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Alternative lipid remodelling pathways for glycosylphosphatidylinositol membrane anchors in *Saccharomyces cerevisiae*

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Glycosylphosphatidylinositol (GPI)-anchored membrane proteins of *Saccharomyces cerevisiae* exist with two types of lipid moiety—diacylglycerol or ceramide—both of which contain 26:0 fatty acids. To understand at which stage of biosynthesis these long-chain fatty acids become incorporated into diacylglycerol anchors, we compared the phosphatidylinositol moieties isolated from *myo*-[2-^{3}H]inositol-labelled protein anchors and from GPI intermediates. There is no evidence for the presence of long-chain fatty acids in any intermediate of GPI biosynthesis. However, GPI-anchored proteins contain either the phosphatidylinositol moiety characteristic of the precursor lipids or a version with a long-chain fatty acid in the *sn*-2 position of glycerol. The introduction of long-chain fatty acids into *sn*-2 occurs in the endoplasmic reticulum (ER) and is independent of the *sn*-2-specific acyltransferase *SLC1*. Analysis of ceramide anchors revealed the presence of two types of ceramide, one added in the ER and another more polar molecule which is found only on proteins which have reached the mid Golgi. In summary, the lipid of GPI-anchored proteins can be exchanged by at least three different remodelling pathways: (i) remodelling from diacylglycerol to ceramide in the ER as proposed previously; (ii) remodelling from diacylglycerol to a more hydrophobic diacylglycerol with a long-chain fatty acid in *sn*-2 in the ER; and (iii) remodelling to a more polar ceramide in the Golgi.

Keywords: ceramide/diacylglycerol/glycosylphosphatidylinositol/remodelling/*Saccharomyces cerevisiae*

Introduction

Numerous glycoproteins of *Saccharomyces cerevisiae* become attached to a glycosylphosphatidylinositol (GPI) anchor and many ultimately appear in the cell wall or at the plasma membrane (Hardwick *et al.*, 1992; Stratford, 1994; Cawley *et al.*, 1995; Komano and Fuller, 1995). The biosynthesis of GPI-anchored proteins follows the same basic rules in all eukaryotes, including yeast (Conzelmann *et al.*, 1990; Englund, 1993; Fankhauser *et al.*, 1993; Nuoffer *et al.*, 1993). Nevertheless, different organisms contain widely differing kinds of lipid moieties in their GPI anchors which, in most organisms, do not at all reflect the lipid moieties present in phosphatidylinositol (PI) which would appear as a natural starting point for the GPI biosynthesis in the endoplasmic reticulum (ER) (Roberts *et al.*, 1988b; Luhrs and Slomiany, 1989; Bütikofer *et al.*, 1990, 1992; Walter *et al.*, 1990; McConville and Ferguson, 1993; Patnaik *et al.*, 1993; Doering *et al.*, 1994; Redman *et al.*, 1994; Brewis *et al.*, 1995; Serrano *et al.*, 1995; Treumann *et al.*, 1995; Kapteyn *et al.*, 1996). In principle, this difference could come about by the specificity of the GlcNAc-transferase which initiates GPI biosynthesis by transferring GlcNAc onto PI or by the prevalence of a rare kind of PI in the environment of this enzyme. Alternatively, lipid moieties might become exchanged either before or after transfer of the GPI onto proteins (Englund, 1993). This latter model has been found to apply to the blood stage form of *Trypanosoma brucei* where longer fatty acids are replaced by myristic acid through sequential deacylation/reacylation reactions on *sn*-2, then *sn*-1 shortly before the GPI is attached to the protein (Masterson *et al.*, 1990). A remodelling mechanism has also been proposed to explain the prevalence of *sn*-1-alkyl, 2-acylglycerol in mammalian cells (Singh *et al.*, 1994). The situation in yeast is peculiar since two very different types of lipid moieties can be found: ceramides and another lipid which is sensitive to mild base treatment and can be removed by PI-specific phospholipase C (PI-PLC), probably diacylglycerol. The ceramides are found on the majority of yeast anchors; they mainly consist of C18:0 phytosphingosine and a C26:0 fatty acid and are different from the main ceramide found in the abundant inositolphosphoceramides (IPCs) (Smith and Lester, 1974; Lester and Dickson, 1993). Alternatively, Gas1p, a well-characterized GPI protein of yeast, is made with a C26:0 fatty acid (FA)-containing, base-sensitive lipid (Fankhauser *et al.*, 1993). In both types of lipid moieties the C26:0 may be hydroxylated on C2. Complete GPIs (CPs) ready to be transferred to proteins are present only in very low amounts in wild-type cells but can be detected in lipid extracts of [2-^{3}H]mannose-labelled *pmi40*, a strain with a conditional defect in mannose biosynthesis incorporating elevated amounts of exogenously added [^{3}H]mannose at 37°C (Sipos *et al.*, 1994). CPs also become detectable in extracts from [^{3}H]inositol-labelled *gaa1* and *gpi8* cells which both have a conditional mutation affecting the addition of CPs to proteins (Benghezal *et al.*, 1995; Hamburger *et al.*, 1995). In all these cases, the CPs are completely susceptible to mild base treatment, supporting the notion that the ceramide moieties may be introduced at a later stage by lipid remodelling of protein anchors. The work presented here addresses the question of whether the C26:0 long-chain FAs of mild base-sensitive anchors are present already at the beginning of GPI biosynthesis, or whether they are introduced only at a later stage.

It appeared impossible to obtain GPI intermediates in

sufficient quantity and purity as required for GC–MS. We therefore considered radiolabelling with lipid precursors such as acetate or palmitate in order to increase the sensitivity of detection. Acetate, although uniformly labelling all possible lipid moieties to comparable specific activity, was considered inappropriate because GPI intermediates would represent only a very scanty fraction among all labelled lipids and their purification seemed out of reach. As to the use of palmitate, we anticipated the same problem and in addition that it would introduce a heavy bias towards palmitate-containing lipid moieties. To avoid these inconveniences we resorted to metabolic labelling with [^{3}H]inositol. In contrast to the other possible labels, inositol labels only a subset of phospholipids, namely the various PIs, IPCs and GPI intermediates. Inositol labelling also was expected uniformly to label all possible GPIs irrespective of their lipid moieties and thus, we could be confident not to bias the result of our analysis by the label. The disadvantage of [^{3}H]inositol labelling consisted of the fact that direct isolation of the isolated lipid moiety was not feasible, so that only more complex fragments containing the lipid and the [^{3}H]inositol, i.e. the PI or IPC moieties could be analysed. Nevertheless, this approach clearly identified two novel types of lipid remodelling in yeast GPI anchors.

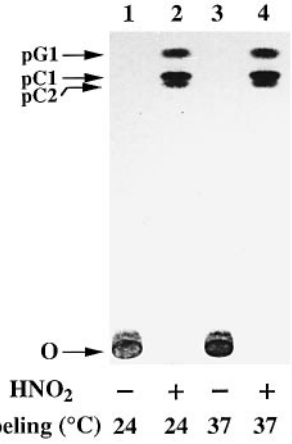

Fig. 1. The standard protocol produces GPI anchor peptides free of any lipid contamination. 20 OD of X2180 cells (harvested at OD_{600} of 3–4) were preincubated at 24°C or 37°C for 10 min and pulse-labelled with [^{3}H]inositol using 10 μCi/OD for 30 min. After a 4-fold dilution with SDC medium the incubation was continued for another 60 min. GPI peptide purification yielded 200×10^3 and 265×10^3 c.p.m. at 24 and 37°C, respectively. Aliquots of 30×10^3 c.p.m. were treated with HNO_2 or control incubated, desalted and analysed by TLC. The TLC was developed in solvent system 1 and the fluorogram was exposed for 4 weeks. pG1, protein-derived Glycerophospholipid 1; pC1 and pC2, protein-derived Ceramide-containing anchor lipids 1 and 2; O, origin.

Results

Early GPI intermediates contain a more polar PI moiety than mature proteins

Previously established methods (Conzelmann *et al.*, 1992) were optimized to ascertain complete delipidation of GPI-anchored proteins without major losses of proteins and quantitative cleavage of GPIs or GPI-containing peptides with nitrous acid (see Materials and methods). The standard procedure used in all further preparations yielded pronase-treated inositol-labelled anchor peptides which were free of any contaminating lipids (Figure 1, lanes 1 and 3) since all labelled material remained close to the origin of the TLC. By contrast, all known GPI intermediates except for the complete GPI precursors CP1 and CP2 move off the origin in the solvent system used here (not shown). The protocol used routinely for nitrous acid treatment quantitatively liberated the [^{3}H]inositol-labelled lipid moieties of these anchor peptides (Figure 1, lanes 2 and 4). After a long pulse, three lipid species were detectable in this TLC system whereby only the fastest-migrating species (pG1) was base-sensitive, the other lipid moieties (pC1, pC2) being ceramides (see below). This type of analysis also revealed that, within the limits of the resolution of our TLC analysis, cells use the same types of lipids for GPI anchoring, whether labelled at 24°C or 37°C (Figure 1). To compare the PI moieties obtained from anchor peptides with those present in early GPI intermediates we had to label mutant cells, since no GPI intermediates accumulate to detectable levels in wild-type cells (Puoti *et al.*, 1991; Sipos *et al.*, 1994). *sec53* cells have a ts mutation affecting GDP-Man biosynthesis and, at 37°C, accumulate a very hydrophobic lipid which also is seen in other mutants such as *dpm1*, *sec59*, *gpi4*, *gpi5*, *gpi6* and *gpi9* (Benghezal *et al.*, 1995). Its partial characterization strongly suggested that it consists of GlcN–acylPI (Orlean, 1990; Conzelmann *et al.*, 1992). To isolate the PI moiety from this GlcN–acylPI precursor we had to remove the GlcN and the FA which is linked to the inositol ring. [All GPI intermediates of *S.cerevisiae* contain an acyl chain on the inositol, although this acyl has not been found on protein-bound GPI anchors (Fankhauser *et al.*, 1993)]. As shown in Figure 2A, the removal of the GlcN by nitrous acid reduces rather than increases the mobility of GlcN–acylPI (Figure 2A, lane 4 versus lane 2). This apparent increase in hydrophilicity may be observed because the free amine of GlcN neutralizes the negative charge of the phosphodiester. This hypothesis is supported also by the finding that N-acetylation of the GlcN–acylPI significantly decreases rather than increases its mobility (not shown). The removal of GlcN by nitrous acid is quantitative, since the residual acylPI moiety is resistant towards GPI-specific phospholipase D (GPI–PLD), an enzyme which only acts on GlcN-containing intermediates (Davitz *et al.*, 1989) (Figure 2A, lanes 3 and 4). In contrast, the purified GPI-intermediate (Figure 2A, lane 2) was quantitatively hydrolysed by GPI–PLD (Figure 2A, lane 1). Additional proof for the proposed GlcN–acylPI structure of the *sec53* intermediate was obtained by demonstrating that the [^{3}H]inositol-labelled lipid after purification still is resistant to PI–PLC (not shown), that it reduces its mobility upon N-acetylation, and that its GPI–PLD product (Figure 2A, lane 1) increases its mobility (i.e. its hydrophobicity) in TLC upon N-acetylation or nitrous acid treatment (not shown). Partial deacylation by methanolic ammonia has been reported to preferentially cleave the acyl chain on the inositol (Roberts *et al.*, 1988a) and generated fragments co-migrating with PI, lyso-PI and inositolphosphoglycerol (Figure 2A, lane 5). These assignments were confirmed by treating these fragments with PI–PLC, an enzyme which is expected to hydrolyse both PI and lyso-PI, but is unable to cleave inositol-acylated forms of this intermediate (Figure 2B,

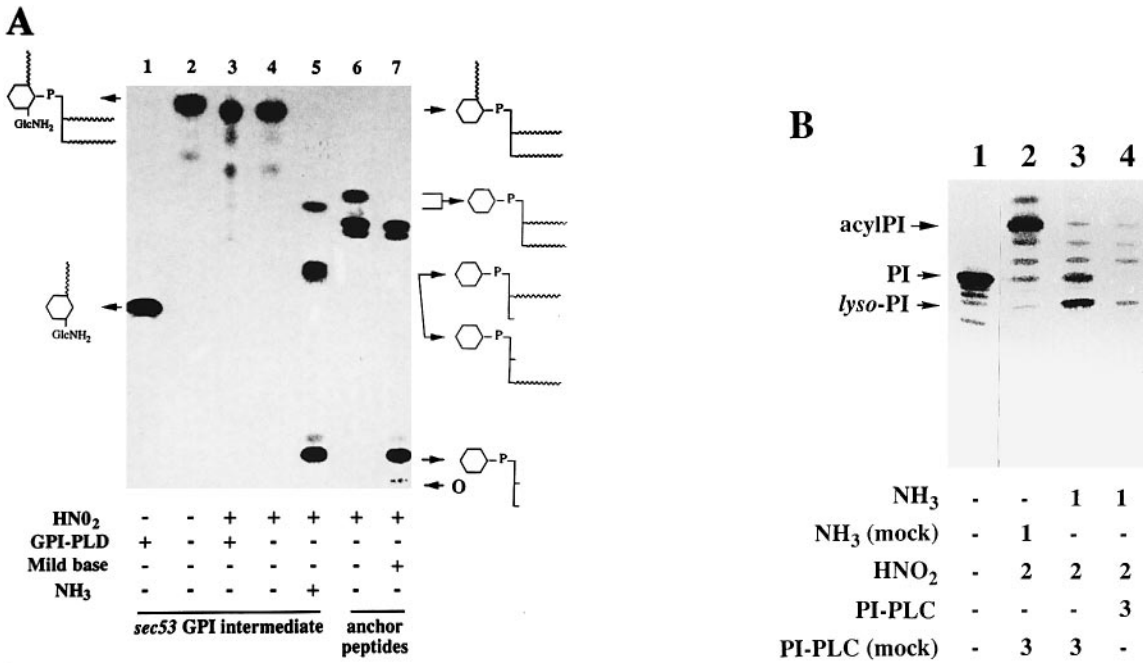

Fig. 2. (**A**) GPI anchors contain a more hydrophobic PI moiety than the GlcN–acylPI intermediate of *sec53*. Lanes 1–5: 20 OD of *sec53* cells were labelled at 37°C under identical conditions as described in Figure 1. In order to purify the GlcN–acylPI intermediate the total lipid extract was treated with 0.02 U of PI–PLC for 5 h. Remaining lipids were extracted by butanol/water partitioning and the at this stage major and most hydrophobic lipid corresponding to GlcN–acylPI was purified to radiochemical purity by two rounds of preparative TLC. Aliquots (20×10^3 c.p.m.) of purified GlcN–acylPI were treated with HNO_2 (lanes 3–5) or control incubated (lanes 1 and 2). The release of the GlcN was verified by GPI–PLD treatment (lanes 1 and 3). Treatment of material shown in lane 4 with methanolic NH_3 yielded the partially deacylated products shown in lane 5. Lanes 6 and 7: 30×10^3 c.p.m. aliquots of GPI peptides from X2180 labelled at 37°C from the experiment described in Figure 1 were first treated with HNO_2 and then with or without mild base. The TLC was developed in solvent system 1 and was exposed for 4 weeks. The presumed structures corresponding to the various bands are depicted on the margins. (**B**) The [^{3}H]inositol-labelled GlcN–acylPI of *gpi9* cells was purified to radiochemical homogeneity as before. Aliquots were then subjected to various simple or sequential treatments with HNO_2, PI–PLC, methanolic NH_3 for partial deacylation as indicated. If several treatments were done, that denoted by '1' preceded that denoted by '2', etc. 'Mock' designates control incubations done without NH_3 or without PI–PLC. Finally, all samples were desalted by water/butanol partitioning and analysed by TLC using solvent system 2. Storage of TLC-purified GlcN–acylPI or acylPI resulted in the generation of several minor bands, as will be discussed below. Lane 1 contains the total lipid extract of [^{3}H]inositol-labelled wild-type cells.

lanes 2–4). As expected, the species migrating with PI and lyso-PI were reduced to inositol-phosphate by PI–PLC (not shown). When the partially deacylated lipid co-migrating with PI in lane 3 was once more treated with methanolic ammonia, it was transformed into the lower band of lane 3 which co-migrates with lyso-PI (not shown). These data clearly demonstrate that the partial deacylation products of lane 5 co-migrating with PI and lyso-PI carry no acyl chains on the inositol.

Importantly, the PI moiety generated from GlcN–acylPI of *sec53* had a lower R_f, i.e. was apparently less hydrophobic than the PI moiety obtained from the GPI anchors of mature proteins (Figure 2A, lanes 5 and 6). Mild base treatment of anchor peptides generated inositolphosphoglycerol from the PI moiety, whereas anchor-derived IPCs (pC1 and pC2) were not hydrolysed (Figure 2A, lane 7). The difference between the major and a minor, slightly faster migrating, deacylation product could not be identified.

To exclude strain differences in the utilization of fatty acids for GPI biosynthesis, we compared the mobilities of the GlcN–acylPI intermediate of various mutants. As shown in Figure 3A, although coming from different genetic backgrounds, all mutants analysed produced a GlcN–acylPI of identical mobility. The products of methanolic ammonia also migrated similarly (Figure 3B) and were all PI–PLC-sensitive (Figure 3C).

Fatty acids in sn-2 of the GlcN–acylPI intermediate and the mature GPI anchor are different

To obtain more information on their structure, the PI moieties from the early *sec53* intermediate and mature anchors were treated with phospholipase A_2 (PLA_2). Working with the deamination product of the early *sec53* intermediate was difficult because, during storage of the purified lipid at –20°C or at 4°C, this early intermediate became transformed into apparently more hydrophobic and less hydrophobic species. When the major species was repurified, the other species reappeared upon storage (not shown). This leads us to believe that the various forms of acylPI may be generated by transesterification, resulting in the spontaneous migration of the acyl group on the inositol ring. Transesterification of acetyl groups on sialic acids and FA migration on lyso-glycerophospholipids at neutral or mildly basic pH are commonly observed (Plückthun and Dennis, 1982; Kamerling *et al.*, 1987; Butor *et al.*, 1993) and the appearance of multiple forms of the GlcN–acylPI has been observed previously by others (Costello and Orlean, 1992). However, the more and less hydrophobic species may also arise through oxidative degradation of unsaturated FAs. Although the acylPI moiety of the early *sec53* GPI intermediate thus migrated as one major and three minor species (Figure 4, lane 2), all these bands yielded mainly PI, lyso-PI and glycerophospho-inositol upon mild deacylation with meth-

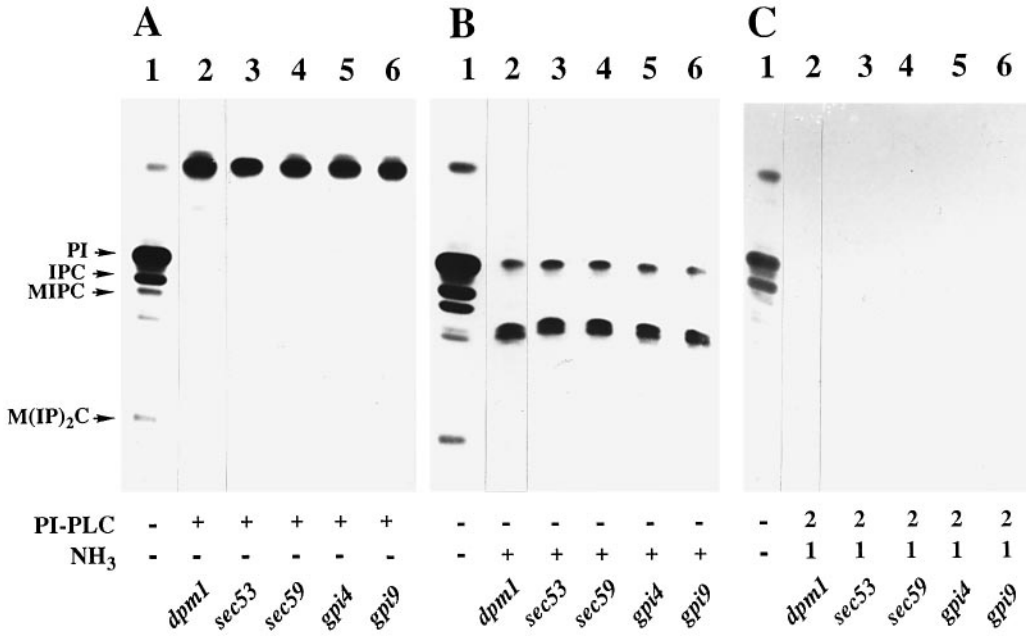

Fig. 3. The GlcN–acylPI from mutants generated in different genetic backgrounds has the same mobility in TLC. *dpm1*, *sec53*, *sec59*, *gpi4* and *gpi9* mutants were preincubated and labelled for 2 h at 37°C with [^{3}H]inositol, lipid extracts were run on TLC and the hydrophobic GlcN–acylPI intermediate was purified by two rounds of preparative TLC. Purified lipids were treated with PI–PLC (**A**), subjected to partial deacylation with methanolic NH_3 (**B**), or treated with both, methanolic NH_3, then PI–PLC (**C**). Before TLC, the PI–PLC-treated samples were desalted by butanol/water partitioning. The only product of PI–PLC treatment, phosphoinositol–GlcN, was detected by the TLC analysis of the aqueous phase of this partitioning (not shown). Lanes 1 contain total lipid extract from [^{3}H]inositol-labelled *gpi9* cells. *dpm1*, *sec* and *gpi* mutants were generated in different genetic backgrounds.

anolic NH_3 (Figure 4, lane 4). The identity of PI was further confirmed by PLA_2 treatment whereby PI was degraded to lyso-PI, whereas all forms of acylPI were resistant to PLA_2 (lanes 1 and 5). PLA_2 treatment of the anchor lipid moieties obtained from mature anchor peptides yielded a lyso-PI which migrated to the same position in our TLC system (Figure 4, lane 6). This indicates that the FA in the *sn*-1 position of mature diacylglycerol-containing anchors is the same as in the early precursor of *sec53*. This leaves us with the expectation that it is the length of the FA in *sn*-2 which is responsible for the higher mobility of the anchor-derived PI as compared with the PI from the early *sec53* intermediate (Figure 2A, lane 6 versus lane 5; Figure 4, lane 7 versus lane 4). Indeed, by treating anchor lipid moieties with methanolic NH_3, a treatment which hydrolyses the acyls in *sn*-1 and *sn*-2 at comparable rates, we generated two forms of lyso-PI, one co-migrating with the PLA_2 product of the early *sec53* intermediate and a second one which has a higher R_f (Figure 4, lanes 8 and 9). This second, more hydrophobic lyso-PI is completely resistant to PLA_2 (Figure 4, lane 10). It is noteworthy, that this second more hydrophobic lyso-PI is not generated from the early *sec53* intermediate (Figure 4, lanes 4 and 5). A band with somewhat higher mobility (Figure 4, lanes 3–5, lozenge) must be a different fragment since it is susceptible to PLA_2. These results indicate that the early GPI precursor of *sec53* and mature mild base-sensitive anchors contain diacylglycerol moieties which differ by the length of the FA in *sn*-2. This finding implies the existence of some form of remodelling step in between these two biosynthetic stages.

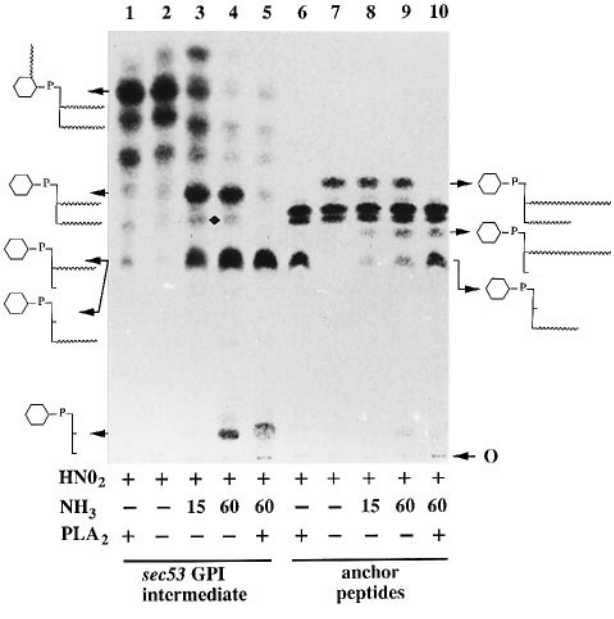

Fig. 4. Anchor peptides contain a long-chain FA in *sn*-2. Acyl-PI and PI moieties generated by HNO_2 treatment in the experiments shown in Figures 1 and 2 were further analysed: Aliquots (20×10^3 c.p.m.) of purified GlcN–acylPI from *sec53* (lanes 1–5) and aliquots (30×10^3 c.p.m.) of X2180 GPI anchor peptides (lanes 6–10) were from cells labelled at 37°C. After extraction of HNO_2 treated lipids by butanol/water partitioning the samples were stored for a few days at –20°C, and then treated with methanolic NH_3 (lanes 3–5 and 8–10) or control treated (lanes 1, 2, 6 and 7) at 30°C for 15 or 60 min as indicated. Finally, some samples were subjected to digestion with PLA_2 (+) or mock-incubated (–). The TLC was developed in solvent system 1 and exposed for 4 weeks. The presumed structures corresponding to the various bands are depicted on the margins.

Lipid extracts of wild-type cells contain large amounts of PI–PLC-resistant GPI structures

The mannosylation defect of *sec53* is known to have pleiotropic effects on protein glycosylation, protein translocation into the ER and vesicular traffic from the ER to the Golgi (Ferro-Novick *et al.*, 1984a,b; Feldman *et al.*, 1987; Kepes and Schekman, 1988). Therefore, we were concerned that the early precursors accumulating in *sec53*, *sec59*, *dpm1*, *gpi4* and *gpi9*, all mutants with defects in mannosylation reactions, may contain different FAs than the true intermediate of wild-type cells. Although until now we had been unable to detect any GPI intermediate in the extracts of [^{3}H]inositol-labelled wild-type cells, we decided to investigate if any GPI intermediate may go undetected because it is hidden by co-migrating PI, IPCs or mannosylated forms of IPC. In developing a suitable strategy we assumed that potential GPI intermediates of wild-type cells, like the previously described abnormal GPIs accumulating in GPI biosynthesis mutants, would resist PI–PLC treatment (Orlean, 1990; Conzelmann *et al.*, 1992; Benghezal *et al.*, 1995). Thus, starting with [^{3}H]inositol-labelled lipid extracts, PI–PLC was used to remove the multiple species of PI which represent the most abundant inositol-labelled lipids. The subsequent nitrous acid treatment was expected to liberate the acylPI moiety from all potential GPI intermediates. Indeed, as shown in Figure 5A, PI–PLC treatment removed the bulk of PI so that only 5–11% of c.p.m. of the initial lipids were left after PI–PLC treatment (Figure 5, lanes 2 and 5). The subsequent nitrous acid treatment led to the appearance of at least six very hydrophobic bands migrating to the high R_f region expected for acylPIs (Figure 5A, lanes 3 and 6). These bands did not appear if the PI–PLC-treated lipid extract was treated with GPI–PLD before nitrous acid (not shown). Importantly, these bands accounted for a significant fraction of counts not only in *gpi8* (Figure 5, lane 6), a mutant which accumulates late GPI intermediates because of its deficiency in the addition of GPIs to proteins (Benghezal *et al.*, 1995), but also in W303 and X2180 wild-type cells (Figure 5, lanes 3, 7 and 9). The material shown in lanes 3 and 6 was further compared with a similarly treated extract from *sec53* (Figure 5B, lane 10) in a solvent system which yielded fewer but more widely separated bands, which could easily be purified for further analysis (see below). Whereas all strains contained bands A, B′/C′ and B″/C″, *sec53* contained an additional two species labelled D′ and D″ (Figure 5B). It should be noted that nitrous acid attacks the free amine of GlcN, a sugar component which is highly characteristic of GPIs. Bands A–D were therefore considered to be derived from hitherto unrecognized, inositol-acylated GPIs since only this kind of inositol-containing lipids can be expected to be PI–PLC resistant and to significantly increase its mobility on TLC upon nitrous acid deamination. Until now, we have not analysed the glycan moieties of these putative GPI intermediates of wild-type cells. Since lipids B′/C′ and B″/C″ represent potential GPI anchor precursor lipids of wild-type cells, we decided to purify all these novel GPI-derived acylPIs and to compare their deacylation products with the ones from mature anchors.

Fatty acids in sn-2 differentiate GPI precursors and mature GPI anchors in wild-type cells

The putative acylPIs were purified by preparative TLC whereby the upper and lower half of the two major species

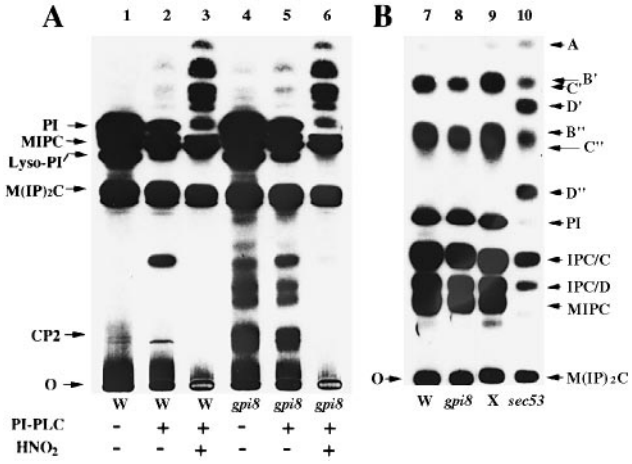

Fig. 5. Previously undetected GPI intermediates are unravelled by nitrous acid treatment of total lipid extracts. 40 OD of cells (W303; *gpi8*; X2180; *sec53*) were preincubated for 30 min at 37°C and labelled with [^{3}H]inositol for 15 min (5 μCi/OD), were diluted 4-fold with SDC medium and further incubated for 60 min. Lipids were extracted and first treated with 0.02 U of PI–PLC for 16 h. Samples were then dried and treated with HNO_2 for 12 h. (**A**) At each stage, 1/20 of the samples were desalted for TLC. Lanes 1 and 4 represent nontreated aliquots of the lipid extracts. Lanes 2 and 5 are representative of PI-PLC resistant lipids. Lanes 3 and 6: lipids after HNO_2 treatment. Lanes 1–3 correspond to samples isolated from W303 cells; lanes 4–6 represent samples from the *gpi8-1* mutant. The TLC was developed in solvent system 2 and exposed for 4 days. (**B**) After HNO_2 treatment, the lipids were desalted by butanol/water partitioning, butanol phases were dried and the HNO_2 treatment was repeated once more for 6 h. Lipids were extracted again with butanol and 1/20 of the counts were analysed by TLC developed in solvent system 3. The plate was exposed for 4 days. IPC/C and IPC/D are subclasses of IPC presumably made in the ER and Golgi respectively (Puoti *et al.*, 1991). MIPC, mannosyl-IPC; $M(IP)_2C$, inositolphospho-mannosyl-IPC; CP1 and CP2, complete precursors 1 and 2. When compared with counts in the initial lipid extract the lipids migrating faster than PI after HNO_2 treatment amounted to: W303 and *gpi8* = 1%: X2180 = 2.4%; *sec53* = 3.5%. A major band midway between $M(IP)_2C$ and CP2 appearing in lanes 2 and 5 corresponds to inositol-phosphate remaining in the butanol phase after extraction with water.

of wild-type cells were scraped separately so as to yield B′, C′, B″ and C″ as separate fractions. Upon rechromatography of the purified lipids we observed that B′ rechromatographed as B′ plus a significant amount of B″ and a trace of A; and that B″ rechromatographed as B″ plus a significant amount of B′ and a trace of A. Thus, we had to accept the fact that these lipids were not stable, but able to interconvert. The same interconversion was observed for the pair C′/C″ and the pair D′/D″. This phenomenon is analogous to the instability of the deamination products of the early GPI intermediate of *sec53* (Figure 4, lane 2). Interconverting lipids were pooled and pools were further analysed after deacylation with methanolic NH_3 and PLA_2 treatment (Figure 6A). The former treatment produced relatively homogeneous populations of PI and lyso-PI from each pool (Figure 6A, lanes 5 and 7) which co-migrated with similarly treated, regular PI from the same cells (Figure 6B). The fact, that a homogeneous PI and a homogeneous lyso-PI pool was obtained from each pool of interconverting lipids supports the idea that interconversion among members of these pools arises through transesterification of the FA on the inositol which is changing its site of attachment. The PI populations derived from B′/B″ and C′/C″ have slightly

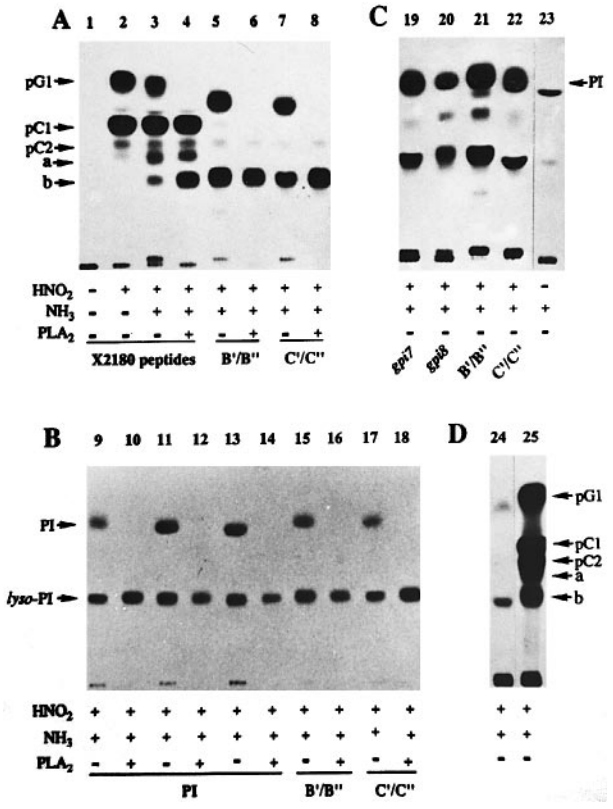

Fig. 6. GPI anchors contain a more hydrophobic PI moiety than intermediates of GPI biosynthesis. (**A**) GPI peptides of X2180 cells from the experiment described in Figure 5 were purified and treated with HNO_2 (lanes 2–4) or control incubated (lane 1). The major acylPIs generated by HNO_2 from GPI intermediates in the same experiment (Figure 5) were purified twice by preparative TLC using solvent system 3 and pooled as indicated at the bottom. Lipids from GPI intermediates (lanes 5–8) and anchor peptides (lanes 3 and 4) were then treated with methanolic NH_3 for 2 h at 30°C. Subsequently, samples were digested with PLA_2 or mock-treated. Different forms of lyso-PI are denoted by a, b. (**B**) The partially deacylated GPI intermediates shown in (A), lanes 5–8 were compared with three different PI pools obtained from the same [^{3}H]inositol-labelled X2180 cells. For this, the lipid extract was run in TLC and the region of PI was arbitrarily divided into three zones of higher, intermediate or lower R_f, the zones were scraped and the three PI pools were purified by two additional rounds of preparative TLC. The pools were further treated as indicated with HNO_2, methanolic NH_3 and PLA_2 in order to make samples comparable. (**C**) CP2 from *gpi8* and the four mannoses containing intermediate (M4 lipid) accumulating in *gpi7* (Benghezal *et al.*, 1995) were purified by PI–PLC treatment of the lipid extract of [^{3}H]inositol-labelled cells, reisolation of lipids over octyl–Sepharose followed by two consecutive, preparative TLC runs. Purified lipids were treated with HNO_2 and methanolic NH_3 and run together with similarly treated partially deacylated GPI intermediates from the corresponding wild-type cells. Lane 23 contains partially deacylated trypanosomal PI containing myristic acid as the only FA. (**D**) Purified CP2 from [^{3}H]inositol-labelled *gpi8* was treated with HNO_2 and methanolic NH_3 and compared with lipid moieties of GPI anchor peptides from X2180. Samples in all panels were analysed by TLC using solvent system 3.

different R_fs (Figure 6A, lanes 5 and 7), indicating that the difference between these two GPI precursor lipids resides in the diacylglycerol moieties, although additional differences in the inositol-bound fatty acyl chain cannot be ruled out. Since the partial deacylation with methanolic NH_3 is not specific for *sn*-1- or *sn*-2-acyls, the relative homogeneity of the lyso-PI population generated from each pool suggests that any given GPI precursor has two FAs of similar length in *sn*-1 and *sn*-2. To compare the PI moieties derived from pools B′/B″ and C′/C″ of wild-type cells with the PI moieties derived from the GPI protein anchors of the same cells, the lipids were hydrolysed by PLA_2 treatment and methanolic NH_3. This analysis led to the same conclusion as the comparison with the GlcN–acylPI intermediate of *sec53*: (i) protein anchors contain a more hydrophobic PI moiety (pG1) than GPI intermediates (Figure 6A, lanes 2, 5 and 7); (ii) partial deacylation with methanolic NH_3 produces two distinct lyso-glycerophospholipids from anchor PIs (Figure 6A, lane 3, lipids a and b) whereas a single homogeneous population is obtained from GPI intermediates (Figure 6A, lanes 5 and 7, lipid b); and (iii) the more polar lyso-PI from anchor peptides (lipid b) co-migrates with the lyso-PI from GPI intermediates. This indicates that GPI intermediates and diacylglycerol-containing GPI anchors of wild-type cells differ with respect to the length of the FA in *sn*-2 whereas, by the resolution power of our TLC system, the FA in *sn*-1 does not appear to be different.

We also prepared [^{3}H]inositol-labelled late GPI intermediates from *gpi7* and *gpi8* which accumulate both the close-to-complete and complete GPI precursor ready for attachment to cells (Benghezal *et al.*, 1995). These late GPI intermediates also have a PI moiety which migrates similarly as the PI from the earlier intermediates (Figure 6C, lanes 19–22). The PI moiety of CP2 from *gpi8* is less hydrophobic than pG1, and its only lyso-form co-migrates with the lyso-PI of GPI intermediates (Figure 6D). This suggests that GPI intermediates up to the completed GPI do not have a long-chain FA at position *sn*-2.

The remodelling of GPI anchors in sn-2 does not require Slc1p

IPCs contain the largest lipid moiety among the phospholipids of *S.cerevisiae* as their ceramide comprises a long-chain base and a C26 fatty acid (Smith and Lester, 1974). Strains unable to make long-chain bases like *lcb1::URA3* require some kind of long-chain base (dihydrosphingosine or phytosphingosine) in their growth medium. Yet, a mutation in *SLC1* (*slc1-1*) suppresses this auxotrophy (Dickson *et al.*, 1990). Slc1p can function as a 1-acyl-*sn*-glycerol-3-phosphate acyltransferase in *Escherichia coli* but Slc1p is not essential for growth of *S.cerevisiae* (Nagiec *et al.*, 1993). The *lcb::URA3 slc1-1* strain makes anomalous mannosylated forms of PI with a C26 in the *sn*-2 position which mimic the lacking mannosylated forms of IPC (Lester *et al.*, 1993). Therefore, the *slc1-1* suppressor allele is thought to encode a variant enzyme with an altered substrate specificity that enables it to use C26 instead of C18 or C16 FAs when acylating the *sn*-2 position of inositol-containing glycerolipids (Nagiec *et al.*, 1993). We decided to test the hypothesis that *SLC1* is a component of an enzyme complex involved in the remodelling of GPI anchors at *sn*-2. For this, the PI moieties from the GPI anchor peptides of the *SLC1* deletion strain were compared with the same material from the corresponding wild-type. As shown in Figure 7, the deletion strain still made normal amounts of diacylglycerol-type GPI anchors, the R_fs of which were identical to those of wild-type cells. Thus, it appears that *SLC1* is either not involved in the *sn*-2-remodelling of diacylgly-

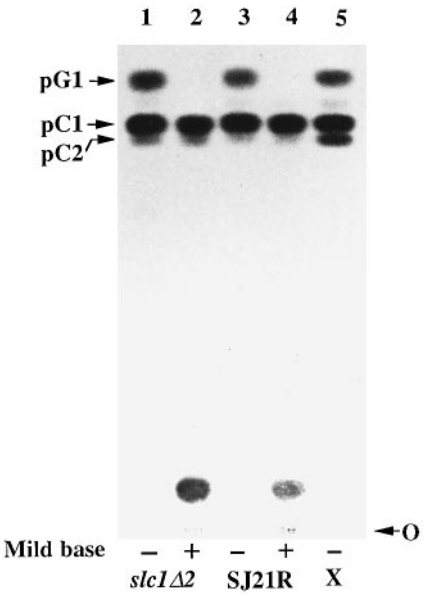

Fig. 7. Analysis of PI moieties of GPI anchors in a *SLC1* deletion strain. YNN5 (*slc1Δ2*), SJ21R (corresponding wild-type) and X2180 (X) cells were labelled with [^{3}H]inositol (15 μCi/OD) for 30 min, cells were diluted 4-fold and incubation was continued for a further 60 min. GPI peptides were purified and subjected to HNO_2 treatment. After butanol extraction, counts were split into two equal aliquots and mild base-treated or control-incubated. Samples (25×10^3 c.p.m./lane) were analysed by TLC developed in solvent system 1.

cerol-containing GPI anchors or that a redundant gene can compensate for its deletion.

Long-chain FAs are introduced at sn-2 rapidly after addition of GPIs to proteins

To study the kinetics of the appearance of long-chain FAs in *sn*-2 of GPI anchors, a pulse–chase experiment was performed. As shown in Figure 8A, after 5 min of pulse, two different PI moieties, pG1 and pG2, were detected. pG1 represents the mature anchor lipid with a long-chain FA in *sn*-2, whereas the less polar pG2 migrates close to the PI from pool C′/C″ (see below) and co-migrates with the free PI of these cells (not shown). pG2 was easily detectable after short pulses and when labelling with a high ratio of [^{3}H]inositol/OD. For chase, only cycloheximide is effective in blocking the further synthesis of labelled anchors, as the radiolabelled PI remains available for GPI synthesis for some time even if further incorporation of [^{3}H]inositol into PI is blocked by the addition of an excess of non-radioactive inositol. In the presence of cycloheximide, pG1 and pG2 were disappearing whereas anchor-derived IPC moieties pC1 and pC2 increased (Figure 8). Also, a lysoform of PI was discovered which disappeared with similar kinetics as pG1 and pG2. The quantitative evaluation of this experiment is shown in Figure 8B. It can be seen that the total counts are still strongly increasing during the first 5–10 min of chase in the presence of cycloheximide. This can be rationalized by the fact that part of proteins is translocated only post-translationally (Schekman, 1994) and that GPI anchor addition may not be instantaneous after translocation is accomplished. Moreover, for the preparation of anchor peptides, efficient delipidation of GPI proteins required affinity chromatography on concanavalin A–Sepharose.

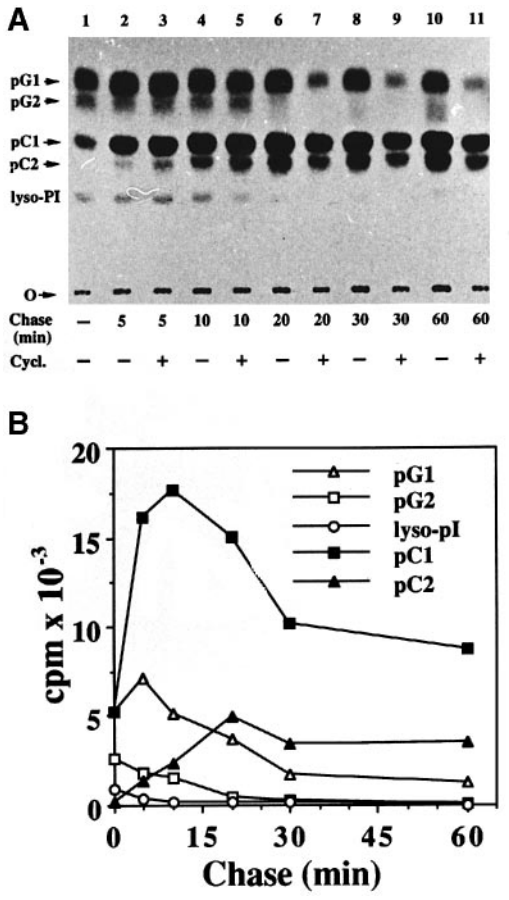

Fig. 8. Changes in GPI anchor lipids of X2180 during chase. (**A**) X2180 cells were pulse- labelled with [^{3}H]inositol (15 μCi/OD) at 30°C for 5 min and the label was chased by the 4-fold dilution with SDC medium containing cold inositol and cycloheximide (+) or inositol alone (–). Inositol and cycloheximide were added to final concentrations of 100 μM and 100 μg/ml, respectively. Labellings were stopped by the addition of NaF and NaN_3 and chilling on ice. Cells were washed with ice-cold water and anchor peptides were prepared. The anchor lipid was then released using HNO_2, desalted and run on TLC in solvent system 3. (**B**) Radioscanning of the TLC plate allowed for the quantitation of the results of (A). Only the data for samples chased in the presence of cycloheximide are shown.

Thus, the rise in total counts during the first minute of chase may also be due to a delay in the transfer of *N*-glycans onto newly made proteins. From the quantitation it appears that different anchor lipids peak at different times: pG1 is increasing during the first 5 min of chase, while lyso-PI and pG2 are already decreasing. Similarly, pC1 peaks at 5–10 min of chase while pG2 and pG1 are decreasing. Finally, pC2 is peaking at 15 min when all other lipid moieties are decreasing. With regard to the introduction of long-chain FAs into *sn*-2, the kinetic data are compatible with the idea that lyso-PI and pG2 may be precursors of pG1, although the kinetics by themselves cannot prove any such relationship. PLA_2 treatment of a mixture of anchor lipids containing pG1 and pG2 and lyso-PI (Figure 9B, lane 6) results in a single lyso-PI which co-migrates with the lyso-PI detectable among anchor lipids after short pulses (Figure 9B, lane 7). This indicates that pG1, pG2 and lyso-PI from anchor peptides have a similar FA in *sn*-1, but differ at *sn*-2 (Figure 9B). These data are compatible with the idea that pG2-type anchors are remodelled to pG1-type anchors through acyl

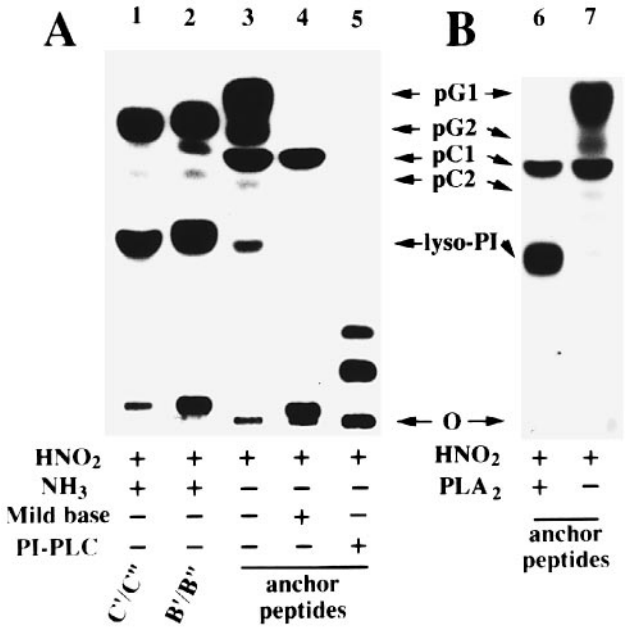

Fig. 9. Comparison of pG2 with PI moiety of GPI intermediates. (**A**) 30 OD of X2180 cells were labelled with [^{3}H]inositol (15 μCi/OD) for 15 min at 24°C. GPI anchor peptides were purified and their lipid parts were released by HNO_2 treatments. 80×10^3 c.p.m. was split into three aliquots and treated with 0.05 U of PI–PLC for 2 h (lane 5) or treated with mild base (lane 4) or left untreated (lane 3). Lanes 1 and 2 contain PI and lyso-PI obtained from pool C′/C″ (lane 1) and pool B′/B″ (lane 2) by HNO_2 and methanolic NH_3 (same material as in lanes 7 and 5 of Figure 6A). TLC was developed in solvent system 3. (**B**) Material prepared similarly to that in lane 3 of (A) was digested with PLA_2 (lane 6) or was mock-incubated (lane 7) and products were run on TLC, solvent system 3.

exchange in *sn*-2 and that the lyso-PI observed after short pulses is an intermediate in this reaction. Side-by-side comparison of pG2 with PI moieties from pools B′/B″ and C′/C″ (Figure 9A) showed that pG2 did not exactly co-migrate with either one, but rather migrated slightly less than pool C′/C″. Similarly, the lyso-PI appearing during short pulses on GPI anchors (Figure 9A, lane 3) was found to co-migrate with the lower half of the lyso-PI forms derived from the partial deacylation of pools B′/B″ and C′/C″ (Figure 9A, lanes 1 and 2). This however might derive from the fact that the latter contain a mixture of two lyso-PI forms having the monoacyl substituent in either *sn*-1 or *sn*-2, whereas the natural lyso-PI appearing in short pulses might contain only one of such substituents. It should also be borne in mind that the partial deacylation may release the FAs from numerous unlabelled phospholipids and that the presence of unlabelled lipids may slightly influence the mobility of the labelled lipids. Thus, we feel that we reach the limits of resolution of our TLC system and cannot at present decide whether pG2 still carries the same lipid moieties as the GPI intermediates, or if pG2 is already the product of a remodelling reaction.

Subcellular localization of lipid remodelling events can be studied using secretion mutants

To determine the subcellular localization of the introduction of long-chain FAs in the *sn*-2 position of diacylglycerol-containing GPI anchors and of other remodelling events, we labelled secretion mutants in which the secretion is conditionally blocked at various stages of the secretory pathway (Novick *et al.*, 1980; Esmon *et al.*, 1981). Figure 10A indicates that, in wild-type cells, the majority of pG1- and pG2-type anchors disappear during a chase of 30 min (lanes 2 and 3). Also, pC2 is appearing only during chase and this is in agreement with data in Figure 8. In *sec12* and *sec18*—two mutants in which secretory vesicles either cannot bud off the ER or cannot fuse with the subsequent *cis* Golgi compartment respectively—pG1 seems to be stable during chase. Thus, the data suggest that pG1- and pG2- and pC1-type anchors can be made in the ER, whereas the pC2-type anchors are made in the Golgi. In an analogous experiment with a longer pulse and a higher amount of radioactivity, data could be quantitated by radioscanning of TLC plates, as shown in Figure 10B. This experiment confirmed the findings of the previous experiment and showed that pG1 is stable also in *sec7* and remains partially stabilized in *sec14* cells which are blocked after the *cis* or the mid Golgi, respectively (Novick *et al.*, 1980; Franzusoff and Schekman, 1989). pC2-type anchors seem to be made efficiently in *sec14* but not *sec7*, thus suggesting that pC2-type anchors may be generated only in the mid Golgi. When passing the pronase digest of the delipidated protein extract over octyl–Sepharose, we always observe a run-through fraction of labelled peptides which does not adhere to octyl–Sepharose. Some soluble forms of GPI peptides might be expected based on the postulated cleavage of certain GPI-anchored proteins at the cell surface as they become integrated into the cell wall (Muller and Bandlow, 1993; Lu *et al.*, 1994, 1995; Muller *et al.*, 1996), but here we also find some soluble inositol-labelled material when labelled proteins are retained in the ER. The soluble fraction has not yet been structurally analysed and the data have been included only to allow for a complete account of labelled material. The interpretation of pulse–chase experiments in secretion mutants thus allows us to conclude that pG1 and pG2 anchors are made in the ER and that at least part of them seem not to be further remodelled in this organelle. However, the data do not indicate why pG1- and pG2-type anchors are labile in the mid Golgi and later compartments. This lability may be due to exchange of lipid moieties during a further remodelling step, to rapid turnover of proteins with this type of anchor, or to loss of the inositolphospholipid moiety during integration into the cell wall.

Discussion

The spectrum of FAs in glycerophospholipids of *S.cerevisiae* is relatively narrow and comprises mainly C16:1, C18:1, accompanied by minor amounts of C14:0, C16:0 and C18:0 (Hunter and Rose, 1972; Wagner and Paltauf, 1994). Yet, analysis of FAs shows very significant differences in the relative frequency of different FAs among the major phospholipid classes and also between the *sn*-1 and *sn*-2 positions (Wagner and Paltauf, 1994). On the other hand, very long C26:0 FAs have been found in sphingolipids and in the diacylglycerol-containing GPI anchor of Gas1p (Smith and Lester, 1974; Fankhauser *et al.*, 1993). In this report we used metabolic labelling with [^{3}H]inositol in order to compare the lipid moieties of different forms of GPI anchors and GPI intermediates. Although this method does not allow for a direct analysis

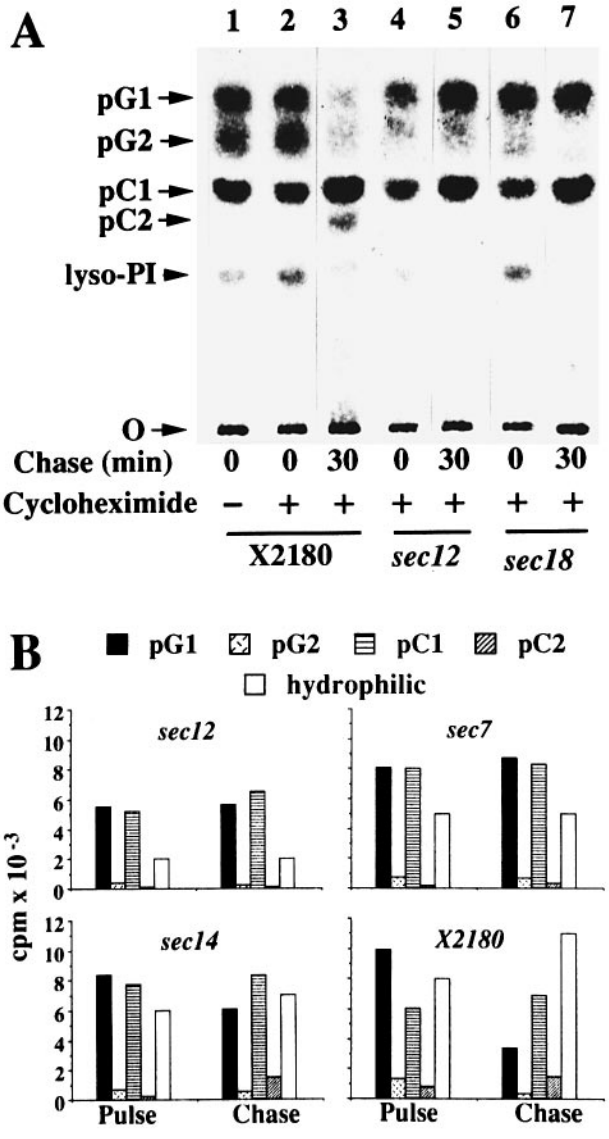

Fig. 10. Nature of GPI anchor lipids on proteins retained by secretion blocks. (**A**) 40 OD of X2180, *sec12* or *sec18* cells were preincubated for 20 min at 37°C and pulse-labelled for 5 min using 15 μCi/OD. Cells were diluted 4-fold with SDC medium and labellings were stopped by the addition of NaF and NaN_3 (lane 1), stopped with NaF and NaN_3 but adding cycloheximide in addition (lanes 2, 4 and 6) or further incubated at 37°C in the presence of cycloheximide, 100 μg/ml (lanes 3, 5 and 7). GPI anchor peptides were prepared and treated with HNO_2. The anchor-derived lipids were desalted and analysed by TLC in solvent system 3. (**B**) The same protocol was used for a similar experiment except that the pulse labelling was for 15 min and the chase for 25 min. The data represent the quantitation by radioscanning of TLC plates. 'Hydrophilic' refers to the fraction of labelled peptides which did not bind to octyl–Sepharose during anchor peptide preparation.

of the lipid component, it was chosen because it was not expected to influence the cell in its choice of lipid components for GPIs. Nevertheless, we have to consider that, in inositol-free medium, inositol production is the rate-limiting step in the formation of PI and that the addition of a bolus of [^{3}H]inositol might transiently increase PI synthesis. Therefore, we cannot be sure that the FA profile of PIs produced during labelling is exactly identical with that of the PI produced during exponential growth. To minimize this problem, we used a standard protocol in which no more than 10 μCi were used to label 1 OD_{600} unit of cells. Our calculation indicates that, in SDC medium, 1 OD_{600} unit of exponentially growing cell generates ~2×10^{-11} mol PI per min. When adding a bolus of 10 μCi of [^{3}H]inositol/OD of cells equivalent to 3.3×10^{-10} mol of inositol, 80% of the counts are incorporated into PI within 10 min. Assuming that endogenous inositol synthesis is not down-regulated by the exogenously added [^{3}H]inositol, this means that during 10 min, the rate of PI synthesis might increase transiently by 130% at most. Thus, we cannot exclude that our labelling procedure introduces a small distortion of the lipid profile of GPIs but that this effect—at least under standard conditions—is probably only minor. We also took care to avoid differences stemming from variations in the genetic background, since all mutant strains were compared with the corresponding wild-type strains. At the level of the resolution of our TLC systems, our analysis failed to unravel any difference in PI moieties of GPI anchors between W303 and X2180 or between cells grown at 24 and 37°C, respectively. Temperature has been reported to have virtually no influence on the type of FAs used for phospholipid biosynthesis by *S.cerevisiae* (Hunter and Rose, 1972).

Our studies revealed the existence of previously unrecognized GPIs in the lipid extract of [^{3}H]inositol-labelled cells. Further analysis showed that these GPIs are heterogeneous and migrate in TLC (solvent system 2) at various positions between PI and $M(IP)_2C$ (unpublished results). It would appear that the amount of this material is not less abundant in wild-type cells than in *gpi8* or *gpi7* (not shown), i.e. in mutants which accumulate some form of GPI intermediate due to a defect in GPI biosynthesis. At present, we do not have an explanation for the appearance of pool D′/D″ in *sec53*. We believe that PIs from pool D′/D″ have very short FAs but this material has not yet been fully characterized. The appearance of pool D′/D″ seems to be specific for mannosylation mutants and not to be a secondary effect of the secretion block of *sec53* since it was also found in *pmi40* but not in *sec12* (not shown).

Our studies establish the existence of two previously unrecognized types of lipid remodelling in yeast. One type is postulated in order to explain the presence of pG1-type PI moieties on GPI anchors, a species which is clearly absent from all free GPIs. This finding raises a number of questions: (i) what kind of primary anchor lipid is acted upon to give rise to pG1-type lipids?; (ii) what is the nature of the FA that is introduced in *sn*-2?; (iii) what part of the GPI does get exchanged during remodelling?; and (iv) what enzymes are mediating this remodelling? In analogy to Gas1p, the mild base-sensitive GPI anchor of which has been characterized, it seems reasonable to assume that pG1-type anchors contain a C26:0 FA in *sn*-2 (Fankhauser *et al.*, 1993). Studies on the rate of incorporation of radiolabelled FAs demonstrated a very rapid non-random incorporation of [^{3}H]palmitic and [^{3}H]oleic acid into either the *sn*-1 or the *sn*-2 position of different classes of glycerophospholipids of *S.cerevisiae* and led to the suggestion that yeast sustains an *sn*-2-specific deacylation/reacylation cycle similar to that of mammalian cells (Wagner and Paltauf, 1994). An analogous reaction might be responsible for the presence of

long-chain FAs in the *sn*-2 position of pG1-type anchors. However, at present we cannot exclude that remodelling is achieved through the replacement of diacylglycerol, phosphatidic acid or an even larger moiety. Exchange of a PI moiety for a different PI moiety however seems unlikely since we could not find any hydrophobic PI of the same TLC mobility as pG1 in the lipid extracts of [^{3}H]inositol-labelled cells (not shown). It must also be stressed that remodelling in *sn*-2 is detected by our experiments because the remodelling exchanges a short FA against a long one. However, our data cannot exclude concomitant remodelling events resulting in more conservative substitutions. Indeed, the exchange of myristate for myristate (myristate exchange) on the mature, GPI-anchored VSG of *Trypanosoma brucei* has recently been described (Buxbaum *et al.*, 1996).

A further type of remodelling was established through the use of secretion mutants. Previous studies had already established that the ceramide moieties of mature GPI anchors of X2180 mainly contain C18:0 phytosphingosine and C26:0 FAs with minor amounts of C20:0 phytosphingosine and monohydroxylated C26:0 (Fankhauser *et al.*, 1993). It is clear from the present and previous studies (Conzelmann *et al.*, 1992) that pC1, the main IPC derived from GPI anchors of mature proteins is different, i.e. is more hydrophobic than the main cellular IPC (IPC/C, IPC-II), the latter consisting of phytosphingosine with a C2-hydroxylated C26:0 (Smith and Lester, 1974; Hechtberger *et al.*, 1994) and is presumably synthesized in the ER (Puoti *et al.*, 1991). Our study also confirms that some remodelling reaction which introduces ceramides can occur in the ER (Conzelmann *et al.*, 1992). This study now demonstrates that only pC1, i.e. the more hydrophobic type of IPC, is introduced in the ER whereas pC2—the anchor-derived IPC co-migrating with IPC/C—only appears on GPI proteins reaching the mid Golgi. This suggests that GPI anchors and IPCs made in the ER ultimately contain different ceramides. The exact nature of the ceramide added onto GPI anchors in the ER is currently under study. Based on the results of this study we have to postulate a further remodelling step to explain the appearance of pC2 in anchors reaching the mid Golgi apparatus. This postulate raises the same type of questions as referred to earlier. In particular, it is not clear what type of GPI anchor lipids become substituted by pC2. Our present working hypothesis is that pC1 contains either C18 phytosphingosine with a C26:0 non-hydroxylated FA or dihydrosphingosine with a hydroxylated C26:0 FA and that the Golgi remodelling step is introducing a further hydroxyl group into the lipid moiety. Studies to establish the exact nature of pC2 are under way.

In several experiments analysing material from cells labelled by only a short pulse, nitrous acid treatment not only released pG1, pG2 and pC1 but also traces of acylPI from anchor peptides. This suggests that inositol-acylated GPIs can be transferred to proteins in yeast. On the other hand, the low amount of this species and the absence of non-acylated CPs in yeast (Sipos *et al.*, 1994) suggest that acyls are removed from inositols either shortly before, during, or immediately after transfer of CPs to proteins. A similar situation seems to prevail in trypanosomes (Güther and Ferguson, 1995).

At present, the role of lipid remodelling on yeast GPI anchors is not clear. One might be led to think that lipid remodelling of anchors is of minor importance, since mutants which can grow in the absence of ceramides have been described (Nagiec *et al.*, 1993). We do not subscribe to this view since first, these mutants grow very poorly in the absence of exogenous long-chain bases (unpublished results). Second, they only can grow when harbouring the *slc1-1* allele which allows them to introduce C26 FAs into *sn*-2 of ordinary PIs (Lester *et al.*, 1993). It may be more than a coincidence that all remodelling pathways introduce long-chain FAs into the lipid moiety of GPI-anchored proteins either by introducing C26-containing ceramides or by introducing the long-chain FA in the *sn*-2 position of glycerophospholipid anchors. At present the data indicate that, with the possible exception of pG2-type anchors, all GPI-anchored proteins are becoming remodelled in a way as to receive a long-chain FA. It may be speculated that the introduction of long-chain FAs is required for the sorting of GPI proteins into specialized membrane domains or transport vesicles. We hope that mutants with defects in the remodelling enzymes will shed more light on the functional aspects of this phenomenon.

Materials and methods

Strains, growth conditions and materials

S.cerevisiae strains were W303-1A (*MAT***a** *ade2-1 can1-100 ura3-1 leu2-3,112 trp1-1 his3-11,15*), X2180-1A (*MAT***a** *SUC2 mal gal2 CUP1*), SJ21R (*MAT***a** *ura3-52 leu2-3,112 ade1 MEL1*), YNN5 (*MAT***a** *ura3-52 leu2-3,112 ade1 MEL1 slc1Δ2::LEU2*), SF 294-2B (*MAT***a** *sec7-1*), SF 226-1C (*MAT***a** *sec12-4*), HMSF169 (*MATa sec14-1*), HMSF176 (*MAT***a** *sec18-1*), HMSF331 (*MAT***a** *sec53-6*), SF402-4D (*MAT***a** *sec59-1*), FBY12 (*MAT***a** *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 gpi4-1*), FBY15 (*MAT*α *ade2-1 leu2-3,112 trp1-1 his3-11,15 gpi7-1*), FBY11 (*MAT*α *ade2-1 leu2-3,112 gpi8-1*), FBY16 (*MAT*α *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 lys2 gpi9-1*), XD6-2B (*MAT*α *dpm1::LEU2 ura3-52 leu2-3,112 trp1-Δ1 lys2-801*) harbouring *DPM1* on plasmid pDM6, and XD6-2B harbouring the thermosensitive *dpm1-6* allele on plasmid pDM8-6 instead of pDM6. Cells were kept on YPD plates and were cultured in SD medium containing salts, vitamins (but no inositol), trace elements and 2% glucose as a carbon source; SDC medium is the same but contains 1% casein hydrolysate in addition. Uracil and adenine, each at 40 μg/ml, were added where required. The OD of dilute cell suspensions was measured in a 1 cm cuvette at 600 nm; '1 OD' refers to cells contained in 1 ml of a cell suspension having an OD_{600} of 1 and is equivalent to ~10^7 cells. *myo*[2-^{3}H]inositol (20 Ci/mmol) was from ARC Inc., St Louis, MO, USA. 1000-fold purified GPI–PLD from bovine serum was a kind gift of Dr Urs Brodbeck, Institut für Biochemie und Molekularbiologie, University of Bern, Switzerland. Phospholipase A_2 isolated from bee venom was from Sigma. 7N NH_3 in methanol (methanolic ammonia) was bought from Janssen Chimica (Geel, Belgium). Concanavalin A–Sepharose and octyl–Sepharose 4B were from Pharmacia (Uppsala, Sweden). PI–PLC from *Bacillus cereus* and Pronase from *Streptomyces griseus* were from Boehringer-Mannheim, Germany. TLC silica gel 60 plates were from Merck ABS, Zurich.

Metabolic labelling with [^{3}H]inositol

Exponentially growing cells were pulse labelled for 5–30 min with [^{3}H]inositol in SDC medium at 10 OD/ml with 5–10 μCi/OD of cells or, to visualize better pG2, at 15 μCi/OD. For chase, cells were diluted 4-fold with SDC.

Preparation of anchor peptides

Radiolabelled cells were broken with glass beads and extensively delipidated with chloroform:methanol:water (10:10:3). The lysate was then resuspended in 200 μl of ethanol:water:diethylether:pyridine:25% NH_3 (15:15:5:1:0.018) by sonication for 15 s and the suspension was left for 15 min at room temperature (Hanson and Lester, 1980). Samples were dried in a Speed-Vac evaporator and glycoproteins isolated over concanavalin A–Sepharose after resolubilizing proteins in sample buffer as described (Conzelmann *et al.*, 1990). Concanavalin A–Sepharose

beads were washed until the effluent contained <500 c.p.m./ml. GPI peptides were eluted by extensive Pronase treatment and subsequent boiling of the concanavalin A beads. Anchor peptides were further purified over octyl–Sepharose as described (Sipos *et al.*, 1994).

Purification of GPI intermediates

Total lipid extracts were digested with 0.02 U of PI–PLC in 20% propanol as described (Puoti and Conzelmann, 1993) for 5 h or overnight when extracts from more than 20 OD were to be treated. After drying the samples, the total lipid extracts were treated by nitrous acid as described (Güther *et al.*, 1994); controls contained 0.5 M NaCl instead of 0.5 M $NaNO_2$. Lipids were then extracted twice with 150 µl of water-saturated butanol and back-extracted by 50 µl of butanol-saturated water. The HNO_2-generated lipids (more hydrophobic than any other [^{3}H]inositol-labelled lipid) were purified twice by preparative TLC using solvent system 3 (see below).

Analytical methods

Specific release of inositol-linked acyl groups was performed using methanolic NH_3 in 200 µl for 2 h at 30°C in an air-heated incubator; controls were incubated only with methanol. Mild base treatment for complete deacylation of glycerophospholipids was done using 8 N NH_3 in 300 µl methanol:water (1:1) for 6 h at 37°C or using NaOH as described (Becker and Lester, 1980). Nitrous acid treatments were done as described (Güther *et al.*, 1994). GPI–PLD and PI–PLC treatments were performed as described (Davitz *et al.*, 1989; Puoti and Conzelmann, 1993). For PLA_2 treatment, lipids were resolubilized in 30 µl buffer (25 mM Tris–HCl, pH 7.5, 2 mM $CaCl_2$, 0.1% sodium deoxycholate) and treated with 2 U of enzyme for 5 h at 37°C. For TLC, lipids were desalted by butanol extraction if significant salt concentrations had to be removed, lipids were dried and resolubilized in chloroform:methanol (1:1) for application. Pre-equilibrated tanks with the following solvent systems were used for ascending TLC: solvent system 1 [chloroform:methanol:0.25% KCl (55:45:10, v/v)]; solvent system 2 [chloroform:methanol:water (10:10:3, v/v)]; solvent system 3 [chloroform:methanol:0.25% KCl (55:45:5, v/v)]. In most cases, radioactivity on TLC plates was monitored and quantitated by one- and two-dimensional radioscanning using a Berthold radioscanner.

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