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Two different types of lipid moieties are present in glycophosphoinositol-anchored membrane proteins of *Saccharomyces cerevisiae*

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Numerous glycoproteins of *Saccharomyces cerevisiae* are anchored in the lipid bilayer by a glycophosphatidylinositol (GPI) anchor. Mild alkaline hydrolysis reveals that the lipid components of these anchors are heterogeneous in that both base-sensitive and base-resistant lipid moieties can be found on most proteins. The relative abundance of base-resistant lipid moieties is different for different proteins. Strong alkaline or acid hydrolysis of the mild base-resistant lipid component liberates C18-phytosphingosine indicating the presence of a ceramide. Two lines of evidence suggest that proteins are first attached to a base-sensitive GPI anchor, the lipid moiety of which subsequently gets exchanged for a base-resistant ceramide: (i) an early glycolipid intermediate of GPI biosynthesis only contains base-sensitive lipid moieties; (ii) after a pulse with [^{3}H]*myo*-inositol the relative abundance of base-sensitive GPI anchors decreases significantly during chase. This decrease does not take place if GPI-anchored proteins are retained in the ER.

Key words: ceramide/glycophosphatidylinositol/*myo*-inositol/*Saccharomyces cerevisiae*/secretion

Introduction

Glycophosphatidylinositols (GPIs) are a recently discovered class of glycolipids that can become covalently attached to the C-terminus of glycoproteins thereby anchoring these proteins to cellular membranes (for review see Ferguson and Williams, 1988; Low and Saltiel, 1988; Cross *et al.*, 1990; Doering *et al.*, 1990; Thomas *et al.*, 1990). GPIs are attached to proteins in the endoplasmic reticulum (ER) within seconds or minutes of their translation and translocation (Bangs *et al.*, 1985; Ferguson *et al.*, 1986; Conzelmann *et al.*, 1987). The structures of the carbohydrate moiety of one mammalian and two protozoan GPI anchors have been reported (Ferguson *et al.*, 1988; Homans *et al.*, 1988; Schneider *et al.*, 1990) and comparison of these shows that the linear core oligosaccharide linking the protein to the lipid moiety is conserved between mammalian and protozoan organisms. In contrast, the lipid moieties of GPI anchors are quite different in different proteins (reviewed by Ferguson and Williams, 1988; Thomas *et al.*, 1990; also Luhrs and Slomiany, 1989). Individual proteins usually contain either diacylglycerols or alkylacylglycerols. Evidence for a ceramide-like lipid has been found in one case, namely the contact site A protein of *Dictyostelium discoideum* (Stadler *et al.*, 1989). While acyls and alkyls with chain lengths ranging from C14 to C26 and containing from zero up to six double bonds have been described on different proteins from different species, a more limited heterogeneity can also be observed on individual proteins (Schneider *et al.*, 1990; Thomas *et al.*, 1990). There are no firm data as to whether the lipid moieties found on a protein are determined by the species, the cell type or the protein itself. Some GPI anchors also contain an additional acyl chain attached to the *myo*-inositol (Roberts *et al.*, 1987, 1988a,b; Field *et al.*, 1991). The range of quite unusual lipid moieties found on many GPI anchors might be explained either by a preference of the UDP-GlcNAc: *myo*-inositol-phospholipid α-1,6-*N*-acetylglucosaminyl transferase for phosphoinositides with unusual lipid moieties, or else by some remodeling step during which acyl chains commonly found on phosphatidylinositol (PI) are replaced later on during anchor biosynthesis. A case for the latter model has recently been provided by a pioneering study in *Trypanosome brucei* (Masterson *et al.*, 1990). In this system the acyl chains of the mature GPI are replaced by myristic acid (C14:O) shortly before the transfer of the GPI onto the variant surface glycoprotein. In this article we provide evidence suggesting another type of lipid remodeling which seems to occur after the GPI has been attached to proteins.

Results

GPI-anchored yeast proteins are made with two different lipid moieties

Upon incubation of *Saccharomyces cerevisiae* with [^{3}H]-*myo*-inositol, the numerous GPI-anchored glycoproteins (GPIgps), but no other proteins, become radiolabeled (Conzelmann *et al.*, 1990). The [^{3}H]*myo*-inositol-labeled anchor peptides prepared from the pool of all GPIgps were found to be partially resistant to mild alkaline hydrolysis. Since mild alkaline hydrolysis of GPIs removes all hydroxy-esterified fatty acids from glycerol and *myo*-inositol (Roberts *et al.*, 1988a) we concluded that some fraction of the anchors did not contain PI but some other mild base-resistant lipid. It was clear, however, that the [^{3}H]*myo*-inositol in these anchor peptide preparations was linked to the lipid through a phosphodiester bond since 95% of the label became water soluble when treated with PI-specific phospholipase C (PI-PLC) (Conzelmann *et al.*, 1990).

To find out what proteins contain base-resistant anchor lipids, cells were labeled with [^{3}H]*myo*-inositol. After delipidation and separation of GPIgps by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), individual proteins were recovered from the gel and anchor peptides were prepared and subjected to mild alkaline

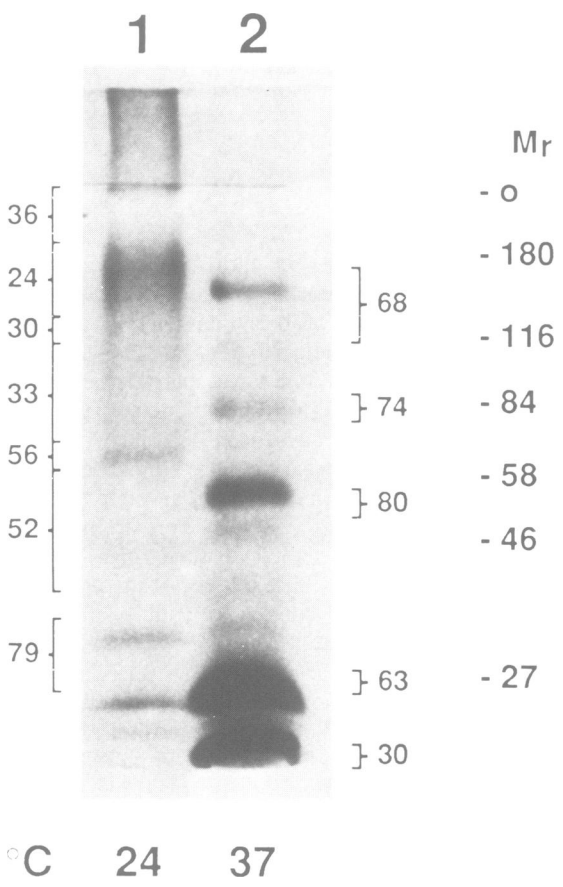

Fig. 1. Mild base sensitivity of GPI anchors of individual proteins. *Sec18* cells were preincubated for 20 min and labeled with [^{3}H]*myo*-inositol at either 24°C (lane 1) or 37°C (lane 2) for 2 h. GPIgps were extracted and purified by affinity chromatography on Concanavalin A−Sepharose (Con A−Sepharose) (procedure A). Samples were split (20:80) and eluted glycoproteins were separated by gel electrophoresis on an 8% polyacrylamide slab gel. The lanes containing 20% of material were processed for fluorography (lanes 1 and 2) whereas lanes containing 80% of counts were cut into 3 mm wide slices from which the label was eluted using pronase digestion (procedure F). Aliquots of anchor peptides from individual slices or pools (as indicated by brackets) were subjected to mild alkaline hydrolysis or control incubated (both incubations in quadruplicate) and products were partitioned in Triton X-114 (TX-114). The percentage of base-sensitive anchors corresponding to bands or zones of lane 1 are given on the left; for bands of lane 2 they are given on the right. Figures represent the percentage of c.p.m. found in the aqueous phase of the TX-114 phase separation system after mild base treatment after subtraction of the percentage of anchor peptides which partitioned into the aqueous phase before base treatment (3.8% of total on average). The mobilities of molecular mass (M_r) standard proteins (in kDa) are indicated on the right. o = top of the running gel.

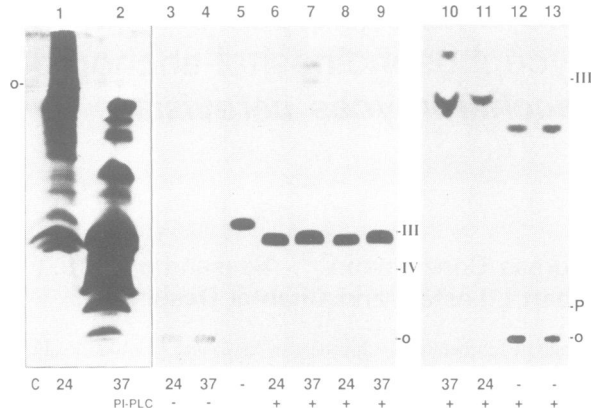

Fig. 2. TLC analysis of lipid moieties of [^{3}H]palmitic acid-labeled GPI anchors. *Sec18* cells were preincubated at the labeling temperature and labeled with [^{3}H]palmitic acid at either 24°C or 37°C for 2 h as indicated at the bottom. Cells were broken, proteins were extracted (procedure C) and delipidated, and Con A-binding glycoproteins were further freed of potential contaminating lipids by preparative SDS−PAGE in a 12% polyacrylamide gel (procedure F). While analytical lanes were processed for fluorography (lanes 1 and 2), the preparative lanes (including stacking gel) were cut into pieces and incubated with pronase. Eluted anchor peptides were partitioned between butanol and water and the butanol phases were analyzed by TLC either directly (lanes 3 and 4) or after treatment with PI-PLC (lanes 6, 7, 10 and 11). (After PI-PLC, counts were recovered quantitatively in the butanol phase.) PI-PLC treated samples subjected to mild alkaline hydrolysis are shown in lanes 8 and 9. IPC/C and MIPC/E were purified from the $CHCl_3-CH_3OH-H_2O$ extracts of the same cells by HPLC and treated with PI-PLC to release the labeled ceramide moieties: ceramide from IPC/C, lane 12; ceramide from MIPC/E, lane 13. Lane 5 contains [^{3}H]palmitic acid as used for labeling. TLC was performed with solvent 95:5 (lanes 3−9) or solvent 40:10:1 (lanes 10−13). Positions of two different ceramides from bovine brain (III and IV) and of phytosphingosine (P) are indicated on the right. o = top of running gel or origin of TLC.

hydrolysis. For this we used a temperature-sensitive secretion mutant in which the vesicular transport of secretory proteins from the ER to the Golgi is blocked at 37°C but is normal at 24°C (Novick *et al.*, 1980, 1981; Esmon *et al.*, 1981). Labeled proteins are smaller when labeling of this mutant is done at 37°C than at 24°C since elongation of *N*-glycans in the Golgi does not take place. As shown in Figure 1, individual proteins contained both base-sensitive as well as base-resistant anchors while the relative proportion of base-resistant anchors varied from protein to protein, and the percentage of base-sensitive anchors was in general lower on proteins labeled at 24°C, i.e. on proteins that had reached the Golgi. Nevertheless, base-resistant anchors could also be detected on proteins which had been labeled at the restrictive temperature (37°C) and such anchors were particularly prevalent on a heavily labeled protein of 21 kDa (Figure 1, lane 2). Contamination of the anchor peptides of the 21 kDa protein by base-resistant inositolphosphoceramides (IPCs, Steiner *et al.*, 1969; Smith and Lester, 1974) was ruled out since the radioactivity contained in the anchor peptide preparation from the 21 kDa protein stayed at the origin when analyzed by thin-layer chromatography (TLC) whereas IPCs migrated with R_f values of 0.3−0.6 (not shown). Stronger hydrolysis conditions (0.1 N NaOH, 2 h, 65°C) did not significantly increase the percentage of base-labile anchors from any protein (not shown). Thus, two classes of lipid moieties were found on most proteins.

Characterization of the lipid moiety of base-resistant GPI anchors

We have shown previously that the bulk of [^{3}H]palmitic acid gets incorporated into GPIgps whereas incorporation into otherwise acylated proteins is quantitatively much less important in *S.cerevisiae* (Conzelmann *et al.*, 1990). In order to analyze the isolated lipid moieties of GPI anchors, anchor peptides were prepared from a pool of all [^{3}H]palmitic acid-labeled proteins of *sec18*, as described in Figure 2. These anchor peptides did not migrate on TLC (Figure 2, lanes 3 and 4), but after treatment with PI-PLC their lipid moieties migrated to a position near the ceramide standards, the mobility being identical whether anchors from *sec18* cells

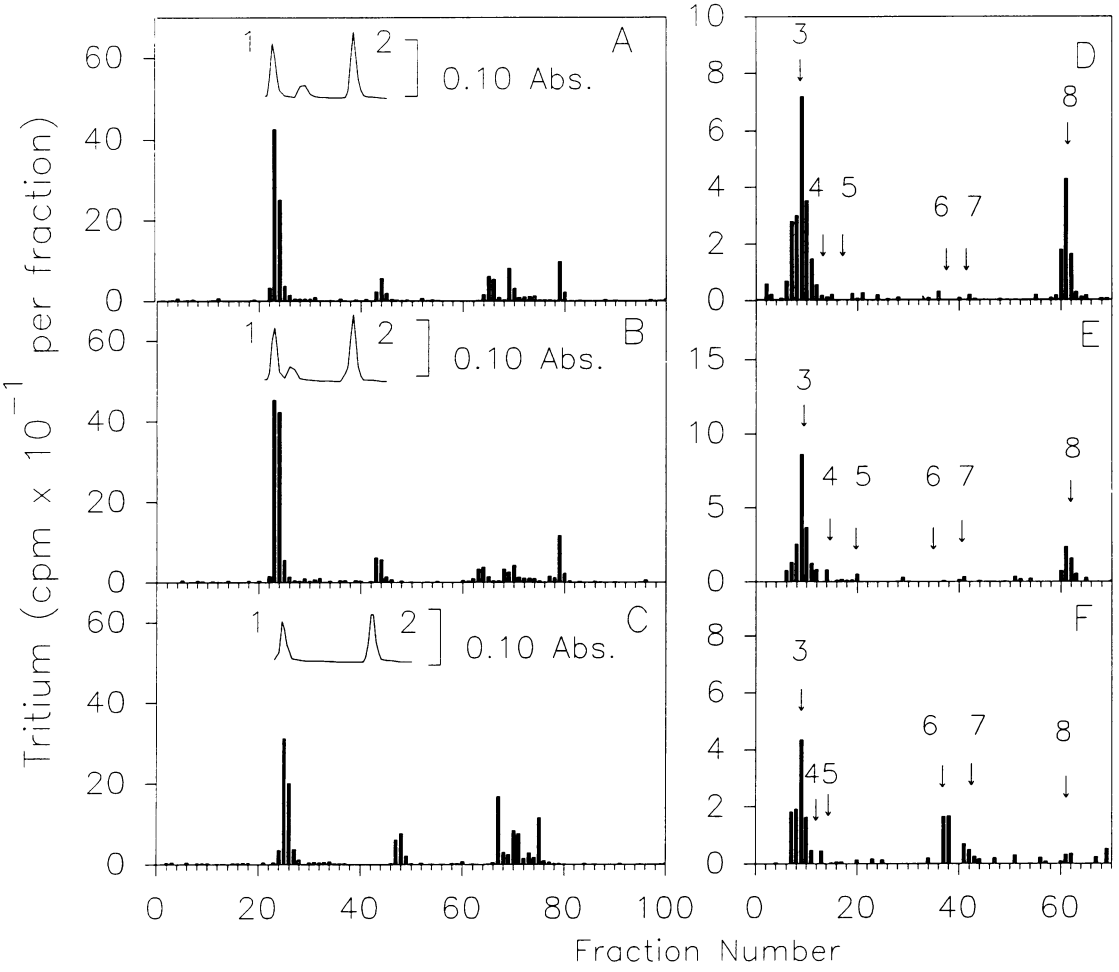

Fig. 3. HPLC analysis of hydrolysates of mild base-resistant anchor lipids. [^{3}H]palmitic acid-labeled anchor peptides from *sec18* cells labeled at 24°C or 37°C were treated with PI-PLC and the main mild base-resistant lipid component (Figure 2, lanes 6 and 7) was isolated by preparative TLC. Aliquots of 8000 c.p.m. were subjected to strong acid hydrolysis and products were converted to diphenylcarbonyl derivatives to allow for detection of long chain bases. Other aliquots of 8000 c.p.m. were subjected to strong alkaline hydrolysis, fatty acids were extracted with ethylether (~20% of counts) and were converted to phenacyl derivatives for fatty acid determination. Derivatized samples were separated by HPLC together with similarly derivatized unlabeled lipids as internal standards. Standards were detected by UV monitoring and the radioactivity of each fraction was determined by scintillation counting. Acid hydrolysates are in panels A–C, fatty acid extracts of base hydrolysates in panels D–F. Panels A and D: cells labeled at 24°C; panels B and E: cells labeled at 37°C. Panels C and F contain hydrolysates from IPC/C (IPC-II by Smith and Lester, 1974) isolated from the same cells. UV absorbance profile (Abs.) of internal standards are shown as inserts in panels A–C: 1 = C18-phytosphingosine; 2 = C20-phytosphingosine and C18-D-erythrodihydrosphingosine which elute at the same position. Internal fatty acid standards (panels D–F) came out in single fractions and are indicated by vertical arrows: 3 = hydroxy-C18:0, 4 = C18:0, 5 = hydroxy-C22:0, 6 = hydroxy-C26:0, 7 = C24:0, 8 = C26:0.

labeled at 24°C or those labeled at 37°C were analyzed (Figure 2, lanes 6 and 7). The ceramide standards used are from bovine brain and therefore do not contain the phytosphingosine typical of yeast ceramides (Smith and Lester, 1974). PI-PLC also released some more hydrophobic lipids migrating close to the solvent front, this material being more abundant in cells labeled at 37°C. Mild alkaline hydrolysis did not change the mobility of the major band produced by PI-PLC treatment (Figure 2, lanes 8 and 9). After strong acid or alkaline hydrolysis, however, a major product comigrated on TLC with phytosphingosine in two different solvent systems (not shown). The major base-resistant lipid obtained by PI-PLC treatment of [^{3}H]palmitic acid-labeled anchors was isolated from a preparative TLC plate and subjected to strong acid or strong alkaline hydrolysis, and derivatized products were analyzed by HPLC together with appropriate internal standards (Figure 3). This analysis revealed the presence of C18-phytosphingosine as a major component in anchors made by *sec18* cells at either

Table I. HPLC analysis of hydrolysis products of [^{3}H]palmitic acid-labeled, mild base-resistant anchor lipids

	Percent ^{3}H of starting material	
	24°C[a]	37°C[a]
Acid hydrolysis		
C18-Phytosphingosine	57.2	54.9
Unidentified peak	7.8	8.1
Ethanol wash	32.3	24.5
% recovered	97.3	87.5
Alkaline hydrolysis		
C26:0	6.1	2.9
Unidentified	14.2	17.4
% recovered	20.3	20.3

This represents the quantification of data shown in Figure 3. Figures indicate the relative amounts of radioactivity coeluting with internal standards as percentage of the starting material subjected to hydrolysis (= 100%).

[a]Labeling temperature.

temperature (panels A and B). The material in fractions 43–46 could not be identified, whereas the material eluting after fraction 60 corresponds to the position expected for fatty acids. Fatty acids were resolved using a system shown in Figure 3 panels D–F. The bulk of material eluted in the less well resolved zone of short chain fatty acids (about fraction 9) and certainly contained several species since standards eluted in single fractions. A lower percentage of counts coeluted with the C26:0 fatty acid standard. Analysis of the major IPC (IPC/C) labeled in the same experiment showed that the [^{3}H]palmitic acid labeling of IPC/C results in a pattern of radioactive fragments similar to the mild base-resistant anchor lipids with the exception of the expected presence of hydroxy-C26:0 fatty acid (panels C and F). The quantification of these results is given in Table I. It is evident that fatty acids were not as strongly labeled as long chain bases. It is conceivable that externally added [^{3}H]palmitic acid is not directed into the elongation pathway involved in the elaboration of the C24 and C26 fatty acids typical of yeast ceramides (Smith and Lester, 1974).

The susceptibility of the major anchor lipid to strong acid or alkaline, but not mild alkaline, hydrolysis conditions suggests that the C18-phytosphingosine is substituted by an acyl group on its amine, since an *O*-acyl is expected to be sensitive to mild base treatment while an *O*-alkyl group would be resistant to the strong acid and alkaline hydrolysis conditions used. Thus, these results strongly suggest that the mild base-resistant anchor component is a ceramide-like lipid.

The [^{3}H]palmitic acid-labeled, PI-PLC-treated anchor lipid was also compared with the ceramides generated by PI-PLC treatment of two major IPCs (IPC/C and MIPC/E; Smith and Lester, 1974; Puoti *et al.*, 1991), both of which were isolated by preparative HPLC from a lipid extract of the same [^{3}H]palmitic acid-labeled cells as were used to prepare the anchor lipids shown in Figure 2. TLC analysis showed that the base-resistant anchor lipid has a higher mobility, i.e. is more hydrophobic than the ceramides derived from IPC/C or MIPC/E (Figure 2, lanes 10–13). Since the ceramides from IPCs also contained [^{3}H]C18-phytosphingosine as the main long chain base (Figure 3, panel C), the difference in mobility on TLC between anchor- and IPC-derived ceramides must be due to a difference in the probable fatty acid substituent on the amine of C18-phytosphingosine.

Since lipid moieties of yeast GPI anchors seem to be heterogeneous it is conceivable that [^{3}H]palmitic acid labels only a minor subclass of anchors. We therefore analyzed the anchor lipids from cells labeled with [^{3}H]*myo*-inositol, a tracer which can be expected to label anchors irrespective of their lipid moiety. To preserve the linkage between the labeled *myo*-inositol and the unlabeled lipid moiety the anchor peptides were treated with nitrous acid under conditions which selectively split GPIs between the non-acetylated glucosamine and the *myo*-inositol residue (Ferguson *et al.*, 1985). This treatment has previously been shown to cleave yeast anchors (Conzelmann *et al.*, 1990). Initially, 55% of labeled anchors partitioned into the water phase in a water/butanol separation but none of this material migrated in TLC (Figure 4, panel C, lanes 1 and 2). Nitrous acid treatment cleaved 63% of anchors and the HNO_2-released lipids which partitioned into the butanol phase were separated by HPLC (to get rid of detergent), as shown in Figure 4, panel B. The main peak (β, 83% of material) eluted just before IPC/C whereas a minor fraction (γ, 5% of material) coeluted with $M(IP)_2C/H$ which represents the most abundant IPC of *S. cerevisiae* (Smith and Lester, 1974). Anchor lipid β was completely resistant to mild alkaline

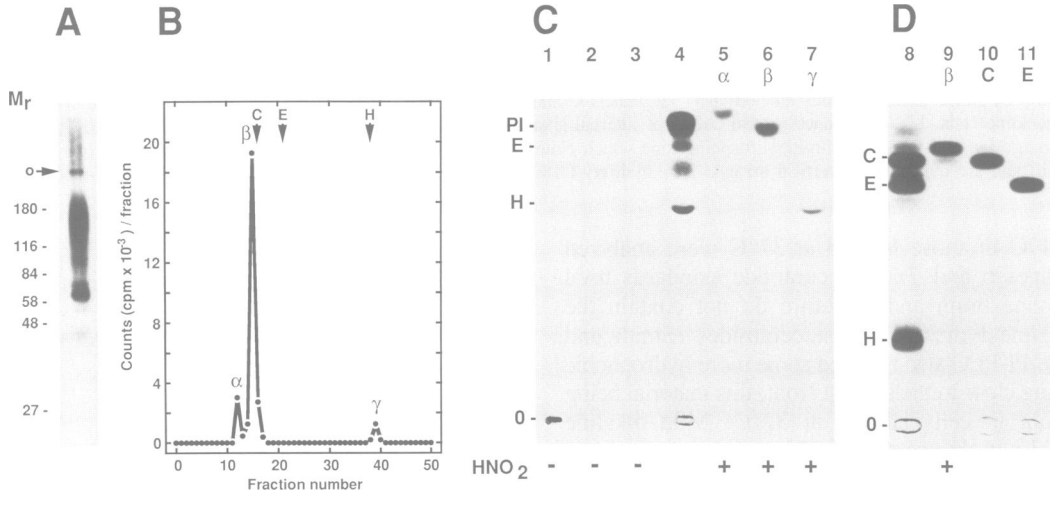

Fig. 4. TLC analysis of [^{3}H]*myo*-inositol-labeled anchor lipids generated by nitrous acid treatment. X2180 cells labeled with [^{3}H]*myo*-inositol at 30°C for 90 min, GPIgps were prepared by procedure D and an aliquot of delipidated proteins was analyzed by SDS–PAGE and fluorography (**panel A**). The bulk of GPIgps were treated with proteinase K. An aliquot of the resulting peptides was partitioned between butanol and water and the counts contained in the water phase (panel C, lane 1, spotted in H_2O) as well as in the butanol phase (lane 2) were analyzed by TLC to show that no free lipids were present after proteinase K treatment of GPIgps. The anchor peptides were then treated with HNO_2, extracted with butanol and separated by preparative HPLC (**panel B**). Elution positions of standard IPCs are indicated: C = IPC/C; E = MIPC/E; H = $M(IP)_2C/H$. HNO_2-released, HPLC-purified anchor lipid in peaks α, β, γ (panel B) were analyzed by TLC (**panel C**, lanes 5–7). Lane 3 of panel C contains the butanol extract of a control incubation at pH 3.5 without HNO_2. In **panel D** we compared lipid β (lane 9) to known lipid standards by TLC in solvent 55:45:10. Standards are: total of [^{3}H]*myo*-inositol-labeled lipids of X2180 before (lane 4) and after (lane 8) mild alkaline hydrolysis; IPC/C (lane 10) and MIPC/E (lane 11) from [^{3}H]*myo*-inositol-labeled yeast cells. o = top of running gel and origin of TLC.

hydrolysis (not shown). The comparison by TLC of anchor lipid β with IPC/C and MIPC/E (Figure 4, panel D) clearly shows that the anchor lipid β has a higher mobility, i.e. is more hydrophobic than these major IPCs. Only a minor base-resistant [^{3}H]*myo*-inositol-labeled lipid of the same mobility as the anchor lipid could be detected in the total lipid extract (Figure 4, lane 8). Thus, with [^{3}H]*myo*-inositol-labeled anchors we reach a similar conclusion to that of [^{3}H]palmitic acid-labeled anchors: the lipid moiety of the base-resistant anchors is not derived from the major IPCs of yeast cells but is slightly more hydrophobic. Incidentally, these results further confirm that the base-resistant components of the yeast anchor peptides do not consist of contaminating IPCs.

GPI anchors of newly made proteins have base-sensitive lipid moieties which become base-resistant during maturation

Previous experiments had shown that [^{3}H]*myo*-inositol-labeled proteins could be found as early as 3 min after addition of [^{3}H]*myo*-inositol to yeast cells and that cycloheximide blocked incorporation of [^{3}H]*myo*-inositol into proteins within minutes after addition of cells (Conzelmann *et al.*, 1990). Thus, to follow the fate of newly made GPIgps, we performed pulse–chase experiments using [^{3}H]*myo*-inositol for pulse labeling and cycloheximide plus cold *myo*-inositol to initiate the chase. Cycloheximide was used in addition to *myo*-inositol since the latter, though competing efficiently with the incorporation of [^{3}H]*myo*-inositol into PI, does not block the continuing incorporation of [^{3}H]PI into GPIgps. In these pulse–chase experiments we found a high percentage of base-sensitive anchors after the pulse, but the relative proportion of base-sensitive anchors significantly decreased during chase (Figure 5). The apparent change in base sensitivity of anchors during chase is compatible with the idea that the lipid moieties of GPIgps get exchanged during maturation, but two other possible explanations have to be considered. First, it seems conceivable that anchors with both types of lipids are indiscriminately transferred onto nascent proteins but that proteins with mild base-sensitive anchors get rapidly degraded so that the relative proportion of base-sensitive anchors decreases with time. Since in our experiments we find no significant increases in the total amount of base-resistant anchors during chase (Figures 5 and 6), we have no experimental evidence at present to rule out this interpretation. Secondly, it seemed possible that through extraction and delipidation we selectively lose proteins with base-sensitive anchors while extracting chased cells. Several control experiments, however, failed to support this. (i) Since the major difference between pulsed and chased GPIgps is brought about by the very extensive elongation of the *N*-glycans in the Golgi (Conzelmann *et al.*, 1990; Figure 2, lanes 1 and 2) we chose to perform pulse–chase experiments in *mnn9* cells in which this elongation of *N*-glycans does not occur (Tsai *et al.*, 1984; Gopal and Ballou, 1987). The result showed that the relative decrease of base-sensitive anchors during chase also occurs in *mnn9* cells (not shown). (ii) Control experiments failed to reveal any loss of GPIgps into the cell wall fraction which is discarded during the extraction procedure (see Materials and methods). (iii) Since through pronase treatment of polyacrylamide gels we did not quantitatively recover the counts of the mature, high molecular weight GPIgps, we also prepared anchor peptides using a delipidation procedure which avoided the SDS–PAGE step. The results shown in Figure 6 again document the relative decrease of mild

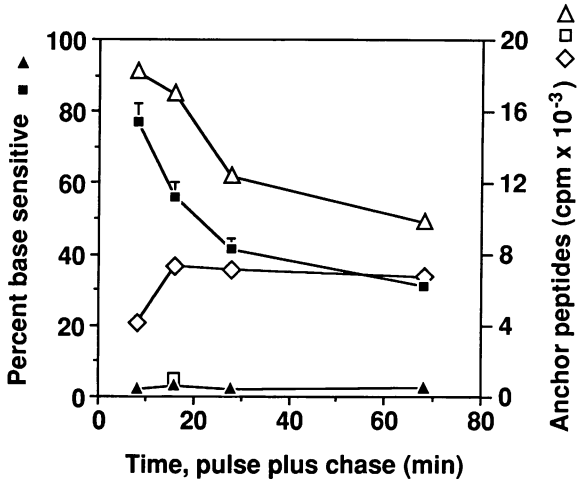

Fig. 5. Change of mild base sensitivity of GPI anchors during chase. X2180 cells were grown at 24°C, labeled with [^{3}H]*myo*-inositol for 8 min and then chased for 0, 8, 20 or 60 min in the presence of cycloheximide. Glycoproteins were isolated using procedure A and were delipidated by SDS–PAGE. Individual sample lanes of the slab gel (including stacking gel) were incubated with pronase in order to recover labeled anchor peptides (procedure F). Base sensitivity of anchor peptides was determined by phase separation in TX-114 after mild alkaline hydrolysis (■) or after control incubations (▲) (*X* values, see Materials and methods) and was plotted as a function of the time elapsed between the start of the pulse and the end of the chase. Standard errors of duplicates (σ_{n-1}) are indicated by vertical bars unless they are so small that they overlap with the main symbols. The total of base resistant anchors (◇) (*Z* values, see Materials and methods) was calculated based on the recoveries of anchor peptides at each time point (△). (□) Total base resistant anchor peptides when cycloheximide was added 10 min before [^{3}H]*myo*-inositol.

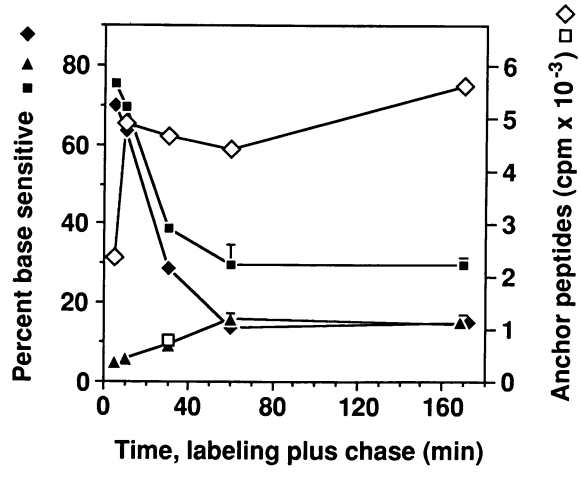

Fig. 6. Change of mild base sensitivity of GPI anchor peptides prepared by chloroform–methanol delipidation. X2190 cells were grown at 30°C and an aliquot of 2.5 OD_{600} units of cells was labeled for 5 min with [^{3}H]*myo*-inositol. Other aliquots were similarly labeled for 10 min and were chased for 0, 20, 50 or 160 min in the presence of cycloheximide and *myo*-inositol. Glycoproteins were prepared by procedure A and delipidated by extraction with chloroform–methanol (procedure E). The glycoproteins were treated with endoglycosidase H and pronase. The percentage of base-sensitive anchor peptides was determined and plotted using symbols as in Figure 5. (◆) Percentage of mild sensitive anchor peptides after subtraction of SHAPs (*Y* values, see Materials and methods).

base-sensitive anchors during chase. Thus, none of the several control experiments indicated that selective loss of proteins is a problem. In four out of six experiments of the type shown in Figures 5 and 6 we observed an increase of spontaneously hydrophilic anchor peptides (SHAPs) during chase, i.e. of peptides which partitioned into the aqueous phase prior to base treatment (Figure 6). The origin of this material is not clear at present. Occasional pretreatment of anchor peptides with endoglycosidase H, an enzyme which removes all *N*-glycans of *S.cerevisiae*, reduced the fraction of these SHAPs suggesting that part of this increase might be due to the elongation during chase of *N*-glycans associated with anchor peptides. In several later experiments, the percentage of SHAPs was very low and showed no tendency to increase during chase (e.g. Figures 5, 7 and 8). Whether or not SHAPs were subtracted, the change in base sensitivity of anchor peptides during chase was very significant (Figures 5 and 6).

Subcellular localization of the GPI anchor modification

To see if the relative decrease of base-sensitive anchors described above can occur while proteins are in the ER, we determined the rate of this decrease in mutants which have a secretion block in the transport from the ER to the Golgi. As shown in Figure 7, it appears that in the three secretion mutants tested, a significant fraction of anchors is base-resistant after a pulse with [^{3}H]*myo*-inositol whether or not secretion is blocked; however, the fraction of base-resistant anchors increases more rapidly during chase when secretion is working normally than when it is blocked. Since the proteins do not change their molecular weight when retained in the ER, we could also investigate the rate of appearance of base-resistant anchors in individual proteins during chase. As can be deduced from the comparison of peak heights in Figure 8 (panel A), GPIgps remain relatively stable while retained in the ER. In keeping with the experiments in Figure 7, the decrease of base-sensitive anchors comes to a halt after only 10 min of chase in most proteins (Figure 8, panel B). Moreover, there are significant differences in the amounts of base-sensitive anchors between different proteins already after the 10 minutes of pulse. The increase of base sensitivity in protein F during chase is probably artifactual since the total amount of c.p.m. in this protein increased during chase (Figure 8, panel A) and protein F might therefore represent a breakdown product of proteins A, B or C.

The only detectable GPI precursor is base-sensitive

Intermediates in GPI biosynthesis have been identified in *T.brucei* (Krakow *et al.*, 1986, 1989; Menon *et al.*, 1988, 1990; Masterson *et al.*, 1989; Mayor *et al.*, 1990a,b) but so far we have been unable to detect similar glycolipids in yeast, possibly because the pool size of the mature anchor glycolipid is too small. A potential precursor of GPIs, however, can be observed in *sec53-6*, a temperature-sensitive glycosylation mutant in which synthesis of GDP-mannose is blocked at 37°C (Kepes and Schekman, 1988). When labeled at 37°C, this mutant accumulated a [^{3}H]*myo*-inositol- as well as a [^{3}H]glucosamine-containing hydrophobic glycolipid (lipid X, Figure 9, lanes 2 and 3). After mild base hydrolysis, none of the [^{3}H]glucosamine-labeled lipid X was left at the original position upon TLC (Figure 9, lanes 9 versus 7). Data obtained with purified [^{3}H]*myo*-inositol-labeled lipid X suggested the structure

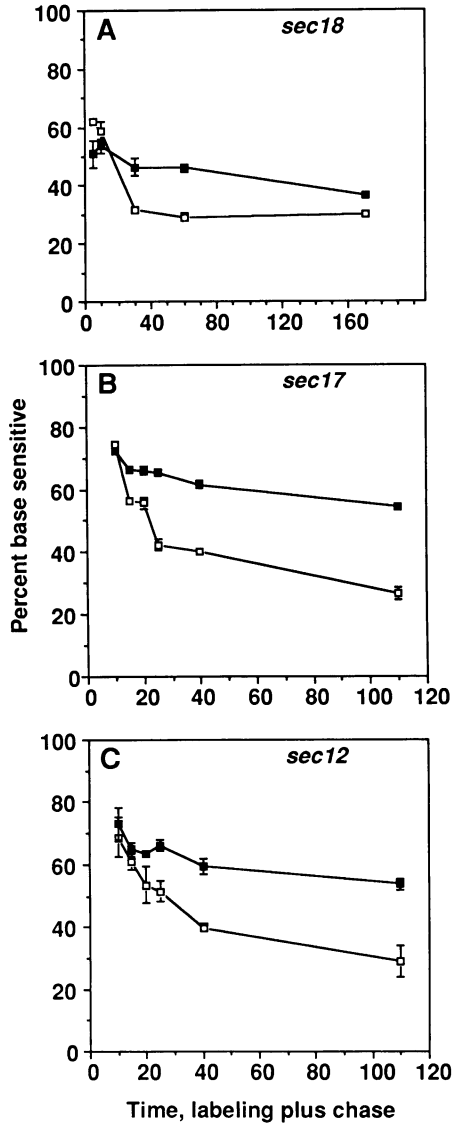

Fig. 7. Change of mild base sensitivity of anchors during chase in secretion mutants. *Sec18* cells, *sec17* and *sec12* cells were preincubated and labeled with [^{3}H]*myo*-inositol at 24°C or 37°C for 5 or 10 min (panel A) or 10 min (panels B and C) and cells labeled for 10 min were chased for 0–160 min in the presence of cycloheximide. Glycoproteins were prepared using procedure A. **Panel A.** glycoproteins were subjected to delipidation with chloroform–methanol (procedure E), endoglycosidase H and pronase treatment. **Panels B and C:** glycoproteins were further purified by SDS–PAGE and eluted from the gel with pronase (procedure F). Anchor peptides were treated with mild alkaline hydrolysis or control incubated and partitioned in TX-114. (□) Labeling and chase at 24°C; (■) labeling and chase at 37°C. SHAPs were low and showed no tendency to increase during chase. (Mean values for SHAPs for 24°C and 37°C curves respectively: panel A, 8.9% and 3.7%; panel B, 2.4% and 1.6%; panel C, 3.8% and 2.4%.) Thus, SHAPs have been subtracted (*Y* values, see Materials and methods).

GlcN-acylinositol-P-diacylglycerol based on the following: (i) mild base treatment removed all hydrophobic components of lipid X; (ii) the mild base treated fragment became uncharged upon treatment with hydrofluoric acid (HF) (Ferguson *et al.*, 1988) and *N*-acetylation; (iii) the HF-generated fragment was resistant to jack bean α-mannosidase but yielded [^{3}H]inositol after treatment with HNO_2. The presence of an acyl chain on the inositol was

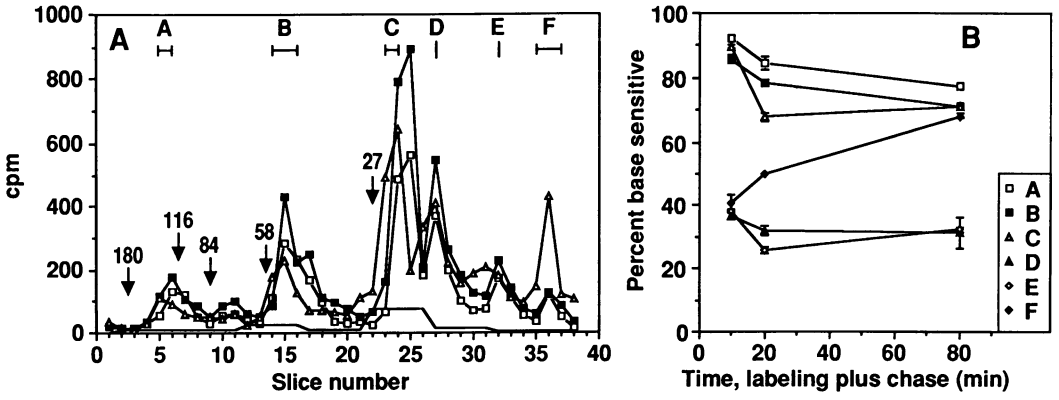

Fig. 8. Change of mild base sensitivity of anchors of individual proteins retained in the endoplasmic reticulum. *Sec18* cells were preincubated and labeled with [^{3}H]*myo*-inositol at 37°C for 10 min and chased in the presence of cycloheximide for 0, 10 or 70 min. Glycoproteins were purified by procedure A and separated by SDS−PAGE in an 11% gel. Individual lanes were sliced into 2 mm wide slices and digested with pronase. 5% of each eluate was counted by β-counting to obtain the profiles of radioactivity shown in **panel A**: (□) no chase; (■) 10 min of chase; (Δ) 70 min of chase; (—) labeling in the presence of cycloheximide (200 μg/ml). Arrows indicate the position of various molecular mass standards in the range of 27−180 kDa as indicated. Eluates were pooled as indicated by horizontal bars (pools A−F) and aliquots from individual pools from each time point subjected to mild base hydrolysis and partitioned in TX-114. **Panel B** shows the change in base sensitivity during chase for the individual proteins contained in pools A−F after subtraction of SHAPs (*Y* values, see Materials and methods). SHAPs were low and showed no tendency to increase during chase. (Mean values for SHAPs in curves derived from peaks A−F were 4.4%, 2.7%, 3.1%, 2.4%, 1.9% and 2.4% respectively.)

suggested since (i) lipid X was PI-PLC resistant; (ii) treatment with NH_3 under conditions which allow the removal of *myo*-inositol-bound acyls (Roberts *et al.*, 1988a) gave rise to several more polar lipids, some of which were PI-PLC sensitive (not shown); and (iii) the migration in TLC indicated that lipid X was more hydrophobic than PI. The structure of lipid X and the presence of non-acetylated glucosamine in particular strongly suggest that lipid X is an intermediate of yeast GPI biosynthesis. The same glycolipid has recenty been described by Orlean (1990). Importantly, other mild base-resistant, [^{3}H]glucosamine-labeled lipids of *sec53* did not comigrate with any [^{3}H]*myo*-inositol-labeled lipids and hence are unlikely to be GPI intermediates (Figure 9, lanes 6−10). The absence of a base-resistant form of lipid X suggests that the biosynthesis of GPIs of yeast is initiated on a base-sensitive lipid.

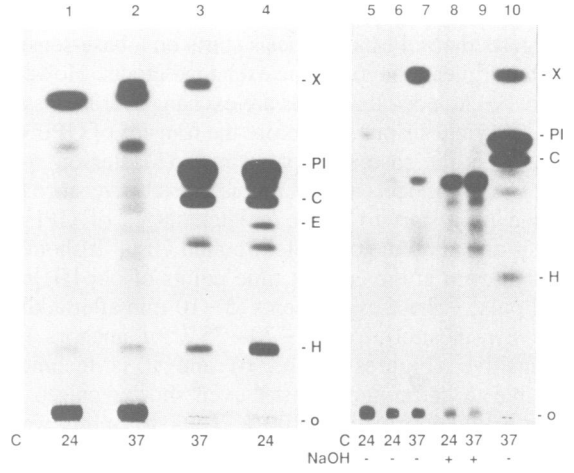

Fig. 9. Accumulation of an early glycolipid intermediate in *sec53*. Lanes 1−4 and 10: aliquots of 5 OD_{600} units of *sec53* cells were preincubated and labeled with either [^{3}H]glucosamine (lanes 1 and 2) or [^{3}H]*myo*-inositol (lanes 3, 4 and 10) at 24°C or 37°C for 60 min as indicated at the bottom. Lipids were extracted using procedure C, desalted and analyzed by TLC (system 55:45:10) and fluorography. Lanes 5−9: 5 OD_{600} units of X2180 cells (lane 5) or *sec53* cells (lanes 6−9) were precultured, preincubated (20 min) and labeled with [^{3}H]glucosamine at 24°C or 37°C for 100 min. 10% of the desalted lipid extract was analyzed directly (lanes 5−7) whereas 90% was analyzed after mild alkaline hydrolysis (lanes 8 and 9). X = lipid X; C, E and H = IPCs as in Figure 4.

Discussion

The data presented herein show that two different lipid components can be found in GPIgps of *S.cerevisiae*. The base sensitivity of one of these components is consistent with it being a conventional diacylglycerol, the kind of lipid found on the variant surface glycoproteins of *T.brucei*. No attempt was made to characterize this base-sensitive lipid since it was not efficiently labeled by [^{3}H]palmitic acid. The other component appears to be a C18-phytosphingosine-containing ceramide which, based on its migration in TLC, is more hydrophobic than the ceramides from IPC/C and MIPC/E. Since the latter also contain C18-phytosphingosine, the difference in hydrophobicity has to lie in the substituent on the amine. The most abundant IPC of yeast (IPC/C, this paper; ICP-II by Smith and Lester, 1974) and MIPC/E contain C26 hydroxylated fatty acids (Smith and Lester, 1974) and it therefore seems possible that the substituent on the GPI anchor is a nonhydroxylated fatty acid. Indeed, the only fatty acid component of the anchor ceramide that could clearly be identified is a nonhydroxylated C26 fatty acid. Direct chemical analysis of yeast anchors is currently being carried out.

The lipid component of the GPI anchor of contact site A protein from *D.discoideum* has been postulated to be a ceramide (Stadler *et al.*, 1989). While this anchor is not cleaved by PI-PLC, we find that the mild base-resistant yeast anchor as well as IPC/C and MIPC/E are sensitive to PI-PLC (Figure 2; Conzelmann *et al.*, 1990; Puoti *et al.*, 1991; Bordier, 1981). The PI-PLC resistance of the ceramide anchor of *D.discoideum* might be due to the presence of an acyl group on the *myo*-inositol. Interestingly, the radioactivity of the [^{3}H]palmitic acid-labeled contact site A anchor is nearly quantitatively recovered in a fatty acid,

while in *S.cerevisiae* it is incorporated into anchors mainly in the form of C18-phytosphingosine (whose synthesis starts with the condensation of serine with palmitoyl-CoA). Thus, it seems that *D.discoideum* and *S.cerevisiae* make quite different uses of this tracer.

The existence of two types of anchor lipids in *S.cerevisiae* leaves us with two possibilities with regard to precursor biosynthesis: either GPIs are assembled on both types of lipids and both types of GPIs are transferred onto newly made proteins ('two precursor model') or GPIs with only one type of lipid are transferred to proteins and this lipid is replaced by another lipid on part of the proteins later on ('exchange model'). The two precursor model implies that the glycosyltransferases, which assemble these anchor precursors by the stepwise addition of monosaccharides (Masterson *et al.*, 1989; Menon *et al.*, 1990), are 'blind' with regard to the lipid moiety onto which they add or else are manyfold. The fact that *S.cerevisiae* does not use ceramides corresponding to the major IPCs but uses a ceramide typical of a minor IPC to make the base-resistant GPI anchors (Figure 4, panel D) may be difficult to reconcile with the possibility that all anchor glycosyltransferases are 'blind' for the lipid moieties. The absence of a base-resistant early intermediate of anchor biosynthesis in *sec53* (Figure 9), suggests that GPI biosynthesis starts on a base-sensitive lipid; this argues in favor of the exchange model. However, it cannot be excluded that lipids are exchanged at later stages of GPI biosynthesis but still before the transfer of GPIs onto proteins as is the case in trypanosomes (Masterson *et al.*, 1990). The two models are not mutually exclusive since lipid exchange may start to occur at later stages of GPI biosynthesis and continue on protein-bound GPIs. It should be noted that even at the earliest time points of our [^{3}H]*myo*-inositol pulse−chase experiments (5−10 min after addition of [^{3}H]*myo*-inositol), only ~70−75% of anchors were base-sensitive (Figures 5 and 6) and it is technically impossible to determine if, after even shorter pulses, this fraction would approximate 100%. Thus, presently we are not able to rule out one or other of these models.

In the two precursor model, we have to interpret the decrease of base sensitivity of anchor peptides during chase (Figures 5 and 6) as the result of a higher turnover rate of GPIgps with base-sensitive anchors. Since most proteins seem to contain both types of anchors, this might even imply a direct influence of the GPI lipid moiety on the protein turnover, e.g. by facilitating the sorting of GPIgps into a degradative compartment. In the context of the exchange model, the decrease of base sensitivity of anchor peptides during chase (Figures 5 and 6) can be interpreted as the replacement of the initially transferred base-sensitive GPI lipids by ceramides. This decrease of base-sensitive anchors is significantly slowed by an ER to Golgi secretion block (Figure 7), a finding which points to a compartmental distribution of biosynthetic events. In terms of the two precursor model, this slowing possibly means that the secretion block interferes with the selective transport of GPIgps with base-sensitive anchors to a degradative compartment or with degradation itself. In the context of the exchange model several concepts can be envisaged to explain this slowing effect of the secretion block. It might indicate: (i) that lipid exchange is severely disturbed when the ER becomes distended or fragmented; or (ii) that lipid exchange occurs for some proteins in the ER, for others in the Golgi; or conceivably (iii) that lipid exchange is bidirectional, i.e. that base-sensitive lipids are exchanged for base-resistant ones at the same time as base-resistant lipids get replaced by base-sensitive ones. The fraction of base-sensitive anchors in proteins would then be dictated by the relative rate constants of the two reactions and the fraction of base-sensitive lipids in the pool of exchanging lipids. Thus the effect of the secretion block might reflect a difference in lipid composition between ER and later compartments.

We can only speculate about the identity of enzymes which possibly might be involved in the exchange of lipid moieties of GPI anchors: (i) yeast microsomes contain an activity which transfers phosphoinositol from PI onto ceramides thus carrying out the first step in the biosynthesis of IPCs (Becker and Lester, 1980). It is conceivable that this enzyme would transfer phosphoinositol groups even if they were substituted with carbohydrate and protein components of a GPIgp. Mammalian tissues contain a PI:*myo*-inositol exchange enzyme for which no specific role has been proposed and which, if present in yeast, might be involved in the remodeling of GPI anchors (Takenawa and Egawa, 1980; Bleasdale and Wallis, 1981).

Lipid remodeling has been described in trypanosomes (Masterson *et al.*, 1990) but may also occur on mammalian GPI anchors since the latter most often contain quite unusual lipid components which are certainly not representative of the lipids found in PI. Due to their low abundance, no lipid structures of biosynthetic intermediates of GPIs have been reported in mammalian cells so far and a comparison of the lipid components of GPI precursors and mature anchors awaits further experimentation.

Materials and methods

Strains, growth conditions and materials

Haploid *S.cerevisiae* strains were used. The secretion mutants were those originally developed by Peter Novick and Randy Schekman: SF266-1C, a *sec12-4*; HSMF175, a *sec17-1*; HMSF176, a *sec18-1*; HMSF331, a *sec53-6* and the corresponding wild-type strains X2180-1A, a *SUC2 mal gal2 CUP1* and X2180-1B, α *SUC2 mal gal2 CUP1* (Novick *et al.*, 1980, 1981; Esmon *et al.*, 1981). Cells were kept on YPD plates containing 1% Bacto yeast extract, 2% casein hydrolysate (peptone 140), 2% Bacto agar and 2% glucose. Wickerham's minimal medium (Wickerham, 1946) with 2% glucose as a carbon source but omitting *myo*-inositol was used. The optical density (OD) of dilute cell suspensions was measured in a 1 cm cuvette at 600 nm. 1 OD_{600} unit of cells corresponds to $1-2.5 \times 10^7$ cells depending on the strain used. Reagents were obtained from the sources described recently (Conzelmann *et al.*, 1990). Partially purified lyticase from *Arthrobacter luteus* was from Sigma, St Louis, MO, or donated by Dr Susan Gasser (this Institute). D-[6-^{3}H]Glucosamine (20−40 Ci/mmol) was from Amersham, Buckinghamshire, UK. Pure PI-PLC from *Bacillus cereus* was from Boehringer, Mannheim, FRG. Ceramides type III and type IV from bovine brain and phytosphingosine from yeast were from Sigma.

Radiolabeling of cells

All cultures were done in a shaking waterbath at 250 r.p.m. For labeling with [^{3}H]*myo*-inositol, cells were precultured overnight in minimal medium, exponentially growing cells were centrifuged and resuspended at 10 OD_{600} units/ml in fresh medium and aliquots put into large 50 ml Falcon tubes. The cells from secretion mutants were preincubated for 10−20 min at 37°C (to induce the secretion block) or at 24°C (for control) before the addition of [^{3}H]*myo*-inositol (0.5−10 μCi/OD_{600} unit, 0.36−7.2 μM). After 20 min the cells were diluted with 4 vol of medium plus 40 μg/ml of *myo*-inositol. In pulse−chase experiments, cells were diluted with 4 vol medium containing cycloheximide (200−300 μg/ml) and, in some experiments, *myo*-inositol (40 μg/ml). Labeling with [9,10-^{3}H]palmitic acid (55 Ci/mmol) was done in minimal medium using 50 μCi/ml and 1 OD_{600} unit of cells per ml. For labeling with [^{3}H]glucosamine cells were cultured either in minimal medium supplemented with 1% casein hydrolysate and the glucose content reduced to 0.1% or in minimal medium adjusted to pH 8.0 in order to facilitate the uptake of label through the plasma membrane. Cells were labeled at 5 OD_{600} units of cells per ml and 10−15 μCi/ml of

[^{3}H]glucosamine. At the end of the incubations, cells were placed on ice, NaF and NaN_3 were added (10 mM final of each), cells were rapidly centrifuged (12 000 g × 30 s) and frozen.

Preparation of anchor peptides

Various protocols were used.

Procedure A. Labeled cells were quickly placed on ice, NaF and NaN_3 were added, cells were centrifuged (12 000 g × 30 s), cells were resuspended and snap frozen in a 2-fold concentrated SDS- and 2-mercaptoethanol-containing sample buffer ('final sample buffer, Laemmli, 1970) (2–10 OD_{600} units/100 μl) containing benzamidine, NaF and NaN_3 (10 mM each) in addition. After thawing, the cells were broken by vortexing with glass beads (4 × 1 min) and boiled for 5 min. After dilution and addition of a 20-fold excess of the detergent Triton X-100 over SDS, cell walls were removed by centrifugation and glycoproteins were adsorbed onto saturating amounts of Con A–Sepharose as described (Conzelmann *et al.*, 1990). After extensive washing, the adsorbed glycoproteins were eluted from beads by boiling in sample buffer.

Procedure B. This was identical to procedure A except that the cell walls of broken cells were washed in water and treated with lyticase (100 U/OD_{600} unit of cells in 50 mM Tris–HCl, pH 7.4, 4 mM $MgCl_2$, 40 mM 2-mercaptoethanol, 0.5% Triton X-100, 50 μg/ml antipain and 40 μg/ml pepstatin for 1 h at 37°C). Lyticase was then inactivated by boiling (20 min), insoluble material was removed by centrifugation, and the supernatant was adsorbed onto Con A–Sepharose and bound material was eluted as above. This was done in an attempt to exclude that the cell wall fraction contained GPIgps which were not extracted by SDS. However, no GPIgps were detected in the cell wall fraction by this method.

Procedure C. Labeled cells were suspended in $CHCl_3$–CH_3OH–H_2O (10:10:3), broken with glass beads, centrifuged and the pellet extracted twice with the same solvent for delipidation. The 10:10:3 extract was used for lipid analysis while the pellet was dried, resuspended in 'final sample buffer', boiled and cell walls were removed by centrifugation. Glycoproteins were further purified over Con A–Sepharose as in procedure A.

Procedure D. Cells were broken and boiled in sample buffer as in procedure A, the cell walls were removed by centrifugation and 1 ml of $CHCl_3$–CH_3OH–H_2O (10:10:3) was added to 100 μl of the supernatant. After vortexing, the phases were separated by centrifugation and the proteins contained in the interphase were further delipidated by four extractions with 1 ml of $CHCl_3$–CH_3OH–H_2O (10:10:3). The delipidated proteins were resuspended by boiling in sample buffer, and GPIgps further purified with Con A–Sepharose as in procedure A.

After elution from Con A–Sepharose the glycoproteins obtained by the various procedures were further delipidated by one of the following procedures.

Procedure E. 100 μg of ovalbumin and 100 μg of cytochrome *c* were added to the eluate, the samples were dried under reduced pressure and proteins precipitated four times with 0.8 ml of $CHCl_3$–CH_3OH (1:1, v/v) for delipidation. Thereafter, glycoproteins were digested with pronase or proteinase K (1 mg/ml in 100 mM Tris–HCl, pH 8.0, 1 mM $CaCl_2$ for 2–4 h at 37°C). In some experiments, *N*-glycans were removed from labeled proteins by incubating them with endoglycosidase H (1 mU, 4 h, 37°C) as described (Owen *et al.*, 1981) before adding pronase.

Procedure F. Proteins eluted from Con A–Sepharose were further delipidated by SDS–PAGE (Laemmli, 1970). After electrophoresis the lipid-containing zone below and up to 2 mm above the dye front was removed. For elution of GPIgps the gel was cut into appropriate slices which were incubated for 16 h with pronase in the buffer described above but containing 0.04% of Triton X-100, 20 μg/ml of gentamycin and 10 mM NaN_3 in addition. In other experiments proteins of gel lanes were electroeluted as described (Bhown *et al.*, 1980). The region below the dye front, which is known to contain the phospholipids, was often cut out and its radioactivity determined by scintillation counting. These controls did not reveal the presence of significant amounts of contaminating lipids after the Con A–Sepharose purification step. Also, no contaminating lipids could be detected in the anchor peptide preparation by TLC (Figure 4, panel C, lanes 1 and 2).

Determination of mild base sensitivity of [^{3}H]myo-inositol-labeled anchor peptides

Duplicate samples of anchor peptides were deacylated in 0.1 N NaOH in $CHCl_3$–CH_3OH–H_2O (10:10:3) for 30 min at 37°C (Becker and Lester, 1980). Control tubes were incubated in solvent 10:10:3 without NaOH. After neutralization with acetic acid and addition of corresponding amounts of sodium acetate to control tubes, the samples were dried in a Speed-Vac evaporator, suspended in 500 μl Triton X-114, 1% in 10 mM Tris–HCl, pH 7.4, 150 mM NaCl by vortexing and using a bath sonicator. After warming to 37°C, the precipitated detergent was sedimented by centrifugation (Bordier, 1981) and the upper, aqueous phase removed to fresh tubes. Then the radioactivity of both phases was determined by scintillation counting. In control separations, 90% [^{3}H]*myo*-inositol and 1% of [^{3}H]PI were recovered in the aqueous phase. The percentage of water soluble radioactivity (X in the following) was calculated for each sample using the following formula: $X = \{[(\text{c.p.m.}^{\text{aqueous}}/\text{c.p.m.}^{\text{detergent}} + \text{c.p.m.}^{\text{aqueous}}) - 0.01]/0.9\} \times 100$. To focus attention onto anchor peptides in which the removal of lipid moieties by mild alkaline hydrolysis is reflected by a transition from the Triton X-114 detergent to the aqueous phase, the X value was modified by subtraction of SHAPs using formula: $Y = [(X_{+\text{NaOH}} - X_{-\text{NaOH}})/(100 - X_{-\text{NaOH}})] \times 100$. All determinations were carried out in duplicate, triplicate or quadruplicate and standard deviations (σ_{n-1}) were indicated by error bars (Figures 5–8). Absolute amounts of base-resistant anchors (Z values) after various times of chase were calculated by formula: Z = c.p.m. recovered in anchor peptides × [(100 − Y)/100].

Structural analysis of labeled anchors and lipids

Anchor lipids or isolated lipids were deacylated by mild alkaline hydrolysis as described (Becker and Lester, 1980). After neutralization with acetic acid, lipids were desalted by partitioning between butanol and water as described (Krakow *et al.*, 1986) and the butanol extract was analyzed by TLC. Treatment of anchor peptides or lipids with PI-PLC from *B.cereus* was done at 37°C for 1 h in the following buffer: 20 mM Tris–HCl, pH 7.4, 0.04% Triton X-100, 0.2–1 mM EDTA (50 μl/tube). Strong acid hydrolysis of [^{3}H]palmitic acid-labeled anchor peptides or lipids was done in 1 N HCl in CH_3OH–H_2O (82:18) for 16 h at 80°C in Teflon-lined glass tubes as described (Dickson *et al.*, 1990). Solvent was evaporated and the products were subjected to Folch partitioning and the dried organic phase was analyzed by TLC. Alternatively, HCl was evaporated and the dried sample was then directly converted to biphenylcarbonyl derivatives and analyzed by HPLC as described (Dickson *et al.*, 1990), the program being completed by washing the column with 100% ethanol at the end. C18-Phytosphingosine, C20-phytosphingosine and C18-erythrodihydrosphingosine were added as internal standards. Strong alkaline hydrolysis was done in 1 N KOH in CH_3OH–H_2O (2:1) at 80°C for 18 h. After acidification, either the ether extract was analyzed by TLC or phenacyl derivatives were prepared and analyzed on HPLC as described (Dickson *et al.*, 1990). Nitrous acid treatment of anchor peptides was done as described (Roberts *et al.*, 1988a), the products were partitioned between water and butanol (Krakow *et al.*, 1986), the butanol phase was dried and lipids were separated by preparative HPLC as described (Puoti *et al.*, 1991). This step proved necessary in order to get rid of detergents before TLC. The labeled lipid standards generated from $CHCl_3$–CH_3OH–H_2O extracts of [^{3}H]*myo*-inositol- or [^{3}H]palmitic acid-labeled cells using HPLC were IPC/C (major IPC among several IPCs which differ from each other with regard to the ceramide moiety) and MIPC/E (mannosyl IPC) and $M(IP)_2C/H$ as defined by Puoti *et al.* (1991). Ascending TLC was performed on 0.2 mm thick silica gel 60 plates (Merck) using the following solvent systems: $CHCl_3$–CH_3OH (95:5); $CHCl_3$–CH_3OH–0.25% KCl in water (55:45:10); $CHCl_3$–CH_3OH–2 N NH_3 (40:10:1). For spotting, lipids were taken up in $CHCl_3$–CH_3OH–water (10:10:3) unless indicated otherwise. This led to the loss of counts during application when complete anchor peptides were spotted. The developed TLC were sprayed with EN^{3}HANCE (NEN) and fluorograms were obtained using X-OMAT film (Kodak) exposed at −80°C.

Liberation of head groups of lipid X from *sec53* cells with HF was done as described (Ferguson *et al.*, 1988). Selective removal of the acyl chain attached to *myo*-inositol with methanolic NH_3 was performed according to Roberts *et al.* (1988a), but incubating for 2 h at 24°C. Isolated head groups were treated with nitrous acid (Roberts *et al.*, 1988a), the products were *N*-acetylated, desalted using mixed bed ion exchange resin (AG-501, Bio-Rad) and analysed by descending paper chromatography as described (Conzelmann and Kornfeld, 1984). SDS–PAGE was carried out under reducing conditions (Laemmli, 1970).

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References

Bangs,J.P., Hereld,D., Krakow,J.L., Hart,G.W. and Englund,P.T. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 3207–3211.

Becker,G.W. and Lester,R.L. (1980) *J. Bacteriol.*, **142**, 747–754.

Bhown,A.S., Mole,J.E., Hunter,F. and Bennett,J.L. (1980) *Anal. Biochem.*, **103**, 184–190.

Bleasdale,J.E. and Wallis,P. (1981) *Biochim. Biophys. Acta*, **664**, 428–440.

Bordier,C. (1981) *J. Biol. Chem.*, **256**, 1604–1607.

Conzelmann,A. and Kornfeld,S. (1984) *J. Biol. Chem.*, **259**, 12528–12535.

Conzelmann,A., Spiazzi,A. and Bron,C. (1987) *Biochem. J.*, **246**, 605–610.

Conzelmann,A., Riezman,H., Desponds,C. and Bron,C. (1988) *EMBO J.*, **7**, 2233–2240.

Conzelmann,A., Fankhauser,C. and Desponds,C. (1990) *EMBO J.*, **9**, 653–661.

Cross,G.A.M. (1990) *Annu. Rev. Cell Biol.*, **6**, 1–39.

Dickson,R.C., Wells,G.B., Schmidt,A. and Lester,R.L. (1990) *Mol. Cell. Biol.*, **10**, 2176–2181.

Doering,T.L., Masterson,W.J., Hart,G.W. and Englund,P.T. (1990) *J. Biol. Chem.*, **265**, 611–614.

Esmon,B., Novick,B. and Schekman,R. (1981) *Cell*, **25**, 451–460.

Ferguson,M.A. and Williams,A.F. (1988) *Annu. Rev. Biochem.*, **57**, 285–320.

Ferguson,M.A.J., Low,M.G. and Cross,G.A.M. (1985) *J. Biol. Chem.*, **260**, 14547–14555.

Ferguson,M.A.J., Duszenko,M., Lamont,G.S., Overath,P. and Cross,G.A.M. (1986) *J. Biol. Chem.*, **261**, 356–362.

Feruguson,M.A.J., Homans,S.W., Dwek,R.A. and Rademacher,T.W. (1988) *Science*, **239**, 753–759.

Field,M.C., Menon,A.K. and Cross,G.A.M. (1991) *J. Biol. Chem.*, **266**, 8392–8400.

Gopal,P.K. and Ballou,C.E. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 8824–8828.

Homans,S.W., Ferguson,M.A.J., Dwek,R.A., Rademacher,T.W., Anand,R. and Williams,A.F. (1988) *Nature*, **333**, 269–272.

Kepes,F. and Schekman,R. (1988) *J. Biol. Chem.*, **263**, 9155–9161.

Krakow,J.L., Hereld,D., Bangs,J.D., Hart,G.W. and Englund,P.T. (1986) *J. Biol. Chem.*, **261**, 12147–12153.

Krakow,J.L., Doering,T.L., Masterson,W.J., Hart,G.W. and Englund,P.T. (1989) *Mol. Biochem. Parasitol.*, **36**, 263–270.

Laemmli,U.K. (1970) *Nature*, **227**, 680–685.

Low,M.G. and Saltiel,A.R. (1988) *Science*, **239**, 268–275.

Luhrs,C.A. and Slomiany,B.L. (1989) *J. Biol. Chem.*, **264**, 21446–21449.

Masterson,W.J., Doering,T.L., Hart,G.W. and Englund,P.T. (1989) *Cell*, **56**, 793–800.

Masterson,W.J., Raper,J., Doering,T.L., Hart,G.W. and Englund,P.T. (1990) *Cell*, **62**, 73–80.

Mayor,S., Menon,A.K. and Cross,G.A. (1990a) *J. Biol. Chem.*, **265**, 6174–6181.

Mayor,S., Menon,A.K., Cross,G.A., Ferguson,M.A., Dwek,R.A. and Rademacher,T.W. (1990b) *J. Biol. Chem.*, **265**, 6164–6173.

Menon,A.K., Mayor,S., Ferguson,M.A., Duszenko,M. and Cross,G.A. (1988) *J. Biol. Chem.*, **263**, 1970–1977.

Menon,A.K., Schwarz,R.T., Mayor,S. and Cross,G.A. (1990) *J. Biol. Chem.*, **265**, 9033–9042.

Novick,P., Field,C. and Schekman,R. (1980) *Cell*, **21**, 205–205.

Novick,P., Ferro,S. and Schekman,R. (1981) *Cell*, **25**, 461–469.

Orlean,P. (1990) *Mol. Cell. Biol.*, **10**, 5796–5805.

Owen,M.J., Kissonerghis,H.F., Lodish,H.F. and Crumpton,M.J. (1981) *J. Biol. Chem.*, **256**, 8987–8993.

Puoti,A., Desponds,C. and Conzelmann,A. (1991) *J. Cell Biol.*, **113**, 515–525.

Roberts,W.L., Kim,B.H. and Rosenberry,T.L. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 7817–7821.

Roberts,W.L., Myher,J.J., Kuksis,A., Low,M.G. and Rosenberry,T.L. (1988a) *J. Biol. Chem.*, **263**, 18766–18775.

Roberts,W.L., Santikarn,S., Reinhold,V.N. and Rosenberry,T.L. (1988b) *J. Biol. Chem.*, **263**, 18776–18784.

Schneider,P., Ferguson,M.A.J., McConville,M.J., Mehlert,A., Homans,S.W. and Bordier,C. (1990) *J. Biol. Chem.*, **265**, 16955–16964.

Smith,S.W. and Lester,R.L. (1974) *J. Biol. Chem.*, **249**, 3395–3395.

Stadler,J., Keenan,T.W., Bauer,G. and Gerisch,G. (1989) *EMBO J.*, **8**, 371–377.

Steiner,S., Smith,S., Waechter,C.J. and Lester,R.L. (1969) *Proc. Natl. Acad. Sci. USA*, **64**, 1042–1048.

Takenawa,T. and Egawa,K. (1980) *Arch. Biochem. Biophys.*, **202**, 601–607.

Thomas,J.R., Dwek,R.A. and Rademacher,T.W. (1990) *Biochemistry*, **29**, 5413–5422.

Tsai,P., Frevert,J. and Ballou,C.E. (1984) *J. Biol. Chem.*, **259**, 3805–3811.

Wickerham,L.J. (1946) *J. Bacteriol.*, **52**, 293–301.

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