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Glycosylphosphatidylinositol membrane anchors in *Saccharomyces cerevisiae*: absence of ceramides from complete precursor glycolipids

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Glycosylphosphatidylinositol (GPI) anchoring of membrane proteins occurs through two distinct steps, namely the assembly of a precursor glycolipid and its subsequent transfer onto newly synthesized proteins. To analyze the structure of the yeast precursor glycolipid we made use of the *pmi40* mutant that incorporates very high amounts of [^{3}H]mannose. Two very polar [^{3}H]mannose-labeled glycolipids named CP1 and CP2 qualified as GPI precursor lipids since their carbohydrate head group, Manα1,2(X$\rightarrow$PO$_4\rightarrow$6)Manα1,2Manα1,6Manα-GlcN-inositol (with X most likely being ethanolamine) comprises the core structure which is common to all GPI anchors described so far. CP1 predominates in cells grown at 24°C whereas CP2 is induced by stress conditions. The apparent structural identity of the head groups suggests that CP1 and CP2 contain different lipid moieties. The lipid moieties of both CP1 and CP2 can be removed by mild alkaline hydrolysis although the protein-bound GPI anchors made by the *pmi40* cells under identical labeling conditions contain mild base resistant ceramides. These findings imply that the ceramide moiety found on the majority of yeast GPI anchored proteins is added through a lipid remodeling step that occurs after the addition of the GPI precursor glycolipids to proteins.

Key words: ceramide/glycosylphosphatidylinositol/membrane attachment/remodeling/*Saccharomyces cerevisiae*

Introduction

Numerous glycoproteins of *Saccharomyces cerevisiae* get attached to a GPI anchor (Conzelmann *et al.*, 1988; Vai *et al.*, 1990; Müller and Bandlow, 1991; Nuoffer *et al.*, 1991). The biosynthesis of GPI anchored proteins in yeast follows the same rules as in mammals and trypanosomes. (i) Similar C-terminal protein sequences are required to tag yeast proteins for GPI anchor addition (Englund, 1993; Nuoffer *et al.*, 1993). (ii) The yeast carbohydrate core structure linking the protein to the lipid is identical to that of higher eukaryotes (Figure 1; Fankhauser *et al.*, 1993). (iii) Yeast anchors are added rapidly to newly translated proteins while they still reside in the endoplasmic reticulum (ER) as is the case in other organisms (Conzelmann *et al.*, 1990). Peculiarities of yeast GPI anchors are (i) the presence of a side chain on the α1,2-linked mannose of the core oligosaccharide consisting of one or two mannose residues (M4 and M5 in Figure 1) and (ii) the presence of a ceramide moiety on the GPI anchor of most but not all GPI-proteins (Conzelmann *et al.*, 1992; Fankhauser *et al.*, 1993). The ceramides found on the majority of yeast anchors consist of C18:0 phytosphingosine and C26:0 fatty acids and are different from the main ceramides found in the abundant inositolphosphoceramides (IPCs; Smith and Lester, 1974). On the other hand, the abundant 125 kDa GPI-glycoprotein (known as gas1, ggp125 or gp115) contains no ceramide but an unusual C26:0 fatty acid-containing, base sensitive lipid (Fankhauser *et al.*, 1993). The question thus arises if the GPI glycolipid precursors are also made with two different lipid moieties or if one of these lipid moieties is added through remodeling of GPI anchored proteins. The only GPI precursor lipid which has been observed so far is an early intermediate with the head group GlcN-acylinositol-PO$_4$ accumulating in *dpm1* and *sec53*. Since it only contains mild base sensitive lipid moieties we and others speculated that the ceramide anchor must be added at a later stage of GPI biosynthesis (Conzelmann *et al.*, 1992; Costello and Orlean, 1992). Here we show that also the complete GPI glycolipids do not contain ceramide moieties, a finding which strongly suggests that lipid moieties of yeast GPIs are indeed remodeled and that in contrast to trypanosomes (Masterson *et al.*, 1990), they are remodeled after rather than before their addition to proteins.

Results

Accumulation of very polar glycolipids in pmi40 is enhanced by tunicamycin and cycloheximide

Due to the absence of endogenously made mannose under restrictive conditions, the incorporation of [2-^{3}H]mannose into lipids is greatly enhanced in *pmi40* cells after shifting cells to 37°C (Figure 2A). As indicated by the results in Table I, at 37°C *pmi40* displays, relative to wild type cells, a 3-fold increase in uptake of the label from the medium and a 30-fold overall increase of incorporation into lipids. This increase brings into view a series of minor lipids the most polar of which were termed CP1 and CP2 and will be shown to correspond to complete anchor precursor (CP) lipids below. It is worth noting that the relative abundance of CP1 is independent of the labeling temperature, but that the generation of CP2 is strongly enhanced at 37°C. An attempt to label CP lipids with [^{3}H]*myo*-inositol is illustrated in Figure 2B. Although some bands comigrating with CP2 could be seen in *pmi40* at 24°C and in several wild type strains, the purification and analysis of [^{3}H]*myo*-inositol-labeled CP lipid candidates proved impracticable because of the very low abundance of these lipids in the [^{3}H]*myo*-inositol-labeled extracts. By scanning, CP1 plus CP2 could account for up to 22% of lipids in [^{3}H]mannose-labeled extracts (Table I) whereas only 0.05-0.15% of counts were found in the corresponding bands from [^{3}H]*myo*-inositol-labeled extracts. Tunicamycin and cyclo-

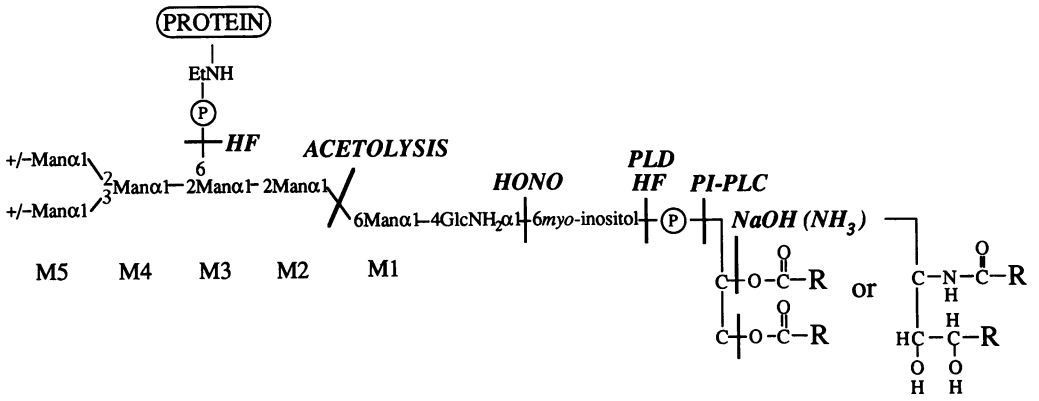

Fig. 1. GPI anchor structures of yeast proteins. The scheme outlines the structural variants found in *S.cerevisiae* (Fankhauser *et al.*, 1993). The mannoses are indicated by M1 to M5. Eighty percent of the GPI anchors of wild type cells contain only M1−M4. A fifth mannose (M5) is present in 20% of GPI anchors and is linked either α1,2 or α1,3 to M4. The sites of cleavage obtained by HF dephosphorylation, nitrous acid (HONO) deamination, phosphatidylinositol-specific phospholipase C (PI-PLC), phospholipases D (PLD) or methanolic NH_3 are indicated. EtNH = ethanolamine; R = alkyl chain.

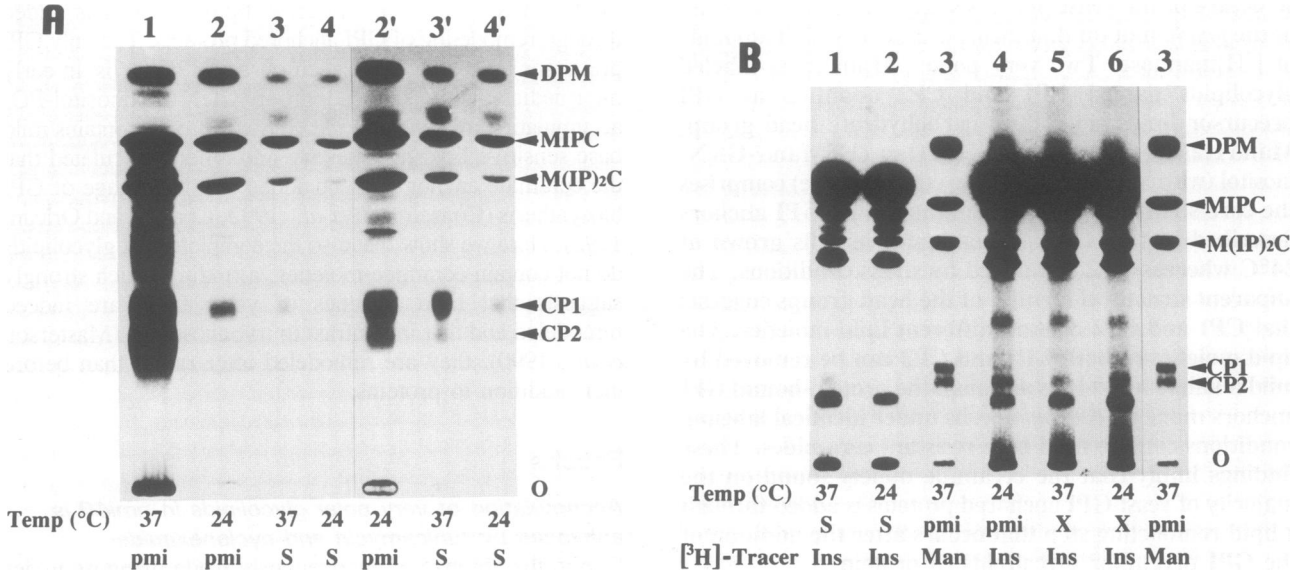

Fig. 2. *pmi40* cells incorporate high amounts of [^{3}H]mannose. (**A**) 2.5 A_{600} units of *pmi40* cells or corresponding S-150 wild type cells (S) were labeled with [2-^{3}H]mannose at 24°C or 37°C in the presence of 20 μg/ml of tunicamycin (added at the start of the preincubation) and 100 μg/ml of cycloheximide (added together with the radiolabel). Lipids were extracted, desalted by butanol/water partitioning, separated by TLC (4×10^7 cell equivalents per lane) and visualized by fluorography. Exposure of fluorogram for lanes 1−4: 4 days, for lanes 2′−4′: 28 days. o, origin; MIPC, mannosylinositolphosphoceramide; $M(IP)_2C$, inositolphospho-MIPC (Smith and Lester, 1974); DPM, dolicholphosphomannose. (**B**) *pmi40*, S-150 and X-2180-1A (X) were labeled with 25 μCi of [^{3}H]mannose or 25 μCi of [^{3}H]*myo*-inositol in the presence of cycloheximide (100 μg/ml) at the indicated temperatures. (For labeling with [^{3}H]*myo*-inositol we had to use glucose-containing SDCU medium, since inositol is not taken up efficiently in the pyruvate-containing medium which is optimal for labeling with [^{3}H]mannose.) Half of the [^{3}H]mannose-labeled lipid extract corresponding to 130 000 c.p.m. was spotted onto TLC (lanes 3) whereas the total of [^{3}H]*myo*-inositol-labeled extracts (7×10^6−1.6×10^7 c.p.m.) were spotted in the other lanes. Exposure of fluorogram: 4 days.

Table I. Incorporation of [^{3}H]mannose into *pmi40* cells (quantification of radioactivity on the gel shown in Figure 2A)

	Temp. of labeling (°C)	c.p.m. in medium after labeling ($\times 10^{-6}$)[a]	c.p.m. in desalted lipid extract ($\times 10^{-3}$)[b]	% of total lipids[c]			
				CP1	CP2	MIPC	$M(IP)_2C$
S-150	24	25.0	16	4.2	0.9	56.0	6.1
S-150	37	22.0	37	5.3	2.6	60.2	7.6
pmi40	24	17.2	132	3.5	2.0	58.5	19.0
pmi40	37	3.8	1150	5.1	16.6	31.5	18.2

[a]3×10^7 c.p.m. (= 25 μCi) of radioactivity were added at the beginning of labeling.
[b]Derived from 2.5 A_{600} units of cells.
[c]100% = sum of radioactivity in all peaks of the corresponding TLC lane.

heximide both increased the overall incorporation into lipids as shown qualitatively in Figure 3 and quantitatively in Table II. The enhancing effect of tunicamycin and cycloheximide can be explained by an increased availability of mannose for lipid synthesis since tunicamycin blocks the incorporation of mannose into the *N*-glycans of glycoproteins whereas cycloheximide blocks glycoprotein biosynthesis altogether. It is worth noting that cycloheximide doubles the relative amount of label in CP1 and CP2, an effect that might be due to the failure of these lipids to be used for anchoring of proteins when protein synthesis is blocked.

Structural characterization of CP lipids

CP1 and CP2 were purified and their hydrophilic head groups released by partial deacylation with NH_3 followed by treatment with PI-PLC. Analysis of these head groups as documented in Figures 4 and 5 gave identical results for CP1 and CP2 and, therefore, only the results obtained with CP1 are shown. As shown in Figure 4A, the HF fragment, after *N*-acetylation, comigrated with the Man_4-GlcNAc-Ins standard (Ins = inositol) suggesting that CPs contain only four mannoses, although on mature proteins 20% of anchors

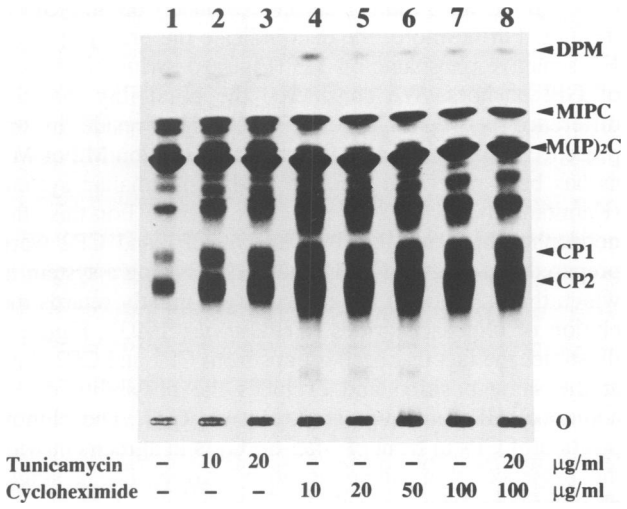

Fig. 3. Tunicamycin and cycloheximide increase the incorporation of [^{3}H]mannose into CP1 and CP2. *pmi40* cells were preincubated at 37°C for 20 min and then labeled with [^{3}H]mannose in the presence or absence of various concentrations of tunicamycin or cycloheximide as indicated. Lipids were analyzed as in Figure 2.

contain a fifth mannose (Figure 1). Treatment of this HF fragment with jack bean α-mannosidase (JBAM; Figure 4C) yielded only free [^{3}H]mannose, suggesting that no HF resistant groups are attached to its α-linked mannoses.

Since complete anchor precursor glycolipids are expected to contain an HF sensitive phosphoethanolamine group on the third mannose (M3, Figure 1), the PI-PLC-generated head groups were subjected to digestion by JBAM prior to HF dephosphorylation (Figure 4B). The resulting fragment comigrates with Man_3-GlcNAc-Ins. This result confirms the presence of an HF sensitive group on the third mannose which hinders the activity of JBAM. The fourth mannose that was removed is linked α1,2 since not only JBAM but also ASAM, an α1,2-specific α-mannosidase, can remove this mannose residue (Figure 4E). ASAM treatment of the *N*-acetylated, desalted HF fragment generated a peak comigrating with Man_2-GlcNAc-Ins (Figure 4D). Thus, the anchor contains at least two α1,2-linked mannoses of which only one is in terminal position before HF dephosphorylation.

Acetolysis preferentially cleaves α1,6-linked mannose residues and thus is expected to cleave GPI structures between M1 and M2 (Figure 1; Schneider *et al.*, 1990). To avoid the cleavage of other bonds, we used very mild acetolysis conditions which result in only partial fragmentation of the starting material. After acetolysis of the HF fragments of CP1 and CP2 we observed a predominant Man_1-GlcNAc-Ins fragment in addition to the uncleaved Man_4-GlcNAc-Ins starting material (Figure 4F). Taking into account that the specific activity of the starting Man_4-GlcNAc-Ins is four times higher than the one of Man_1-GlcNAc-Ins we calculate that the acetolysis procedure cleaved 89% and 92% of the head groups of CP1 and CP2, respectively. For further confirmation of their presumed GPI configuration, CP1 and CP2 were subjected to nitrous acid deamination under conditions that cleave between Ins and glucosamine, the latter being transformed into 2,5-anhydromannitol through subsequent reduction with $NaBH_4$. CP1 and CP2 were both sensitive to nitrous acid deamination as their migration was shifted to the expected Man_4-anhydromannitol standard after treatment (Figure 5C) but not after control incubation (Figure 5D). The presence of a free amine group on the HF fragments of CP1 and CP2 could be demonstrated independently by a significant shift in mobility after *N*-acetylation with acetic anhydride (Figure 5A and B). To obtain the position of the HF sensitive

Table II. Effect of tunicamycin and cycloheximide on incorporation of [^{3}H]mannose into *pmi40* cells (quantification of radioactivity on TLC shown in Figure 3)

	Tunicamycin (μg/ml)	Cycloheximide (μg/ml)	c.p.m. in medium after labeling ($\times 10^{-6}$)[a]	c.p.m. in desalted lipid extract ($\times 10^{-3}$)	% of total lipids			
					CP1	CP2	MIPC	$M(IP)_2C$
1	–	–	2.5	320	3.3	8.0	9.6	37.9
2	10	–	2.7	400	5.1	11.9	9.1	32.9
3	20	–	2.6	460	5.3	12.3	9.4	30.3
4	–	10	2.6	920	5.7	19.2	14.2	30.8
5	–	20	2.4	880	5.5	18.4	14.6	32.9
6	–	50	2.5	990	5.8	19.6	13.6	33.0
7	–	100	2.5	1070	5.2	17.0	14.7	31.3
8	20	100	2.9	1080	5.2	17.4	17.5	30.9

[a]3×10^7 c.p.m. (= 25 μCi) of radioactivity was added at the beginning of the labeling.

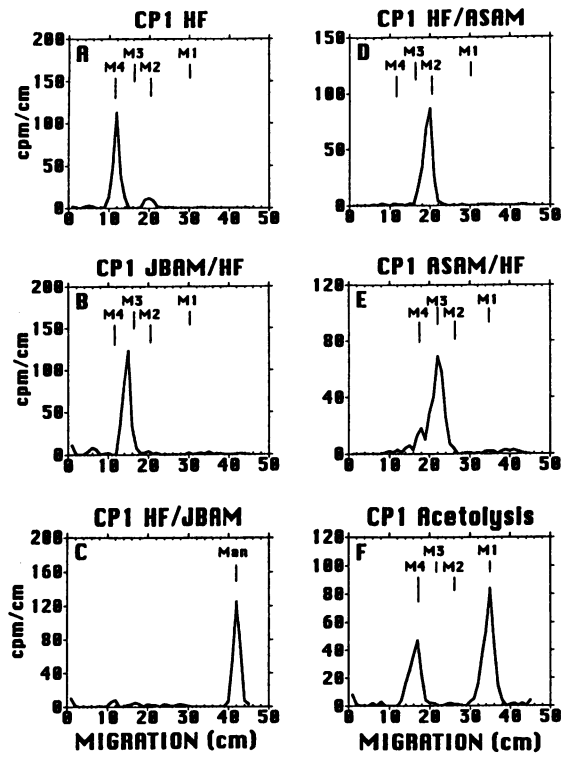

Fig. 4. Fragmentation of CP1 using HF, JBAM, ASAM and acetolysis. Head groups were obtained from purified, [^{3}H]mannose-labeled CP1 and were treated as follows: (**A**) HF; (**B**) JBAM then HF; (**C**) HF, *N*-acetylation, desalting, JBAM; (**D**) HF, *N*-acetylation, desalting, ASAM; (**E**) ASAM then HF; or HF, *N*-acetylation, desalting, acetolysis (**F**). The thus generated fragments were *N*-acetylated, desalted and separated by paper chromatography and the radioactivity in 1 cm wide strips was determined through scintillation counting. The positions of standards run in parallel on the same paper are indicated: M1 to M4 = Man_1- to Man_4-GlcNAc-Ins. Identical results were obtained by TLC separation of labeled fragments.

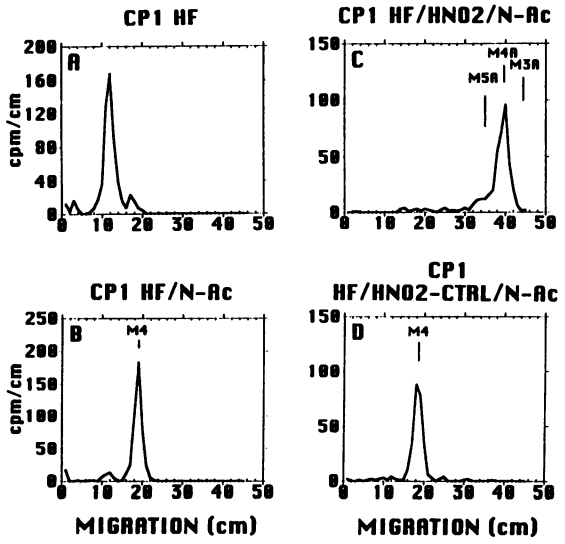

Fig. 5. Nitrous acid treatment of CP1. Head groups from purified, [^{3}H]mannose-labeled CP1 were either (**A**) left untreated or (**B**) *N*-acetylated or (**C**) treated with nitrous acid reduced with $NaBH_4$ and *N*-acetylated, or (**D**) subjected to a control incubation (CTRL) without nitrous acid and *N*-acetylated. All samples were then desalted and spotted onto a single sheet of paper for chromatography. The position of standards run on the same paper are indicated: M3A−M5A = Man_{3-5}-anhydromannitol; M4 = Man_4-GlcNAc-Ins.

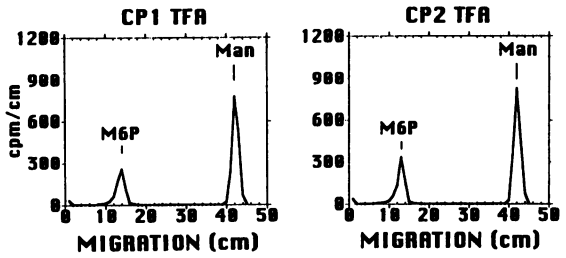

Fig. 6. Trifluoroacetic acid treatment of CP1 and CP2. CP1 and CP2 were hydrolyzed with trifluoroacetic acid and analyzed by paper chromatography in parallel with Man-6-PO_4 (M6P) and [^{3}H]mannose as standards.

group on M3 (Figure 1) which hinders complete degradation of the head groups by α-mannosidase (Figure 4B), CP1 and CP2 were hydrolyzed with trifluoroacetic acid under conditions that cleave all phosphoesters of mannose except for Man-6-PO_4 (Ueda *et al.*, 1993). As shown in Figure 6, mannose and Man-6-PO_4 were obtained at ratios of 2.54 (CP1) and 2.68 (CP2), respectively. This suggests that M3 carries a phosphate on C6.

Collectively, these data demonstrate that the structure of the HF fragment is Manα1,2Manα1,2Manα1,6Manα-GlcN-inositol. Furthermore, the data indicate the presence of an HF sensitive phosphate on M3 (Figure 1) which is typical of GPI anchors. We considered the possibility that the difference between CP1 and CP2 might reside in the presence of an additional HF sensitive group on M2 or M3 as has been described recently in the mammalian system (Kamitani *et al.*, 1992; Ueda *et al.*, 1993). For this, the nondephosphorylated head groups of CP1 and CP2 were prepared and analyzed by Dionex HPLC using a system in which the presence of negative charges greatly retards the elution of oligosaccharides (Ueda *et al.*, 1993). Figure 7 illustrates the fact that head groups from CP1 and CP2 elute at the same position and excludes the possibility of an additional HF sensitive component on CP2. The elution position of CPs in front of Man-6-PO_4 is in agreement with the presence of a single negative charge, i.e. a single phosphoethanolamine substituent on both lipids (Ueda *et al.*, 1993).

The lipid moieties of CP1 and CP2 are sensitive to mild alkaline hydrolysis but resistant to PI-PLC

Although we obtained reasonable amounts of [^{3}H]mannose-labeled CP1 and CP2 from *pmi40* cells, the relatively low abundance of these lipids precluded the purification of CP1 and CP2 radiolabeled in their lipid moiety. Some information about the nature of the lipid moiety of the CPs could, however, be obtained from the [^{3}H]mannose-labeled lipids as shown in Figure 8. Deacylation with mild base leads to the almost complete disappearance of CPs (Figure 8, lanes 1 and 2) as well as all polar lipids migrating more slowly than $M(IP)_2C$ some of which are GPI intermediates (data not shown). Also, no more polar lipids were generated from CPs. By scanning, there still were 2.3% and 1.8% of radioactivity left over in the region of CP1 and CP2, respectively, after base treatment; however, we hesitate to consider this material as mild base resistant CPs, since this activity could also be caused by trailing of bands with higher R_F values. Thus, we cannot find any evidence for the presence of significant amounts of ceramides or *O*-

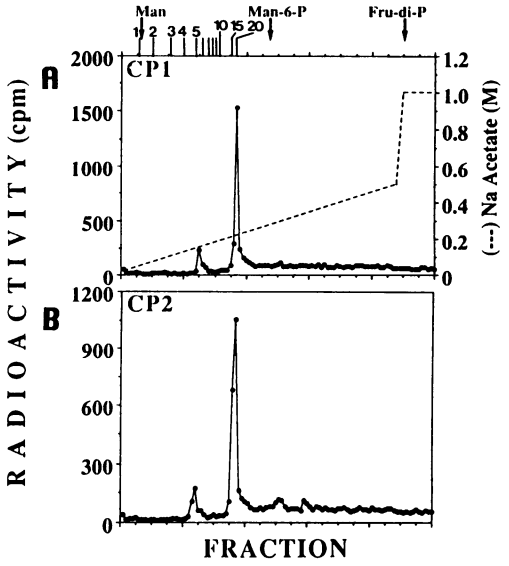

Fig. 7. Comparison of nondephosphorylated head groups of CP1 and CP2 by Dionex HPLC. Lipid moieties were removed from purified CP1 and CP2 by GPI-specific PLD (GPI-PLD) and methanolic NH_3 treatment. Resulting head groups were analyzed in a Dionex HPLC system and radioactivity in each fraction was determined by scintillation counting. The elution positions of glucose oligosaccharides containing from 1 to 20 glucoses and of mannose, Man-6-PO_4 and fructose 1,6-bisphosphate are shown. Injection of a mixture of head groups CP1 and CP2 gave an identical profile (not shown).

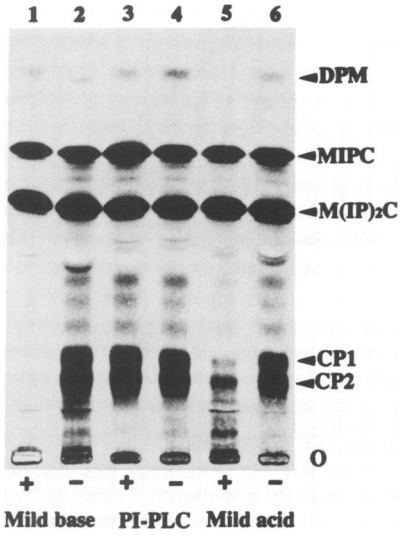

Fig. 8. Analysis of lipid moiety of CP1 and CP2. *pmi40* cells were labeled with [2-^{3}H]mannose at 37°C in the presence of tunicamycin and cycloheximide. Lipid extracts were treated either with mild base (lane 1), or with PI-PLC (lane 3), or with mild acid (lane 5), or subjected to the corresponding control incubations (lanes 2, 4 and 6). Remaining lipids were desalted using Octyl-Sepharose and analyzed by TLC and fluorography.

alkylglycerols on these anchor lipids. On the other hand, CP1 and CP2 are completely resistant to PI-PLC (Figure 8, lanes 3 and 4), although protein-bound GPI anchors of yeast are PI-PLC sensitive (Conzelmann *et al.* 1990). This result is reminiscent of the situation in trypanosomes and mammals and suggests that CP1 and CP2 contain a fatty acid esterified to the inositol which hinders the PI-PLC and which is removed when anchors are attached to proteins. This

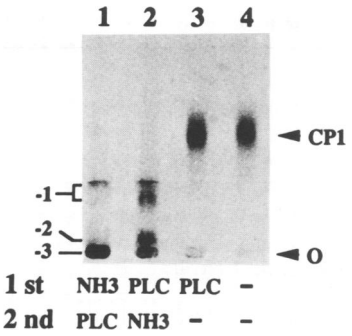

Fig. 9. Partially deacylated CP lipids are sensitive to PI-PLC. Aliquots of 2500 c.p.m. of [^{3}H]mannose-labeled CP1 were sequentially subjected to partial deacylation with NH_3, then PI-PLC or were control incubated in the temporal order indicated at the bottom. The resulting fragments were separated by TLC and fluorography. On the basis of the R_F values, the fragments resulting from partial deacylation are presumed to have lost one (−1), two (−2) or three (−3) acyl chains. Comparable results were obtained with CP2 (not shown).

hypothesis was confirmed by treating partially deacylated CP1 with PI-PLC as described by Costello and Orlean (1992). Partial deacylation of CP1 with NH_3 generated two even more polar lipids lacking either one or two acyl chains which were both PI-PLC sensitive (Figure 9). Incidentally, the sensitivity of CPs to PI-PLC and to GPI-PLD (see above) indicates the presence of Ins in CPs, albeit indirectly. Mild acid hydrolysis largely destroys CPs (Figure 8, lanes 5 and 6).

Analysis of protein anchors from pmi40 cells

Since a ceramide moiety had been found on the GPI anchor of most but not all GPI-proteins (Conzelmann *et al.*, 1992; Fankhauser *et al.*, 1993), it seemed important to verify if *pmi40* cells made base resistant anchors under the conditions used to label CPs. Our methodology only allowed us to obtain radiochemically pure anchor peptides in their [^{3}H]*myo*-inositol-labeled form but it turned out that [^{3}H]*myo*-inositol does not get incorporated efficiently into GPI-proteins under the conditions used for [^{3}H]mannose labeling, very likely because of lack of mannose at 37°C. As can be seen in Table III, CPs made during a 20 min pulse seem to stay largely base sensitive during chase, whereas the GPI anchors made during chase from the added cold mannose and phosphatidyl[^{3}H]inositol are 60% base resistant. When these anchor peptides were treated with mild base and then with nitrous acid and resulting lipids were analyzed by TLC, we observed a heavy band comigrating with the IPC which had previously been identified in GPI anchors of X2180 cells (Conzelmann *et al.*, 1992; not shown). Thus, our experiments clearly indicate that *pmi40* makes base resistant GPI anchors while the CPs remain base sensitive.

Is CP1 a precursor of CP2?

It was apparent from many experiments that CP1 is the main CP in wild type cells (Figure 2) whereas the appearance and accumulation of CP2 were strongly enhanced under various stress conditions such as exposure to 37°C (Table I), deprivation of mannose (Table I) or addition of cycloheximide (Table II). It should be noted that deprivation of mannose results in a secretion block but data obtained with the temperature sensitive *sec18* secretion mutant (Novick *et al.*,

Table III. Mild base treatment of [^{3}H]mannose-labeled CPs and [^{3}H]*myo*-inositol-labeled anchor peptides

	Tracer	Chase conditions	% of total c.p.m. in lipid extract		Base sensitive fraction (%)
			Before mild base treatment	After mild base treatment	
CP1	[^{3}H]mannose	mannose + cyclohex.	11.4	0.4	97
CP2	[^{3}H]mannose	mannose + cyclohex.	13.5	1.1	92
Anchor peptides	[^{3}H]Ins	mannose	–	–	40

pmi40 cells were labeled at 37°C in SPCU in the presence of tunicamycin with the indicated tracers for 20 min and chased by dilution into SDCU containing cycloheximide (100 μg/ml) and/or mannose (10 mM) for 40 min. Lipids from [^{3}H]mannose-labeled cells were either treated with mild base or mock incubated and then analyzed as described in Figure 2 and quantified by scanning. Anchor peptides were obtained from [^{3}H]*myo*-inositol-labeled cells and the fraction of base sensitive anchors was determined by partitioning in Triton X-114 and calculated by correcting for the spontaneously hydrophilic anchor peptides (14% before base treatment) (Conzelmann *et al.*, 1992).

1980) show that blocking the secretory pathway between ER and Golgi by a temperature shift from 24°C to 37°C does not increase the ratio of CP2/CP1 to any greater extent than a similar temperature shift in wild type cells (not shown). Thus, it seems to be the deprivation of mannose rather than the secretion block which induces CP2 synthesis in *pmi40*. Further experiments showed that under stress cells not only upregulate the production of CP2 but diminish synthesis of CP1, i.e. they switch from CP1 to CP2 production within 10 min (not shown). To understand better the relationship between CP1 and CP2, their decay rates were analyzed by pulse–chase experiments. These experiments did not show any precursor product relationship between [^{3}H]mannose-labeled CP1 and CP2 since both CPs accumulated and disappeared with similar kinetics (Figure 10). If pulse and chase were carried out in the presence of cycloheximide, the radioactivity in CPs increased during the first 10–20 min of chase although the incorporation of [^{3}H]mannose into the total lipids was efficiently blocked by the addition of cold mannose (not shown). This can be explained by the biosynthesis of additional CPs from dolicholphospho[^{3}H]mannose during chase since this lipid intermediate accumulated during pulse under cycloheximide (compare Figures 2 and 3) and disappeared within the first 20 min of chase. [Dolicholphospho[^{3}H]mannose is the immediate donor for the addition of M1, M2 and M3 (Figure 1; Menon *et al.*, 1990).] Pulse–chase experiments also demonstrated a profound effect of cycloheximide on the half-lives of CP1 and CP2 (Figure 10). In the absence of the drug the decay of both CPs showed biphasic kinetics with a rapid loss of the bulk of CPs within 15 min and a slow disappearance of the remainder. If cycloheximide was added at the beginning of chase, the decay rate of CPs was rapid only for a few minutes and then slowed down significantly. In contrast, the effect of cycloheximide on the decay of structural lipids such as $M(IP)_2C$ or on the total number of counts in the lipid extract was not very pronounced (not shown). These results can be interpreted to mean that the bulk of CPs accumulated during the pulse remain competent for transfer onto proteins and are rapidly consumed if GPI anchored proteins continue to be made and that arrest of protein synthesis prolongs the half-lives of these CPs by blocking their transfer onto newly made GPI-proteins. Alternatively, the effect of cycloheximide might be explained through the loss of a short-lived regulatory protein which stimulates the decay of free CPs. It may be noteworthy that the amount of radioactivity in CPs after pulse (Figure 10, t = 0) was higher when cells were diluted into SDCU medium containing cycloheximide (●) than into SDCU without cycloheximide (○) or into SPCU (□). This might be explained by a more rapid cessation of the addition of CPs to GPI-proteins upon shift to 0°C and energy deprivation if cycloheximide was present in addition.

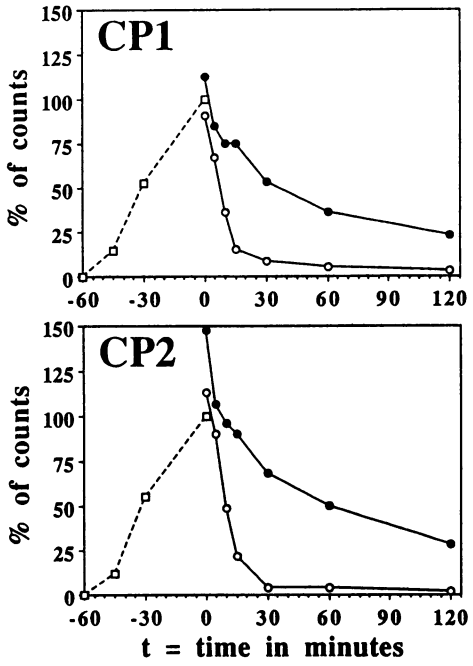

Fig. 10. The half-lives of CP1 and CP2 are increased by cycloheximide. *pmi40* cells were labeled in SPCU medium with [^{3}H]mannose (50 μCi/2.5 A_{600} units) at 37°C for 60 min without any drugs starting at t = −60 min and aliquots were removed for lipid analysis at regular intervals by dilution into prewarmed SPCU containing NaF and NaN_3 and immediately placed onto ice. At t = 0 the remainder of cells was split and one half diluted in SDCU containing cold mannose (10 mM final) (○), the other half into the same supplemented with cycloheximide (●) (10 μg/ml final) to initiate the chase. Chase was performed by further incubation at 37°C for 0–120 min with chases being stopped by the additon of NaF and NaN_3 and chilling. Immediately after chilling cells were washed and organic solvents were added. Lipids were extracted, desalted, separated by TLC and quantified by radioscanning. Relative amounts of radioactivity in CP1 and CP2 during pulse (□) or chase (○,●) are expressed as the percentage of c.p.m. present in each of these lipids at the end of the pulse (□, t = 0 min), i.e. 62 400 and 305 500 c.p.m. for CP1 and CP2 respectively. Very similar results were obtained when the chase was performed at 24°C.

Discussion

GPI anchoring seems to be a post-translational modification that arose early in evolution and allows, for example, the sorting of proteins into specific membrane domains (Brown and Rose, 1992; Cinek and Horejsi, 1992), the regulated release of proteins from the cell surface (Müller and Bandlow, 1993), or the escape of proteins from endocytosis through clathrin coated pits (Lemansky *et al.*, 1990). In yeast, there also is preliminary evidence suggesting that GPI anchors might direct integration of proteins into the cell wall (Schreuder *et al.*, 1993; De Nobel and Lipke, 1994). Here we demonstrate the existence of free GPIs which have the structural features expected for the complete anchor precursor lipid, ready to be transferred onto proteins. Indeed, HF treatment of the GPI moieties isolated from proteins isolated from the ER of yeast yield the same Manα1, 2Manα1,2Manα1,6Manα-GlcN-Ins fragment as found on CPs (A.Puoti, G.Sipos and A.Conzelmann, in preparation). Similar CPs have been described in mammalian cells and trypanosomes where, based on their abundance and their long half-lives, they are considered to be supernumerary CPs which are made in excess of what is required for protein anchoring (Puoti and Conzelmann, 1992; Ralton *et al.*, 1993). We are led to believe that, in contrast to these systems, yeast does not make CPs in significant excess of demand because, when labeling with [^{3}H]*myo*-inositol, one observes massive incorporation into GPI-proteins within minutes (Conzelmann *et al.*, 1990) but only very weak incorporation into CPs. Further findings suggesting that CPs isolated from *pmi40* are representative of the 'true' precursor and are not a further processed form thereof are: (i) the relative increase in labeling of CPs and the increased half-life of CPs in the presence of cycloheximide (Table II, Figure 10), (ii) the presence of CP1 and CP2 in normal wild type cells as well as (iii) the fact that CP1 and CP2 are found in *sec18* at 37°C where the secretory pathway is blocked between ER and Golgi.

The structure of these precursors has several implications for the further study of GPI anchoring in yeast. (i) The presence of only four mannoses suggests that the fifth mannose is added to the GPI-proteins at a later stage of biosynthesis, possibly in the Golgi. (ii) The presence of acylinositol in CPs is analogous to the situation in the mammalian and trypanosomal systems. It predicts that, as postulated for other organisms, yeast contains a deacylase which removes this acyl chain. This activity presumably is localized in a pre-Golgi compartment since GPI anchors of proteins trapped in the ER (isolated from *sec18* or *sec17*) are completely sensitive to PI-PLC, hence, devoid of acylinositol (Conzelmann *et al.*, 1990). (iii) The absence of any structural difference between the head groups of CP1 and CP2 suggests that these two CPs differ with regard to the fatty acids of their diacylglycerol moiety. This difference might concern the degree of hydroxylation, since the base sensitive lipid moiety of ggp125, a GPI anchored glycoprotein, consists of non-hydroxylated and monohydroxylated C26:0 fatty acids (Fankhauser *et al.*, 1993), but differences in chain length or desaturation are also conceivable. The two CP lipids might give rise to the two different mild base sensitive lipid moieties present on GPI-proteins from cells grown at 37°C (see Figure 2, lane 7, in Conzelmann *et al.*, 1992). The exact identities of fatty acids present on CPs and yeast GPI-proteins and the bases for the stress-induced switch from CP1 to CP2 production remain to be investigated. (iv) The most significant implications of the structure of CPs derive from the complete absence of mild base resistant lipid moieties and, therefore, absence of ceramides. In an earlier study, we speculated that ceramides might get incorporated into GPI-proteins by remodeling in order to explain a drastic relative increase of base resistant lipids on GPI-proteins labeled through short pulses with [^{3}H]*myo*-inositol during a subsequent chase period (Conzelmann *et al.*, 1992). However, alternative explanations invoking higher turnover rates of GPI-proteins with mild base sensitive lipids remained equally likely. The absence of ceramides from the complete anchor precursor lipids, however, argues against this latter hypothesis and strongly suggests that lipid moieties of GPI anchored proteins of yeast are remodeled. Remodeling might be carried by an enzyme exchanging diacylglycerol for a ceramide in a manner analogous to the one described for the biosynthesis of IPCs (Becker and Lester, 1980). Since this lipid remodeling is not found in mammalian organisms, it represents yet another interesting target for the design of fungicidal drugs.

The use of *pmi40* for the visualization of the otherwise barely detectable GPI-lipids will be useful for the study of the biosynthetic defect of yeast mutants deficient in GPI-biosynthesis.

Materials and methods

Strains, growth conditions and materials

Saccharomyces cerevisiae strain C4 (*MATa leu2-3,112 ura3-52 pmi40*) and the corresponding parental strain, S-150-2B (*MATa leu2-3,112 ura3-52 trp1-289 his3-1*) were the kind gift of Drs M.A.Payton and D.J.Smith, Glaxo Institute for Molecular Biology, CH-1228 Geneva, Switzerland. The *pmi40* allele encodes a thermolabile phosphomannose isomerase which loses activity within minutes at 37°C (Payton *et al.*, 1991). Cessation of mannose biosynthesis results in a conditional defect in protein secretion which can be corrected by the addition of 10 mM mannose to the culture medium. Other strains have been described (Conzelmann *et al.*, 1992). Cells were kept on SDCU agar plates containing salts, vitamins and trace elements but no *myo*-inositol (Wickerham, 1946), 2% glucose as a carbon source, 1% of casein hydrolysate and uracil (40 mg/l). SDCU medium of the same composition was used for liquid cultures. [2-^{3}H]*Myo*-inositol (55 Ci/mmol) and D-[2-^{3}H]mannose (20 Ci/mmol) were purchased from Amersham (Buckinghamshire, UK). α-Mannosidases from jack bean and *Aspergillus saitoi* were obtained from Oxford GlycoSystems (Oxford, UK). Octyl-Sepharose CL-4B was bought from Pharmacia (Uppsala, Sweden). 1000-fold purified GPI-specific phospholipase D (GPI-PLD) from bovine serum was a kind gift of Dr Urs Brodbeck, Institut für Biochemie und Molekularbiologie, University of Bern, Switzerland.

Radiolabeling with [^{3}H]mannose

For labeling with D-[2-^{3}H]mannose, cells were cultured in SDCU medium. Exponentially growing cells were resuspended in SPCU medium which is identical to SDCU except that it contains 2% pyruvate and only 0.1% glucose. Unless noted otherwise, 2.5 A_{600} units of cells (250 μl of a cell suspension having an absorbance of 10 at 600 nm, containing $\sim 4 \times 10^7$ cells) were preincubated for 20 min and labeled by the addition of 25 μCi of [^{3}H]mannose for 30 min. Similar yields of radiolabeled lipids were obtained when the procedure was scaled up by up to 40-fold. Finally, cells were washed twice in ice-cold water containing 10 mM NaN_3 and 10 mM NaF and extracted with solvent 1 ($CHCl_3/CH_3OH/H_2O$; 10:10:3) as described (procedure C, Conzelmann *et al.*, 1992). Special care had to be taken in order not to lose CP1 and CP2 lipids during desalting: lipids were desalted routinely by partitioning between *n*-butanol and an aqueous solution of 0.1 mM EDTA, 5 mM Tris−HCl, pH 7.5 and back extraction of the butanol phase with water. When large amounts of salt had to be removed, lipids were desalted by chromatography on small Octyl-Sepharose 4B columns (bed volume = 1 ml) equilibrated in buffer A (5% propanol in 0.1 M ammonium acetate, pH 5.5) and run at <6 ml/h. Dried lipid extracts

from 10^8 cells were taken up in 0.5 ml of buffer A and applied to the column. The column was washed with buffer A, the first 2 ml of eluate being reloaded to achieve complete adsorption. The column was washed with 1 ml of water and then eluted first with 25%, thereafter with 50% propanol in water. While IPCs eluted mostly at 25% propanol, complete recovery of CPs required 50% propanol.

Preparative methods

Purification of radiolabeled glycolipids. Lipids were purified by preparative TLC on 0.2 mm thick silica gel 60 plates (Merck, Germany) in solvent 1. Radioactive spots were localized by two-dimensional TLC radioscanning (Berthold LB 2842), scraped and eluted with solvent 1. A second run on TLC was generally necessary to obtain radiochemically pure CPs.

Preparation of [^{3}H]myo-inositol-labeled protein anchors. Labeled cells were broken and proteins were delipidated and purified over Concanavalin A–Sepharose as described (procedure C, Conzelmann *et al.*, 1992). The glycoproteins adsorbed onto Concanavalin A–Sepharose were eluted by digestion with Pronase (1 mg/ml in 100 mM Tris–HCl, pH 8.0, 1 mM $CaCl_2$ for 16 h at 37°C) and anchor peptides were purified by passing over an Octyl-Sepharose minicolumn (see above). The base sensitivity of their lipid moiety was determined and calculated as described (Conzelmann *et al.*, 1992).

Analytical methods

Thin layer chromatography of lipids. Desalted lipid extracts were analyzed by ascending TLC using solvent 1. Radioactivity was located by two-dimensional radioscanning and quantified by one-dimensional radioscanning. TLC plates were sprayed with EN^{3}HANCE (NEN, UK) and fluorograms were obtained using X-Omat film (Kodak) exposed at −70°C.

Analysis of head groups. Soluble head groups were obtained from purified glycolipids through selective removal of acyl groups attached to the *myo*-inositol using methanolic NH_3 (Roberts *et al.*, 1988) followed by PI-PLC treatment in 20% propanol (Puoti and Conzelmann, 1993). Fatty acids and lipids were removed by butanol extraction. Head groups were fragmented by HF dephosphorylation, mannosidase treatments and analyzed by paper chromatography in methylethylketone/pyridine/H_2O (20:12:11) as described (Puoti and Conzelmann, 1993). Before chromatography samples were *N*-acetylated and desalted (Puoti *et al.*, 1991). ASAM treatment was done in 0.1 M sodium acetate buffer, pH 5.0, 10 mM $CaCl_2$ for 24 h at 37°C. Acetolysis was done as described by Schneider *et al.* (1990) except that the peracetylated oligosaccharides were purified by extraction into chloroform before acetolysis. Head groups were treated with nitrous acid as described (Roberts *et al.*, 1988), reduced with $NaBH_4$, desalted with AG-50 (Ferguson *et al.*, 1988) and finally desalted with AG-501-X8 (H^+, OH^-) mixed bed ion exchange resin. Radiolabeled Man_x-GlcNAc-[^{3}H]Ins (where x = 0, 1, 2, 3 or 4) chromatography standards (M1–M4 in Figures 4 and 5) were generated from *S.cerevisiae* GPI anchors as described by Puoti and Conzelmann (1992). The Man_x-2,5-anhydro-[1-^{3}H]mannitol standards (M3A–M5A in Figure 5) were prepared from *S.cerevisiae* GPI anchors as described by Puoti *et al.* (1991). For analysis of the nondephosphorylated head group by Dionex HPLC, purified CP lipids were treated with GPI-PLD for 1 h (Davitz *et al.*, 1989), dried, treated with methanolic NH_3, dried and subjected to butanol/water partitioning in order to remove the cleaved lipid moieties. Dionex analysis of the resulting nondephosphorylated head groups was carried out using gradient 2 exactly as described (Hirose *et al.*, 1992).

Analysis of lipid moiety of GPI lipids and anchor peptides. Complete deacylation of purified lipids or lipid extracts with NaOH was done as described (Puoti *et al.*, 1991). PI-PLC digestion in 20% propanol was done according to Puoti and Conzelmann (1993). Mild acid treatment was done in 0.5 M HCl/propanol (1:1, v/v) at 50°C for 15 min. Partial deacylation of lipids with methanolic NH_3 was done as described (Roberts *et al.*, 1988).

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