v/v/v). Neutral glycans were analyzed by high performance liquid chromatography using a CarboPac PA1 column (4 × 250 mm) connected to a Dionex BioLC Carbohydrate analyzer equipped with a pulsed amperometric detector, anion membrane suppressor, and radioactivity flow monitor (Ramona, Raytest) (Ferguson, 1992b). For gel filtration, neutral glycans were analyzed on a Bio-Gel P4 column (1.5 × 100 cm) held at 55 °C and eluted with water at 0.2 ml min^{-1}. The column was connected to a radioactivity flow monitor and a refractive index monitor (Erma). Radiolabeled samples were analyzed together with β-glucose oligomer standards as described (Ferguson, 1992b).

α-Mannosidase Treatment

Samples were treated with 2.5 units (100 μl) of jack bean α-mannosidase or 0.02 milliunits (20 μl) of *Aspergillus phoenicis* α-mannosidase in 0.1 M NaAc, pH 5.0, for 2 h at room temperature followed by 16 h at 37 °C in a toluene atmosphere. Digests were terminated by heating to 100 °C for 5 min and desalted by passage through 0.2 ml of AG50X12(H^+) and evaporation with toluene (2 × 50 μl).

Acetolysis

Acetolysis was carried out as described (Ferguson, 1992b).

Gas Chromatography-Mass Spectometry (GC-MS)

All analyses were performed with a Hewlett-Packard 5890-MSD system using an SE54 column (30 m × 0.25 mm, Alltech). Methylation analyses were also analyzed using a SP2380 column (30 m × 0.25 mm, Supelco). Inositol, neutral sugar, phosphosugar, and methylation linkage analyses were performed as previously described (Ferguson, 1992b).

Lipid Analysis

An estimated 20 nmol of purified ggp125 or 40 nmol of compound 1 GPI anchors were analyzed for long-chain base and total fatty acid content following strong base hydrolysis as described by Ferguson (1992b), except that 1-heptadecanol was used as an internal standard instead of 1-hexadecyl glycerol. A sample of ggp125 was also analyzed for hydroxyester-linked fatty acids (Ferguson, 1992b). Samples containing hydroxylated fatty acid methyl esters were dried and treated with trimethylsilylation reagent prior to reanalysis by GC-MS.

Nuclear Magnetic Resonance (NMR)

Compound 2 was purified by gel filtration on a Bio-Gel P4 column and further desalted on 0.2 ml of Chelex X100(Na^+) layered over 0.2 ml of AG50X12(H^+) to eliminate di- and monovalent cations. Spectra were recorded at 500 MHz in D_2O as described (Ferguson *et al.*, 1988). Chemical shifts are referenced indirectly to acetone (2.225 ppm at 300 K).

Fast Atom Bombardment (FAB) Mass Spectrometry

20 nmol of compound 12 was permethylated by the method of Ciucanu and Kerek (1984) and the permethylated product recovered from the quenched reaction mixture after loading onto a C18 Sep-Pak cartridge (Waters Associates) from which the product was eluted in 40% (v/v) aqueous acetonitrile. *N*-Acetylation was achieved under conditions previously described (Lederkremer *et al.*, 1991). Hydrazinolysis was carried out using a modification of the method of Bendiak and Cummings (1985) in 1.5 ml of anhydrous hydrazine at 85 °C for 14 h. After hydrazinolysis the sample was lyophilized and *N*-acetylated as above, and the *N*-acetylated product was isolated from peptide fragments and other contaminants by paper chromatography in 1-butanol/ethanol/water (4:1:1, v/v/v) (Takasaki *et al.*, 1982). After elution from the paper and lyophilization, the sample was permethylated and purified on a Sep-Pak cartridge as described above. Glycerol/thioglycerol (1:1, v/v) was used as matrix.

RESULTS

Isolation of Soluble and Lipid-containing Anchor Glycopeptides

Soluble and lipid-containing anchor glycopeptides were purified from a crude membrane fraction in good yield by following the *myo*-[^{3}H]inositol-labeled tracer as outlined in Fig. 1. From 100 g (wet weight) of yeast cells, we typically obtained 25 g of membranes (wet weight) and 4 g of delipidated membrane protein (dry weight). Thorough delipidation of membrane proteins was required, since lipids interfered with the subsequent phase separations in Triton X-114, as well as with octyl-Sepharose column chromatography.

After Pronase digestion, hydrophobic peptides (fraction V) were enriched through phase separations in Triton X-114. Initially, these hydrophobic peptides were directly treated with PI-PLC without going through an Endo H and a second Pronase treatment. Analysis of the resulting SAGP by gel filtration on Bio-Gel P10 showed that about 30% of the tracer was excluded (not shown). When PI-PLC-treated fraction V was treated with Endo H and then loaded onto the P10 column, all of the label was included, while the bulk of (unlabeled) carbohydrate still was excluded (not shown). This suggests that the initially excluded material represents anchor peptides that contain large, Endo H-sensitive *N*-glycans. The presence of *N*-

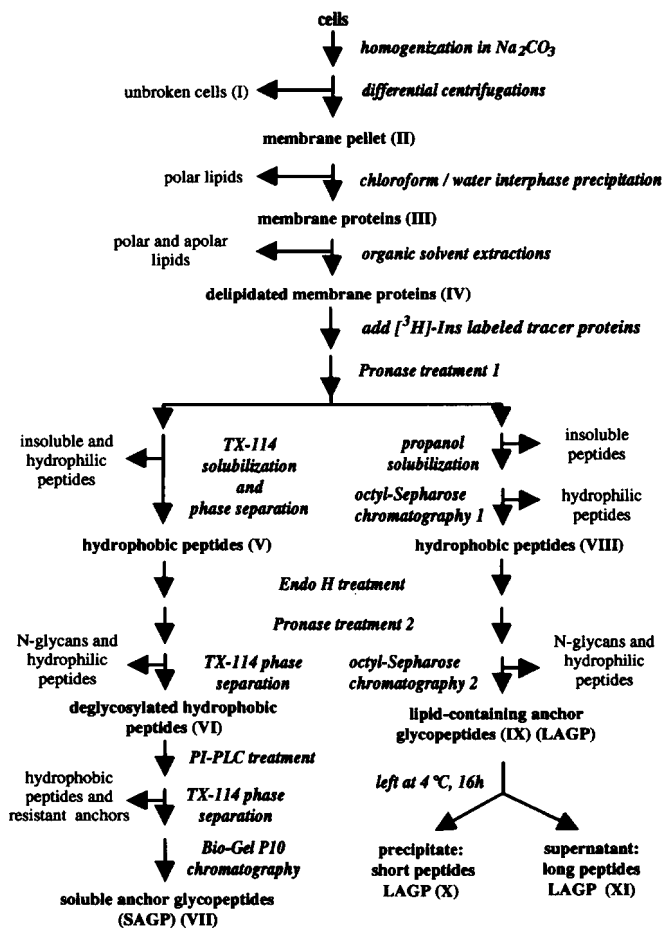


FIG. 1. **Purification scheme for soluble or lipid-containing anchor peptides.**

glycans in the vicinity of the anchor attachment site is expected to hinder proteolytic removal of amino acids close to the anchor. Thus, a second Pronase treatment of fraction V was introduced after the Endo H treatment to reduce the size of peptides on the GPI anchors. SAGP prepared in this way chromatographed on the P10 column as a single peak (Fig. 2*B*) at a position close to the fragment generated from fraction V by treatment with aqueous HF (Fig. 2*A*). In terms of radioactivity, 27% of counts present in the delipidated protein were recovered as SGAP. The major losses were due to incomplete digestion by Pronase (47% of the GPI-anchored proteins were not cleaved into soluble fragments), failure to partition into the Triton X-114 phase after the first Pronase treatment (16%), and incomplete PI-PLC treatment (10%).

We also prepared lipid-containing anchor glycopeptides (LAGP) by a method that retains the lipid moiety (Fig. 1, *lower right*). LAGP could easily be separated from other hydrophobic, Pronase-resistant peptides by reverse phase chromatography on octyl-Sepharose, a matrix that had previously been used to separate free glycoinositol phospholipids and the GPI anchor of a purified protease from *L. major* (McConville and Bacic, 1989; Schneider *et al.*, 1990). This procedure had the additional advantage that no detergent was required to keep anchor peptides in solution; thus, the interference of residual detergent during subsequent lipid analysis was avoided. Most of the LAGP eluted from octyl-Sepharose at a propanol concentration of 45% after Pronase treatment 1, but some minor peaks eluted at lower propanol concentrations (not shown). By analogy to the SAGP preparation, the fraction VIII LAGP were treated with Endo H and a second time with Pronase. The resulting LAGP (fraction IX) eluted from the octyl-Sepharose column as a homogeneous peak at 45% propanol (Fig. 3). LAGP containing on average 2 amino acids precipitated at 4 °C in 45% propanol (fraction X), while LAGP containing more amino acids remained soluble (fraction XI). The final yield of LAGP was 5 μmol from 214 g of yeast cells (wet weight). Of these LAGP, 98% were detergent-binding in a Triton X-114 phase separation assay and could be released into the aqueous phase by PI-PLC treatment (not shown). The purified LAGP were also subjected to mild alkaline hydrolysis, a treatment that hydrolyzes acylglycerols. Most LAGP partitioned into the Triton X-114 detergent phase after this treatment, which is in agreement with previous studies on metabolically labeled yeast GPI anchors (Conzelmann *et al.*, 1992). Based on the recovery of radiolabel, the yield of LAGP was 63% of starting material, the major losses being due to incomplete Pronase digestion (23%).

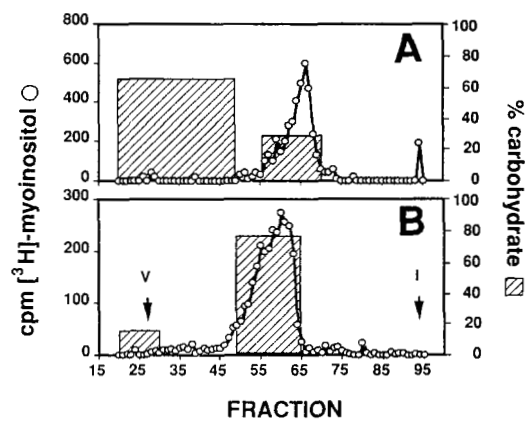


FIG. 2. **Bio-Gel P10 gel filtration of soluble anchor peptides and anchor fragments.** *Panel A*, an aliquot of *myo*-[^{3}H]inositol-labeled fraction V SAGP was first treated with PI-PLC, then dephosphorylated with aqueous HF and analyzed on a Bio-Gel P10 column. *Shaded boxes* represent carbohydrate content in pools of fractions. The width of the boxes delimits the pooled fractions and the height indicates the percentage of total carbohydrate found in that pool. Many fraction V SAGP contain large *N*-glycans (see text), which were separated from the labeled GPI glycan by aqueous HF treatment. *Panel B* shows the liquid chromatography of untreated fraction VII, the final purification step of SAGP. SAGP were monitored by following the radiolabeled tracer. The total carbohydrate content of fraction VII SAGP was much lower than of fraction V SAGP, since *N*-glycans had been removed by Endo H and were lost during the subsequent phase separation in Triton X-114 (Fig. 1). The excluded carbohydrate probably represents Endo H-resistant hydrophobic glycopeptides unrelated to GPI. *V*, void volume; *I*, included volume.

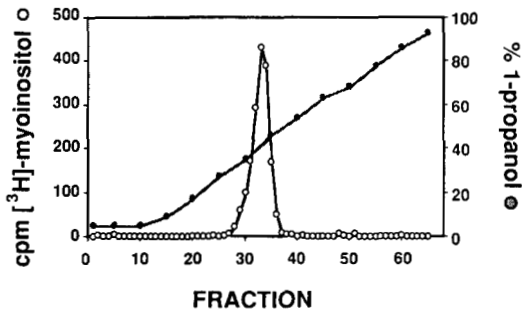


FIG. 3. **Reverse-phase chromatography of lipid-containing anchor peptides on an octyl-Sepharose column.** Fraction IX LAGP were loaded onto an octyl-Sepharose column and eluted with a linear 1-propanol gradient. LAGP were monitored by following the *myo*-[^{3}H] inositol-labeled tracer. The 1-propanol content was determined by the refractive index.

TABLE I
Composition of GPI anchor peptides

Results of neutral sugar analysis, phosphosugar analysis and amino acid analysis of SAGP (fraction VII) and LAGP (fraction X) are expressed as molar fractions of *myo*-inositol, which was set to 1.

Component	SAGP	LAGP
myo-Inositol	1	ND
Mannose	2.9	ND
Mannose 6-phosphate	+[a]	ND
Ethanolamine[b]	1.1	1
Glucosamine[b]	1.4	1.2
Asx[b]	1.1	0.5
Ser[b]	0.6	0.8
Gly[b]	0.5	0.3
Thr[b]	0.4	0.3
Tyr[b]	0.2	0

[a] Present but not quantified.
[b] Determined by amino acid analysis. ND, not determined.

Compositional Analysis of Soluble and Lipid-containing Anchor Glycopeptides

Inositol analysis by GC-MS of the SAGP revealed substantial amounts of *myo*-inositol, a typical constituent of all GPI anchors so far characterized, which is present in 1 mol/mol GPI anchor (Ferguson and Williams, 1988; Low, 1989; Cross, 1990; Thomas *et al.*, 1990; Ferguson, 1991). Thus, to calculate molar ratios of GPI anchor peptide components, we chose *myo*-inositol as reference. Compositional analysis of SAGP revealed only mannose (neutral sugar analysis) and mannose 6-phosphate (phosphosugar analysis) (Table I). Amino acid analysis of SAGP and LAGP revealed substantial amounts of GlcN and EtN and in addition an average of 2.8 (SAGP) or 1.9 (LAGP) amino acids/*myo*-inositol (Table I). *myo*-Inositol and EtN were present in equimolar amounts in SAGP, suggesting that yeast GPI anchors contain only the bridging EtN residue and lack the phosphoethanolamine side chain found in GPI anchors of higher eukaryotes (Ferguson, 1991). Interestingly, only a limited set of amino acids was found in the anchor glycopeptides, with Asx, Ser, Gly, and Thr being most prominent (Table I), suggesting that these amino acids might accommodate the *myo*-inositol-containing membrane anchor in a vast majority of yeast proteins.

Several amino acids were present in amounts of less than 1 residue/*myo*-inositol. This convinced us that the purified material represents a pool of GPI anchor peptides derived from several unrelated proteins. Assuming 1 *myo*-inositol and 1 EtN/anchor, we obtained about 0.8 μmol of SAGP and 2.3 μmol of LAGP from 100 g of yeast cells. Assuming an average molecular mass of 100 kDa for yeast GPI-anchored glycoproteins, we estimated that about 7% of the mass of yeast membrane proteins can be accounted for by GPI-anchored proteins.

Structural Analysis of Yeast Glycolipid Anchors

Fig. 4 shows the reaction scheme and analytical procedures used in this study to determine the structure of yeast glycolipid anchors. SAGP (*compound 2* in Fig. 4) were used for methylation linkage analysis, chemical and enzymatic glycan sequencing, NMR spectroscopy, and fast atom bombardment-mass spectrometry. LAGP (*compound 1* in Fig. 4) were used for lipid analysis and fast atom bombardment-mass spectrometry. *Compounds 1–15* refer to the products derived from GPI peptide material isolated from the X2180-1A strain, whereas *compounds 16* to 20** refer to the analogous products derived from the *pep4-3* strain of *S. cerevisiae*.

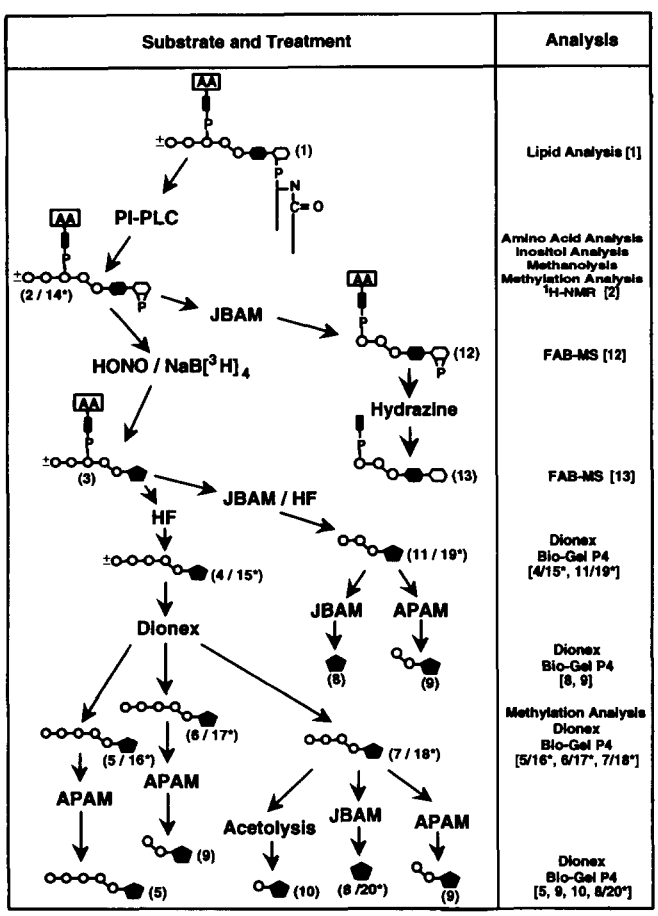


FIG. 4. **Scheme of reactions for analysis of the structure of yeast glycosylphosphatidylinositol anchors.** The order of chemical and enzymatic treatments applied to the isolated anchor peptides is indicated in the *first column*. A reference number in *brackets* is given to each compound. Numbers labeled with * refer to compounds derived from *pep4-3* membrane proteins, whereas unlabeled numbers refer to compounds originating from X2180-1A wild type cells. The *second column* indicates the analytical procedures used to confirm the features of the fragments depicted in the first column. Numbers in *brackets* indicate the compound that has been analyzed. *AA*, amino acid(s); *APAM*, *A. phoenicis* α-mannosidase; *HF*, cold aqueous 50% hydrofluoric acid; *JBAM*, jack bean α-mannosidase; *P*, phosphate; , 2,5-anhydromannitol; , ethanolamine; , glucosamine; ○, mannose; , *myo*-inositol.

TABLE II
Lipid analysis

The amounts of individual components are expressed in nanomolar quantities in the table. Compound 1 (fraction X LAGP) or ggp125 was analyzed by GC-MS as described under "Experimental Procedures." NA, not applicable.

Components	Compound 1	ggp125
Long-chain bases		
Dihydrosphingosine (C18:0)	2	0
Phytosphingosine (C18:0)	28.8	0
Phytosphingosine (C20:0)	5.6	0
Fatty acids		
C26:0	26.4	8.1
C26:0-OH[a]	5.5	10.6
Ratio (long-chain bases/fatty acids)	1.08	NA

[a] C26:0-OH stands for a C26:0 fatty acid with a hydroxyl group on the C2.

The Structure of the Lipid Moiety

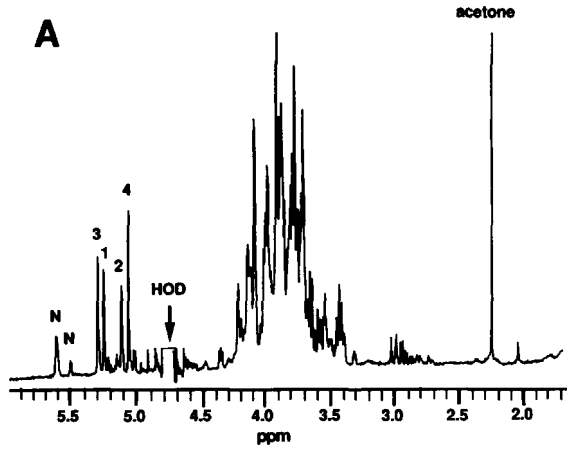
Compound 1 was used for GC-MS lipid analysis, which revealed that the total GPI peptide fraction contained predominantly ceramide structures composed of C18:0 phytosphingosine (with traces of C20:0 phytosphingosine and C18:0 dihydrosphingosine) and amide-linked C26:0 and C26:0 (-2-hydroxy) fatty acids (Table II). In contrast, parallel analyses of GPI peptides derived from purified ggp125 glycoprotein revealed a complete absence of long chain bases. Instead the lipid component contains only base-labile, hydroxyester-linked C26:0 and C26:0 (-2-hydroxy) fatty acids (Table II), suggesting a *lyso-* or diacyl glycerolipid structure.

The Structure of the Carbohydrate Moiety

NMR Analysis—PI-PLC treatment of the GPI peptides released the ceramide moiety and produced compound 2. Analysis of compound 2 by ^{1}H NMR (Fig. 5*A*) revealed four major anomeric proton resonances in the region 5.0–5.4 ppm. The magnitudes of their coupling constants ($J_{1,2}$) with their respective H-2 protons of about 2 Hz suggest that these are α-Man residues. The two additional anomeric proton resonances in the region of 5.5–5.7 ppm (with $J_{1,2}$ values of about 3.5 Hz) are characteristic of α-GlcN (Ferguson *et al.*, 1988; Schneider *et al.*, 1990). The presence of two resonances is due to heterogeneity in the phosphorylation state of the inositol phosphate to which the α-GlcN residue is linked (a mixture of inositol 1, 2-cyclic phosphate and inositol 1-monophosphate).

The sequence of the sugar residues was delineated by ^{1}H-^{1}H COSY and ^{1}H-^{1}H NOESY spectroscopy. Proton resonances were assigned to each residue by analysis of the COSY spectrum (data not shown). The reporter region of the NOESY spectrum is shown in Fig. 5*B*. Inter-residue connectivities are apparent between the following: Man-1 (H-1) and GlcN (H-4), Man-2 (H-1) and Man-1 (H-6), Man-3 (H-1) and Man-2 (H-2), and Man-4 (H-1) and Man-3 (H-2). These data suggest that the major component of compound 2 contains the sequence Manα1–2Manα1–2Manα1–6Manα1–4GlcNH$_2$α1-.

Exoglycosidase Sequencing and Methylation Analysis—The above sequence was confirmed and the nature of the microheterogeneity determined by Dionex HPLC, exoglycosidase sequencing, and GC-MS methylation analyses of various GPI anchor fragments. Deamination and [^{3}H]NaBH$_4$ reduction of compound 2 converted the GlcN residue to [1-^{3}H]anhydromannitol (AHM) to produce compound 3, which was HF-dephosphorylated to form compound 4. Analysis of this neutral glycan core fraction by Dionex HPLC (Fig. 6) showed that three glycan species could be resolved (compounds 5–7) with sizes on Bio-Gel P4 (Table III) comparable with Hex$_5$-AHM (the two minor species, compound 5 and 6) and Hex$_4$-AHM (the major species, compound 7). All three species were degraded to AHM (com-



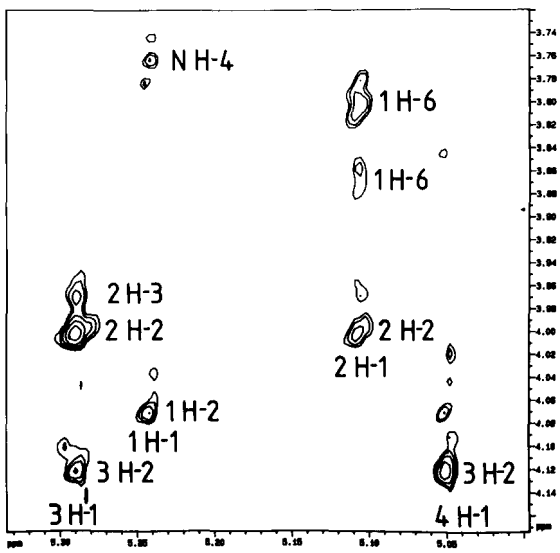


FIG. 5. **NMR analysis of compound 2.** *Panel A*, ^{1}H NMR spectrum of 600 nmol of compound 2. Resonances labeled *1–4* and *N* are due to the anomeric protons of the residues indicated in *panel B* and are assigned on the basis of the ^{1}H-^{1}H COSY spectrum (not shown) and the ^{1}H-^{1}H NOESY spectrum (*panel B*). *HOD* denotes the position of the truncated proton peak originating from water molecules. *Panel B*, reporter region of the ^{1}H-^{1}H NOESY spectrum of compound 2. Cross-peaks that correlate with through-space coupled protons are labeled using the residue descriptors shown above the spectrum, followed by *H-X*, where *X* is the ring position of the relevant proton.

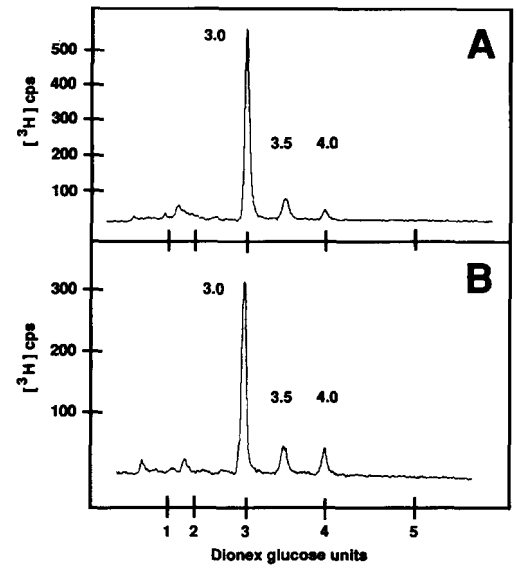


FIG. 6. **Dionex HPLC analysis of deaminated and reduced neutral glycans.** The labeled neutral glycans in compounds 4 (*panel A*) and 15* (*panel B*) were separated by Dionex HPLC. The elution position of β-glucose oligomer internal standards ("Dionex glucose units," or DU) are shown at the *bottom* of each panel. Figures adjacent to each peak indicate the elution position relative to the β-glucose oligomer internal standards. The DU value has no specific meaning but is characteristic of a given structure (Ferguson, 1992b).

pound 8) by jack bean α-mannosidase, confirming that all the hexose residues were α-linked Man. Compound 7 had identical chromatographic properties on the Dionex HPLC and Bio-Gel P4 column to authentic Manα1–2Manα1–2Manα1–6Manα1–4AHM generated from *T. cruzi* GPI molecules (Lederkremer *et al.*, 1991; Güther *et al.*, 1992). Compounds 6 and 7 were sensitive to the Manα1–2Man-specific *A. phoenicis* α-mannosidase, and lost 1 and 2 Man residues, respectively, to form compound 9. This product had identical chromatographic properties to authentic Manα1–6Manα1–4AHM (Ferguson, 1992b). In contrast, compound 5 was resistant to *A. phoenicis* α-mannosidase, suggesting that the terminal α-Man residue is not linked α1–2 to Man-4. All three species produced compound 10 (Manα1–4AHM) after partial acetolysis. A summary of the digestion data is shown in Table III. These data, together with the GC-MS methylation linkage analyses of the individual species (Table IV), define the structures of the neutral glycans as: Manα1–3Manα1–2Manα1–2Manα1–6Manα1–4AHM (compound 5), Manα1–2Manα1–2Manα1–2Manα1–6Manα1–4AHM (compound 6), and Manα1–2Manα1–2Manα1–6Manα1–4AHM (compound 7). The relative proportions of these three species were determined by Dionex HPLC (Fig. 6). The wild type strain contained 5, 15, and 80%, respectively, of these structures, and the *pep4* strain contained 13, 17, and 70%. Methylation analysis of compound 2 after dephosphorylation and N-acetylation (Table IV) shows the presence of 4-*O*-substituted GlcNAc (consistent with the NMR data) and 6-*O*-substituted *myo*-inositol. The latter derivative defines the GlcN-inositol linkage as 1–6. The presence of the EtN phosphate bridge was suggested from the compositional analysis of compound 2 (Table I), which shows the presence of ethanolamine and Man-6-PO_4. The location of the EtN phosphate was estimated by digesting compound 3 with jack bean α-mannosidase prior to aqueous HF dephosphorylation (Table III). The product was exclusively compound 11, a species that co-chromatographs with authentic Manα1–2Manα1–6Manα1–4AHM (Ferguson, 1992b). This result suggests that it is the Man-3 residue which contains the EtN phosphate bridge that prevents further exomannosidase digestion.

Fast Atom Bombardment Mass Spectrometric Analysis—To confirm the structure of the glycan core and to identify the site of the EtN bridge to the protein, compound 2 was treated with jack bean α-mannosidase in order to remove the heterogeneity due to the additional Man residues linked to the core (compound 12). Compound 12 was permethylated without prior *N*-acetylation, resulting in a positively charged species due to quaternization of the GlcN nitrogen atom (McConville *et al.*, 1990). The FAB spectrum of this derivative contains structurally informative fragment ions, which derive from the reducing terminus of the molecule by β-cleavage (Dell, 1987) with charge localization on the quaternized nitrogen (Fig. 7*A*). The fragment ions observed between *m/z* 576 and 1282 confirm the sequence and the substitution pattern of the oligosaccharide

TABLE III
Dionex and Bio-Gel P4 analysis of neutral glycan fragments

Substrate(compound)	Treatment	Compound	Product	Dionex units	GU
SAGP NG (4)		7	Man_4AHM	3.0	5.2
		6	Man_5AHM	3.5	6.2
		5	Man_5AHM	4.0	6.2
SAGP (JBAM) NG (11)[a]		11	Man_3AHM	2.5	4.2
	APAM	9	Man_2AHM	2.2	3.3
	JBAM	8	AHM	ND[b]	1.7
Man_4AHM (7)	APAM	9	Man_2AHM	2.2	3.2
	Acetolysis	10	Man_1AHM	ND	2.4
	JBAM	8	AHM	1.0	ND
Man_5AHM (6)	APAM	9	Man_2AHM	2.2	3.3
Man_5AHM (5)	APAM	5	Man_5AHM	ND	6.2
pep4 SAGP NG (15*)[c]		18*	Man_4AHM	3.0	ND
		17*	Man_5AHM	3.5	6.2
		16*	Man_5AHM	4.0	6.2
	JBAM	20*	AHM	1.0	1.7
pep4 SAGP (JBAM) NG (19*)[a]		19*	Man_3AHM	2.5	ND

[a] These SAGP were digested with JBAM prior to the deamination/reduction treatment.
[b] ND, not determined.
[c] Compounds indicated with asterisks originate from *pep4* SAGP.

TABLE IV
Methylation linkage analysis

Partially methylated alditol acetate	Origin	Compound 2[a]	Compound 7	Compound 6	Compound 5
Mannitol					
(2,3,4,6-Tetra-*O*-methyl-1,5-di-*O*-acetyl)	Terminal Man	1	1	1	1
(3,4,6-Tri-*O*-methyl-1,2,5-tri-*O*-acetyl)	2-*O*-Substituted Man	2.9	2.3	2.9	2.4
(2,4,6-Tri-*O*-methyl-1,3,5-tri-*O*-acetyl)	3-*O*-Substituted Man	0.2	0	0	1
(2,3,4-Tri-*O*-methyl-1,5,6-tri-*O*-acetyl)	6-*O*-Substituted Man	0.9	1	0.7	0.8
2-*N*-Methylacetamido-2-deoxyglucitol (3,6-di-*O*-methyl-1,4,5-tri-*O*-acetyl)	4-*O*-Substituted GlcNAc	0.5	0	0	0
2,5-Anhydromannitol (1,3,5-Tri-*O*-methyl-4-*O*-acetyl)	4-*O*-Substituted AHM	0	+[b]	+	+
Inositol (1,2,3,4,5-Penta-*O*-methyl-6-*O*-acetyl)	6-*O*-Substituted *myo*-inositol	0.4	0	0	0

[a] Methylation analysis of compound 2 was performed following aqueous HF dephosphorylation and *N*-acetylation.
[b] Present but not quantitative (yield variable due to high volatility).

core. The ion at m/z 1339 shows the attachment of the EtN phosphate bridge to the third Man residue, while the next intense ion at m/z 1424 shows the linkage of the COOH-terminal residue (glycine) directly to the amino group of the EtN phosphate bridge. The remaining fragment ions at higher mass are very difficult to interpret since the anchor preparation represents the total array of yeast GPI-anchored proteins and therefore contain heterogeneous peptides. The heterogeneous mixture of COOH-terminal sequences can be further complicated by the overmethylation that occurs during the strong conditions necessary to methylate the anchor. The FAB spectrum in Fig. 7*A* suggests that glycine is one of the major COOH-terminal residues employed by yeast GPI-anchored proteins, and this residue has become mono-overmethylated to yield the ion at m/z 1424.

In order to prove that the complexity at the high mass end of the spectrum is indeed due to the peptide heterogeneity, compound 12 was subjected to hydrazinolysis, a treatment that cleaves amide bonds and is therefore routinely used to release carbohydrate bound to peptide (Takasaki *et al.*, 1982). Studies on the effect of hydrazinolysis on the VSG GPI anchor from *T. brucei* showed that the anchor was released in good yield on hydrazinolysis, bearing a free amino group on the EtN residue, as expected, and lacking the phosphate attached to the inositol residue.[2] The product of hydrazinolysis of the yeast anchor was *N*-acetylated prior to permethylation to prevent quaternization of the two amino groups. The FAB spectrum of the *N*-acetylated permethylated product (Fig. 7*B*) contains a pseudomolecular ion at m/z 1301 for the expected product containing 3 Man residues, GlcNAc, inositol, and *N*-acetylethanolamine phosphate. The fragment ion observed at m/z 1051 corresponds to a single A^+-type cleavage on the reducing side of the GlcNAc residue, and further structurally informative ions are derived from this by secondary β-cleavages to yield information on the sequence and site of attachment of the EtN group (Fig. 7*B*). Importantly, no high mass ions were observed and all ions could be assigned, following removal of the peptide by hydrazinolysis, demonstrating that the heterogeneity in the peptide portion gives rise to the complexity observed in the previous spectrum (Fig. 7*A*).

All data described above are consistent with the structures depicted in Fig. 8.

DISCUSSION

Here we report on the purification and the structures of GPI anchors from *S. cerevisiae* membrane proteins. The GPI anchor peptides were prepared from the total array of yeast GPI-anchored proteins, assuming that they would exhibit the same physicochemical properties as the well known protozoan GPI anchors (Ferguson *et al.*, 1988; Schneider *et al.*, 1990; Güther *et al.*, 1992; McConville and Bacic, 1989). Only a few purification steps (preparation of membranes, protease treatment of delipidated membrane proteins, isolation of GPI anchor peptides by partitioning in Triton X-114 before and after PI-PLC treatment or reverse-phase chromatography on octyl-Sepharose) were sufficient to obtain GPI anchor peptides in good yield and high purity. These procedures circumvent the need of purifying individual GPI-anchored proteins for structural analysis and al-

[2] J. Thomas-Oates, unpublished observations.

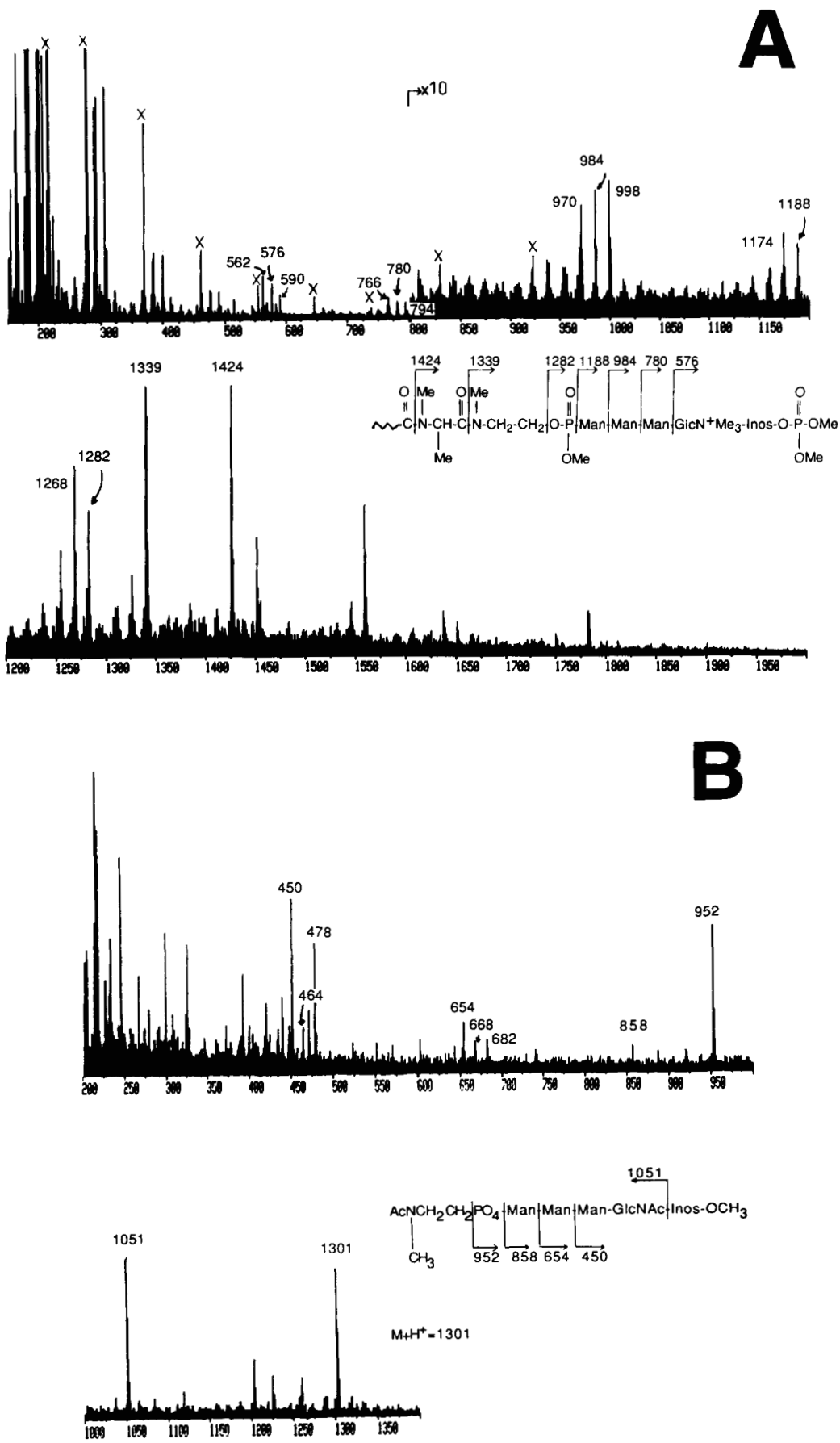

A
x10
984
998
970
1188
1174
562
576
590
766
780
794
1424
1339
1282
1268
1188
984
780
576
B
450
478
464
952
654
668
682
858
1051
1301
M+H+=1301
952
858
654
450

FIG. 7.

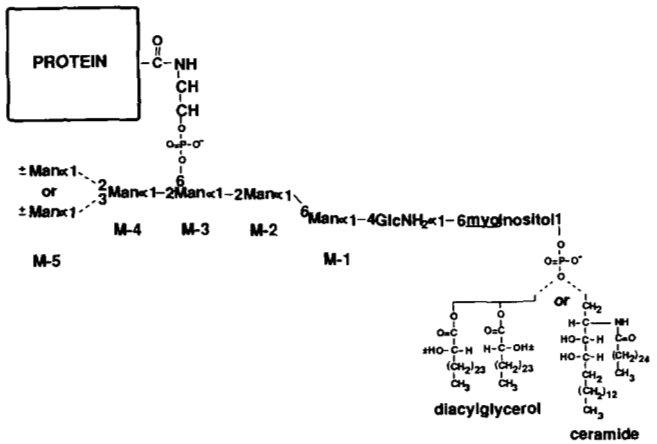


FIG. 8. **Glycosylphosphatidylinositol anchor structures of yeast proteins.** Figure shows the structures of GPI anchors, which can be found on proteins in *S. cerevisiae,* as determined from the experiments described in this paper. The lipid moiety on the majority of GPI anchors is a ceramide. In the case of the ggp125 protein, the GPI anchor contains a *lyso-* or diacylglycerol. The presence of these two classes of lipid moiety is represented by *dashed lines* to the phosphate group on the 1-position of the *myo*-inositol residue. The mannoses are annotated by *M-1* to *M-5*, beginning at the reducing terminus of the glycan. 80% of the GPI anchors contain only the mannoses M-1 to M-4. The M-5 residue, present on some GPI anchors, is linked either α1–2 or α1–3 to M-4 as indicated by the *dashed lines*.

low to obtain information on the whole array of GPI structures made by an organism, although we cannot be certain that all GPI proteins are efficiently extracted and solubilized by our protocol. The isolated anchor peptides contained heterogeneous peptides with a bias for only a limited set of amino acids close to the GPI anchor attachment site. Of the 6 amino acids known to be able to serve as attachment sites for GPI anchors (Gly, Ser, Cys, Ala, Asp, and Asn) (Micanovic *et al.*, 1990; Moran *et al.*, 1991), we only found Asn/Asp, Gly, and Ser in significant amounts in anchor peptides containing on the average 2 amino acids. This suggests that Ala and Cys may not be frequently used in yeast for GPI anchor attachment. In addition, these anchor peptides also contained significant amounts of Thr, an amino acid that has been described to function as a very inefficient acceptor for GPI anchors in mammalian cells (Micanovic *et al.*, 1990; Moran *et al.*, 1991).

To determine the structure of the yeast GPI anchors, we analyzed the anchor peptides using NMR, fast atom bombardment-mass spectrometry, compositional analysis, amino acid analysis, permethylation, chemical modifications, and exoglycosidase digestions. FAB mass spectroscopy formally shows that the glycan is attached to these peptides by an EtN phosphate bridge, which is bound to the carboxyl-terminal amino acid by an amide linkage and to the C6 position of the third Man residue by a phosphodiester linkage. This assignment has been shown for human acetylcholinesterase (Roberts *et al.*, 1988b; Deeg *et al.*, 1992) but was previously only indirectly inferred for the GPI anchors of *T. brucei* VSG (Ferguson *et al.*, 1988), rat brain Thy-1 (Homans *et al.*, 1988), and *L. major* promastigote surface protease (Schneider *et al.*, 1990).

Our results confirm the conservation of a GPI core structure during evolution from *S. cerevisiae* and protozoa up to mammalian organisms, namely ethanolamine-PO_4-6Manα1–2Manα1–6Manα1–4GlcNH_2α1–6*myo*-inositol-1-PO_4-lipid (Ferguson *et al.*, 1988; Homans *et al.*, 1988; Schneider *et al.*, 1990; Güther *et al.*, 1992). In addition, yeast anchors contain a side chain, which consists of 1 or 2 Man residues. The fraction of GPI anchors containing 2 additional Man residues is 20% in the wild type and 30% in the *pep4-3* strain, and the fraction of GPI anchors with a terminal Man-linked α1–3 is 3-fold higher in the *pep4-3* than in wild type. In contrast to the GPI anchors of *T. brucei* VSG (Ferguson *et al.*, 1988), rat brain Thy-1 (Homans *et al.*, 1988), and the scrapie prion protein (Stahl *et al.*, 1992), no other monosaccharides are found associated with the yeast GPI anchors. Similarly, additional EtN phosphate groups, common among higher eukaryotic anchors (Roberts *et al.*, 1988b; Homans *et al.*, 1988; Walter *et al.*, 1990; Stahl *et al.*, 1992), are absent from the yeast GPI anchors and all of the protozoan GPI anchors so far characterized (Ferguson *et al.*, 1988; Schneider *et al.*, 1990). Thus, the presence of these extra EtN phosphate groups may be specific to multicellular organisms.

The lipid analysis of LAGP of yeast anchors yields a ceramide consisting of a C18:0 phytosphingosine and a non-hydroxylated or monohydroxylated C26:0 fatty acid. This chemical analysis thus confirms the conclusions of metabolic labeling studies (Conzelmann *et al.*, 1992), which suggested (i) that the majority of mature GPI anchors contain ceramides and (ii) that these ceramides are different from those present in the major classes of the abundant inositol phosphoceramides of yeast. This study shows that only the minor anchor ceramide (containing a monohydroxylated C26:0 fatty acid) is identical with the ceramide found on one of the major inositol phosphoceramides, namely IPC-II (Smith and Lester, 1974), whereas the ceramide present on the bulk of GPI anchors is not to be found in any of the major inositol phosphoceramides of yeast (named IPC-I, IPC-II, IPC-III, and MIPC) (Smith and Lester, 1974). The fact that the ratio of long chain bases to fatty acids was approximately 1 argues against an additional acyl chain on the *myo*-inositol residue on mature yeast GPI anchors as found, for example, on the human erythrocyte acetylcholinesterase GPI anchor (Roberts *et al.*, 1988a). This is also consistent with the fact that the yeast GPI anchors can be cleaved with bacterial PI-PLC (Conzelmann *et al.*, 1990; Fankhauser and Conzelmann, 1991). Interestingly, however, as in other organisms, early intermediates of yeast GPI anchor biosynthesis contain an acyl group on the *myo*-inositol (Orlean, 1990; Conzelmann *et al.*, 1992).

We were surprised to find that the GPI anchor of the purified ggp125 protein contains no long chain bases, only C26:0 fatty acids. This, together with the fact that the GPI anchor of this protein is easily cleaved by PI-PLC argues for the presence of an unusual mono- or diacylglyceride. The complete absence of ceramide from ggp125 GPI anchors was confirmed with purified protein from an other strain (data not shown). Earlier studies had suggested that both ceramides and mild base-sensitive anchors can be found in immature proteins isolated by preparative gel electrophoresis from a mutant in which labeled GPI proteins were retained in the endoplasmic reticulum. The present findings make it clear, however, that ceramides occur only on some, not all, GPI-anchored proteins, and it remains to be demonstrated that both ceramides and acylglycerols can be present on the same protein in its mature state. Thus, although some limited heterogeneity in the lipid composition of GPI an-

FIG. 7. **Fast atom bombardment-mass spectrometry analysis of compounds 12 and 13.** *Panel A*, positive ion FAB spectrum of permethylated compound 12. Satellite ions observed 14 mass units below and above each β-cleavage ion arise from the mono-methyl esterified phosphate-containing species, and the product of cross-ring cleavage (in which carbon 1 and the ring oxygen atoms from the adjacent sugar residue are retained) (Dell, 1987), respectively. Matrix ions are marked with an *X*. *Panel B*, positive ion FAB spectrum of the *N*-acetylated, permethylated derivative of the hydrazinolysis product, compound 13. Satellite ions observed 28 atom mass units above the β-cleavage ions arise by cross-ring cleavage in which carbon 1 and the ring oxygen atoms from the adjacent sugar residue are retained (Dell, 1987). Smaller satellite ions 14 atom mass units higher than the β-cleavage ions arise by elimination (Dell, 1987) from the molecular species.

chors made by a given organism has been the rule (Thomas *et al.*, 1990), yeast seems to be exceptional in using two widely different lipids for GPI anchoring. It seems worth noting that the C26:0 fatty acids found on ggp125 are quite different from the C16 and C18 fatty acids typical of yeast phospholipids. Clearly, further studies are required to decide whether the anchor precursor glycolipid for ggp125 is built onto a phosphatidylinositol of an unusual fatty acid composition or whether the C26:0 fatty acids are introduced at a later stage through a remodeling step analogous to the one found in trypanosomes (Masterson *et al.*, 1990).

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