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Myoinositol gets incorporated into numerous membrane glycoproteins of *Saccharomyces cerevisiae*; incorporation is dependent on phosphomannomutase (*SEC53*)

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We recently described a 125 kd membrane glycoprotein in *Saccharomyces cerevisiae* which is anchored in the lipid bilayer by an inositol-containing phospholipid. We now find that when *S.cerevisiae* cells are metabolically labeled with [^{3}H]myoinositol, many glycoproteins become labeled more strongly than the 125 kd protein. Myoinositol is attached to these glycoproteins as part of a phospholipid moiety which resembles glycophospholipid anchors of other organisms. Labeling of proteins with [^{3}H]myoinositol for short times and in secretion mutants blocked at various stages of the secretory pathway shows that these phospholipid moieties can be added to proteins in the endoplasmic reticulum and that these proteins are transported to the Golgi by the regular secretory pathway. *sec53*, a mutant which cannot produce GDP-mannose at 37°C, does not incorporate myoinositol or palmitic acid into membrane glycoproteins at this temperature, suggesting that GDP-mannose is required for the biosynthesis of these phospholipid moieties. All other secretion and glycosylation mutants tested add phospholipid moieties to proteins normally.

Key words: acylation/glycosylation/mannose/myoinositol/*Saccharomyces cerevisiae*

Introduction

Numerous membrane glycoproteins of protozoan and mammalian origin are anchored in the lipid bilayer by a glycophosphatidylinositol (GPI) moiety and the significance of this finding has been extensively reviewed (Low, 1987; Ferguson and Williams, 1988; Low and Saltiel, 1988). The complete structure of the carbohydrate moiety of the GPI anchors from the variant surface glycoprotein of *Trypanosoma brucei* and of rat Thy-1, a brain surface glycoprotein, have been elucidated (Ferguson *et al.*, 1988; Homans *et al.*, 1988). The comparison of these two structures shows that the core oligosaccharide which links the phosphatidylinositol (PI) to the C-terminal amino acid of the protein, namely peptide-CO-NH-CH_2-CH_2-P-6Manα1,2Manα1,6Manα1, 4GlcNα1,6myoinositol-phospholipid has been conserved during evolution. Studies in trypanosomes and mammalian cells show that the GPI anchors are added to proteins very rapidly after translation (Bangs *et al.*, 1985; Ferguson *et al.*, 1986; Conzelmann *et al.*, 1987; He *et al.*, 1987). It has recently been demonstrated that the carbohydrate moiety of the anchor is assembled on an inositol-containing phospholipid (Krakow *et al.*, 1986; Masterson *et al.*, 1989). The glycophospholipid thus formed is thought to be transferred '*en bloc*' onto the protein.

Similar membrane anchors might exist in the yeast *Saccharomyces cerevisiae* since a 125 kd membrane glycoprotein can be removed from the Triton X-114 (TX-114) detergent phase by phosphatidylinositol-specific phospholipase C (PI-PLC) and exhibits the CRD-antigen, a carbohydrate antigen commonly found on protozoan and mammalian GPI anchors (Conzelmann *et al.*, 1988a). The structure of the yeast phospholipid anchor is not known yet. When *S.cerevisiae* cells are metabolically labeled with [^{3}H]myoinositol, many proteins become labeled more strongly than the 125 kd protein and it was interesting for us to investigate whether these proteins carry a similar type of phospholipid anchor as that of the 125 kd protein. In this study we characterize these proteins as membrane glycoproteins which contain myoinositol in the form of covalently attached glycophospholipids and correct an earlier view which considers these proteins as being acylated directly on the protein (Wen and Schlesinger, 1984). In addition we report on the biosynthesis of these phosphoinositide moieties in wild-type as well as in well characterized secretion and glycosylation mutants.

Results

[^{3}H]Myoinositol is incorporated into numerous glycoproteins other than the 125 kd glycoprotein

[^{3}H]Myoinositol could reproducibly be incorporated into many proteins other than the 125 kd protein which is known to carry an inositol-containing phospholipid anchor (Figure 1a). Time course studies showed that [^{3}H]myoinositol added to exponentially growing cells was completely taken up and incorporated into lipids within minutes after addition to the culture medium (Figure 1b). The incorporation of [^{3}H]myoinositol into proteins was also very rapid in the beginning but continued at a slower rate even after the incorporation into lipids had ceased (Figure 1b). As shown in Figure 1a, the pattern of [^{3}H]myoinositol-labeled proteins was dramatically changed when the labeling was carried out in secretion mutants in which the vesicle traffic along the secretory route is blocked between the endoplasmic reticulum (ER) and the Golgi apparatus at 37°C (Novick *et al.*, 1980, 1981; Esmon *et al.*, 1981) (Figure 1a). When labeled at 24°C, most proteins had apparent mol. wts of $>$125 kd and considerable amounts of material did not migrate beyond the stacking gel, whereas when we labeled at 37°C, no material bigger than 140 kd could be detected and several major bands of lower mol. wt were observed instead. Other secretion mutants blocked before the Golgi apparatus (*sec12,13,16,20,21,22*) were also labeled with [^{3}H]myoinositol at 37°C and all of them showed the same distinct bands as the mutants shown in Figure 1a (data not shown). All of the [^{3}H]myoinositol-labeled proteins are

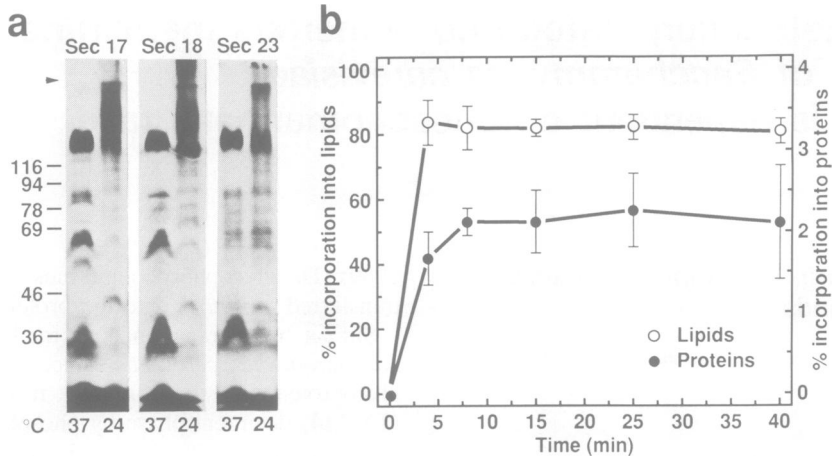

Fig. 1. Panel a: secretion mutants (*sec17*, *sec18* and *sec23*) were pre-incubated for 30 min at 24 or 37°C and labeled with [^{3}H]myoinositol for 1 h at the same temperature. Cells were broken with glass beads, membranes were prepared by ultracentrifugation (procedure E), solubilized in TX-100 and proteins were precipitated with TCA. After extraction of lipids the proteins were analysed by SDS−PAGE/fluorography. The arrowhead on the left indicates the top of the running gel and mol. wt standards are indicated on the left in kd. **Panel b**: X2180-1B cells were labeled at 37°C with [^{3}H]myoinositol (40 μCi/ml). At indicated times triplicate aliquots of 100 μl were removed and incorporation was stopped immediately by adding sample buffer and boiling. To measure incorporation into lipids small aliquots were partitioned in the detergent TX-114 which retains labeled lipids but not the free myoinositol. Protein-associated counts were measured by precipitation of whole cell protein in chloroform/methanol/water (10:10:3) followed by four washes in the same solvent to completely remove non-covalently bound lipids. Percentages of incorporation refer either to total radioactivity added to cells (for lipids) or to counts incorporated into lipids (for proteins). Error bars indicate 2σ values for means of three independent measurements.

glycoproteins since they could be adsorbed onto and specifically be eluted from Con A−Sepharose as shown in Figure 2 (lanes 1−4). (Complete adsorption was easily obtained in other experiments using larger amounts of Con A−Sepharose, see below.) Moreover, the mol. wts of most of the [^{3}H]myoinositol-labeled glycoproteins were reduced by treatment with endoglycosidase H (Figure 2, lanes 5−8) and this reduction was particularly drastic for the high mol. wt glycoproteins labeled at 24°C. This shows that most of these proteins carry N-linked oligosaccharides and that this type of glycan makes up a very large proportion of their mol. wt. This result suggested that the glycoproteins labeled in early secretion mutants at 37°C are precursors of the ones labeled at 24°C and we tried to confirm this hypothesis further by pulse−chase experiments. When we pulse labeled *sec18* cells with [^{3}H]myoinositol at 37°C and chased at 24°C, the chase produced proteins of the same high mol. wt as the ones made at 24°C (Figure 3). These high mol. wt proteins appeared with kinetics reflecting the slow recovery of *sec18* cells from the heat induced secretion block (Tschopp *et al.*, 1984). This experiment, however, is not a true pulse−chase experiment. As will be shown below, [^{3}H]myoinositol is incorporated into proteins as part of a phospholipid. The cold myoinositol added to initiate the chase only blocks the incorporation of [^{3}H]myoinositol into proteins by blocking its incorporation into the obligatory lipid intermediate but does not interfere with the incorporation into proteins of phospholipids which have been labeled with [^{3}H]myoinositol during the pulse. Therefore we tried to do pulse−chase experiments where we also could block protein synthesis with cycloheximide during the chase. Upon addition of this inhibitor to cells, the incorporation of [^{3}H]myoinositol into proteins does not stop instantaneously but comes to a halt only 5 min later (Figure 3, lanes 6, 7). (It is not clear whether this is due to the slow penetration of cycloheximide into cells or to the existence of a lag period between the entry of the newly made glycoproteins into the

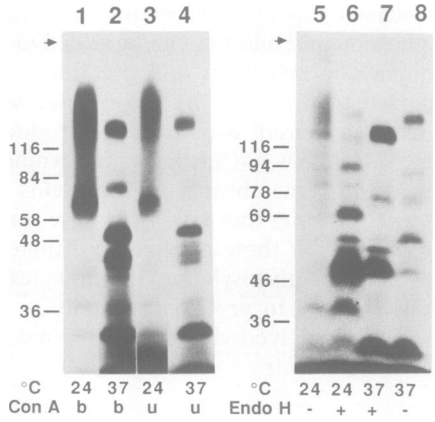

Fig. 2. 15 OD_{600} units of *sec18 pep4* cells (**lanes 1−4**) and *sec17* cells (**lanes 5−8**) were labeled with [^{3}H]myoinositol at 24°C (**lane 1,3,5,6**) or 37°C (**lane 2,4,7,8**). **Lanes 1−4**: cells were lysed in TX-114 (procedure A). The TX-114 detergent phase was adsorbed onto 60 μl of Con A−Sepharose and the bound proteins (b) were eluted with α-methylmannoside (**lanes 1,2**) whereas the unbound (u) material was precipitated with TCA (**lanes 3,4**). Proteins were then visualized by SDS−PAGE followed by fluorography. **Lanes 5−8**: labeled cells were broken and membranes were isolated (procedure C) and solubilized in SDS before incubation with (+) or without (−) endoglycosidase H. Subsequently the samples were processed for SDS−PAGE/fluorography.

ER and the addition of phosphoinositide moieties.) When pulse labeling cells for short times, we obtained mainly proteins of the same size as the ones which accumulated when the transfer from ER to Golgi was blocked (Figure 3, compare lanes 8′ with 9, 10). During chase, these low mol. wt proteins faded whereas high mol. wt proteins increased drastically. As expected, the amount of labeled proteins increased during chase of a 3 min pulse since incorporation into proteins continues for a few minutes even after addition of cycloheximide (compare lane 9 with lane 12). Yet, the high mol. wt proteins observed after chase of a 3 min pulse

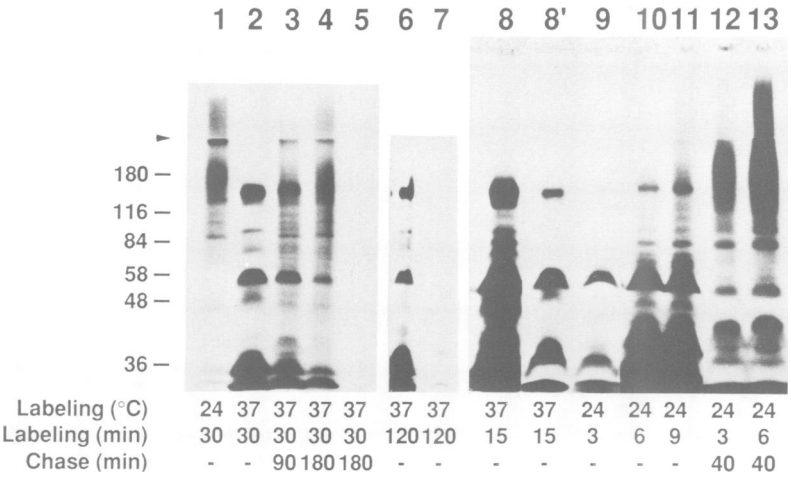

Fig. 3. *sec18* cells were labeled with [^{3}H]myoinositol (15 μCi/tube) at 24 or 37°C and for various periods as indicated at the bottom of the figure. Subsequently some tubes were further incubated at 24°C under chase conditions to allow for recovery from the secretion block and further maturation of labeled proteins. In tubes 3 and 4, chase was initiated by adding 10 volumes of medium containing cold myoinositol (200 μM) whereas in tube 5, cold myoinositol was present from the beginning of the labeling period. In **lane 7**, cycloheximide (100 μg/ml final) was added from a concentrated stock 5 min before the addition of [^{3}H]myoinositol. In **lanes 12** and **13** chase was initiated by 10-fold dilution with medium containing 100 μg/ml of cycloheximide. Cells were broken by procedure F and processed for SDS–PAGE/fluorography. **Lane 8'** is a shorter exposure of **lane 8** (2 versus 10 days).

(lane 12) cannot have been labeled during the few minutes required to stop [^{3}H]myoinositol incorporation into proteins, since even after a long pulse of 9 min (lane 11) there were only small amounts of labeled high mol. wt proteins. We interpret these data to suggest strongly that the high mol. wt glycoproteins are largely derived from lower mol. wt precursors which become labeled in the ER. This implies that the proteins which we observe when labeling *sec18* at 37°C do not represent proteins which would not normally become labeled but become labeled due to the artificially prolonged exposure to phospholipid-attaching enzymes. Rather these proteins represent the accumulation of normal biosynthetic intermediates. These findings also indicate that the [^{3}H]myoinositol-labeled proteins are ectoproteins which travel from the ER to the Golgi by the same secretory vesicles as the other secretory and plasma-membrane glycoproteins (Holcomb *et al.*, 1988). The fact that endoglycosidase H treatment of mature and ER forms of these proteins did not generate the same profile is probably due to further post-translational modifications of the mature forms such as elongation of *O*-glycosides and proteolytic processing.

All of the [^{3}H]myoinositol-labeled proteins were completely sedimented with the cell membranes when ultracentrifuged in 0.1 M Na_2CO_3 at pH 10.5.

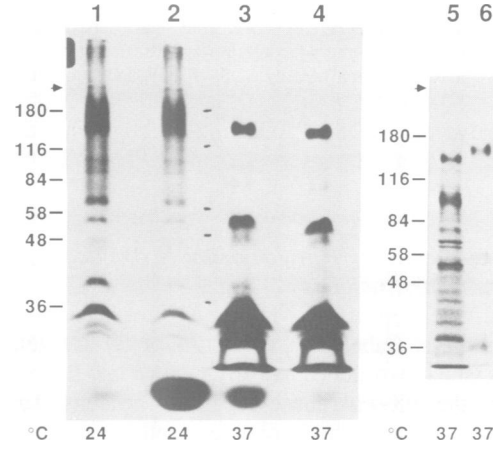

Fig. 4. *sec18* cells were pre-incubated for 15 min and labeled with either [^{3}H]myoinositol (5 OD_{600} units and 50 μCi/tube, **lanes 1,4**) or [^{3}H]palmitic acid (5 OD_{600} units and 300 μCi/tube, **lanes 2,3**) for 2 h at 24°C (**lanes 1,2**) or 37°C (**lanes 3,4**). Cells were broken in SDS (procedure C) and the extracts incubated with Con A–Sepharose. The bound material was eluted by boiling in sample buffer and analysed by SDS–PAGE and fluorography. **Lane 5** contains glycoproteins prepared in the same way from *sec18* cells labeled for 1 h at 37°C with $^{35}SO_4$. **Lane 6** contains the same material as **lane 4**.

Labeling of phospholipid moieties with fatty acids

If [^{3}H]myoinositol is incorporated into proteins as part of an inositol-containing phospholipid the same proteins should also be labeled by radioactive fatty acids. That this is indeed the case is shown in Figure 4. In fact, there are virtually no differences in the profiles of glycoproteins labeled with either [^{3}H]myoinositol or [^{3}H]palmitic acid (Figure 4, lanes 1–4). When labeling at 37°C, both labels were found in major bands of 140, 54, 22 and 17 kd. The $^{35}SO_4$-labeled proteins prepared under the same conditions showed a completely different profile (Figure 4, lane 5 versus 6). This result strongly suggests that glycoprotein-associated [^{3}H]palmitic acid and [^{3}H]myoinositol are part of the same post-translational modification. This is confirmed by the finding that the label of most of these proteins is completely removed by PI-PLC from either denatured (Figure 5, lanes 1–6) or native (Figure 5, lanes 7–10) proteins. This disappearance of label cannot be due to protein degradation by a protease contaminating the PLC preparation since no degradation of proteins was observed previously using the same enzymes on $^{35}SO_4$-labeled yeast proteins and mammalian proteins (Conzelmann *et al.*, 1986, 1987, 1988a), nor in the experiment shown in Figure 8. In the experiment shown in Figure 4, almost no [^{3}H]myoinositol- or [^{3}H]palmitic acid-labeled proteins were detected in the fraction of proteins which did not bind to Con A–Sepharose

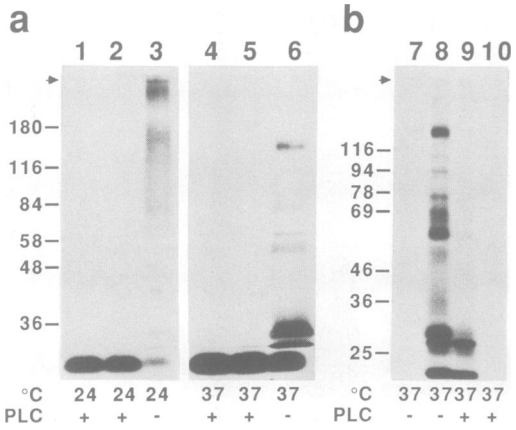

Fig. 5. Panel a: *sec18* cells were pre-incubated for 30 min and labeled with [^{3}H]palmitic acid at 24 or 37°C for 2 h, lysed in SDS (procedure C) and proteins were adsorbed onto Con A–Sepharose. Bound material was eluted with α-methylmannoside (0.5 M in 5 mM EDTA, 0.1% TX-100, 10 mM glycine, pH 2.0). The eluted material was neutralized and treated with PLC from *B.cereus* (**lanes 1,4**) or glycophosphatidylinositol-specific phospholipase C from *T.brucei* (**lanes, 2,5**) or incubated without enzyme (**lanes 3,6**). **Panel b**: *pep4 sec18* cells were pre-incubated for 5 min and labeled for 2 h at 37°C with [^{3}H]palmitic acid. Broken cells were extracted with TX-114 (procedure A) and membrane proteins were incubated with (+) or without (−) PLC from *B.cereus*. Then, after phase separation, proteins partitioning into the aqueous (**lanes 7,10**) or the detergent (**lanes 8,9**) phase were analysed by SDS–PAGE and fluorography.

when analysed by SDS–PAGE and fluorography (data not shown).

Protein-associated [^{3}H]myoinositol is exclusively found in phospholipid moieties

Protease digestion of the [^{3}H]myoinositol-labeled proteins from *sec* cells (labeled at either 24 or 37°C) generated peptides which partitioned to a large extent into the detergent phase of the TX-114 phase separation system (Table I). Treatment of these hydrophobic peptides with PI-PLC resulted in the release of most of the label from the detergent into the aqueous phase. These results strongly suggest that most if not all of the protein-associated myoinositol is part of a phospholipid moiety. After strong acid hydrolysis of labeled peptides, all the radioactivity co-migrated with myoinositol by paper chromatography (not shown).

Phospholipid moieties of S.cerevisiae are nitrous acid sensitive

The presence of the CRD carbohydrate antigen on the 125 kd glycoprotein of *S.cerevisiae* as well as the evolutionary conservation of the core structure of the GPI anchor (Homans *et al.*, 1988) raises the possibility that yeast phospholipid moieties are structurally similar to the GPI anchors of trypanosomes and mammals. A characteristic feature of these GPI anchors is the non-acetylated glucosamine residue glycosidically linked to the myoinositol of the PI. This glycosidic bond is selectively cleaved by mild nitrous deamination (Ferguson *et al.*, 1985), a reaction which liberates PI. As shown in Figure 6, nitrous acid treatment of [^{3}H]palmitic acid-labeled peptides from yeast glycoproteins released a labeled lipid which migrated close to the PI standard. Although we do not know the exact structure

Table I. Sensitivity of [^{3}H]myoinositol-labeled peptides to PI-PLC

Experiment	Cells	Labeling (°C)	Enzyme	% of counts in aqueous phase	
				+ enzyme	− enzyme
1	*sec17*	37	*B.cereus*	93	3.5
				100	6.2
2	*sec18*	24	*B.cereus*	91	6.1
			B.thuringiensis	94	
		37	*B.cereus*	98	5.6
			B.thuringiensis	95	

Cells were labeled with [^{3}H]myoinositol at 24 or 37°C, glycoproteins were separated by SDS–PAGE and molecules of 60–110 kd (experiment 1) or 37–300 kd (experiment 2) were electroeluted and digested with proteinase K. The resulting peptides were treated with PLC from *B.cereus* or PI-PLC from *B.thuringiensis* (+ enzyme) or incubated without enzyme (− enzyme) and subsequently partitioned in TX-114. Counts in the detergent and aqueous phases respectively were determined by scintillation counting.

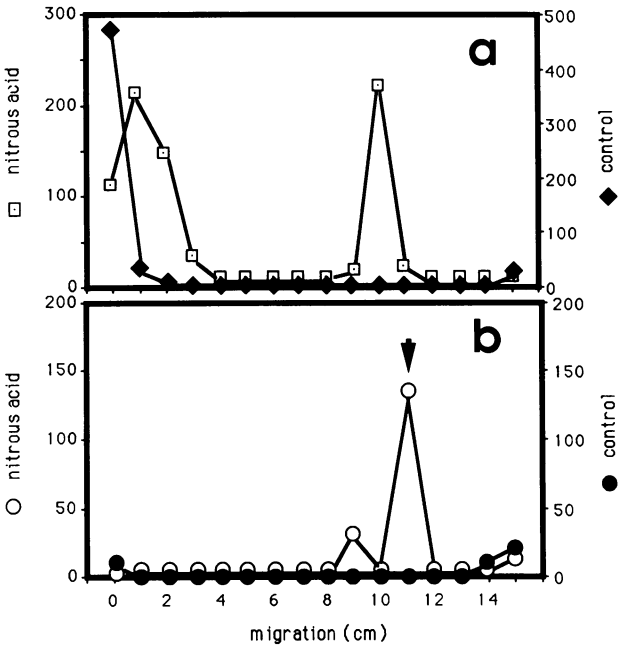

Fig. 6. Panel a: *sec18* cells were labeled with [^{3}H]palmitic acid at 24°C, broken (procedure G) and extensively delipidated with chloroform/methanol/water (10:10:3). After solubilization in SDS the proteins were adsorbed onto Con A–Sepharose and bound glycoproteins were separated in preparative SDS–PAGE. The gel was cut into slices which were treated with pronase and the eluted [^{3}H]palmitic acid-labeled peptides were pooled and incubated with (open symbols) or without (closed symbols) nitrous acid in the presence of 0.1% TX-100 as described (Ferguson *et al.*, 1988). Lipids were extracted with butanol, separated by TLC and segments of 1 cm were scraped off the plate for the detection of radioactivity as described (Krakow *et al.*, 1986). **Panel b**: 400 c.p.m. of [^{3}H]myristic acid-labeled p63 protease (a well characterized GPI-anchored surface protease from *Leishmania major*) was similarly treated as a control. The radiolabel of this protease partitioned into the butanol phase only after treatment with HNO_2 (36% of total) while most of the label of the yeast peptides partitioned into the butanol phase even without treatment. The position of PI from *S.cerevisiae* is indicated by a vertical arrow. Ordinate indicates c.p.m. per cm.

of the liberated lipid, the result suggests that the yeast phospholipid moieties under study contain a non-acetylated glucosamine residue as do mammalian and protozoan GPI anchors.

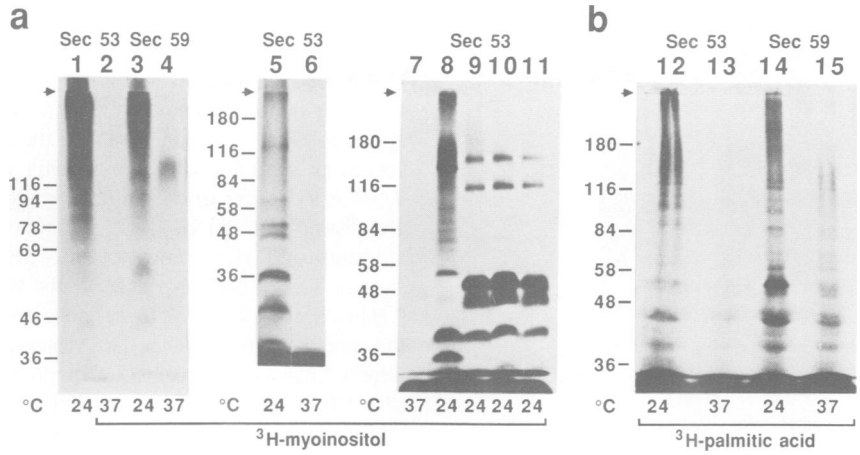

Fig. 7. Panel a: **lanes 1–4**: *sec53* cells and *sec59* cells were pre-incubated for 15 min and then labeled with [^{3}H]myoinositol at 24 or 37°C. Cells were broken and membranes were prepared by ultracentrifugation at pH 10.5 (procedure D). Washed membranes were processed for SDS–PAGE and fluorography. **Lanes 5,6**: 2 OD_{600} units of *sec53* cells were labeled for 15 min only, broken with glass beads (procedure B) and processed for SDS–PAGE. **Lanes 7–11**: *sec53* cells were labeled with [^{3}H]myoinositol in the presence of tunicamycin at final concentrations of 0 μg/ml (**lanes 7,8**), 3 μg/ml (**lane 9**), 10 μg/ml (**lane 10**), 30 μg/ml (**lane 11**). Tunicamycin was added 30 min before the [^{3}H]myoinositol. Cells were broken in SDS (procedure C) and processed for SDS–PAGE. **Panel b**: *sec53* and *sec59* cells were pre-incubated for 30 min and labeled with [^{3}H]palmitic acid for 2 h at 24 or 37°C, broken and membranes were prepared (procedure E). The membrane proteins were delipidated and analysed by SDS–PAGE.

sec53 and sec59 cells do not add phospholipid moieties at the restrictive temperature

Biochemical analysis of the [^{3}H]myoinositol-labeled phospholipid moieties strongly suggests the presence of several mannose residues (unpublished). If these mannoses form a link between protein and the phospholipid as they do in classical GPI anchors from other organisms, then a deficiency affecting mannose incorporation into the phospholipid moiety would block the attachment of [^{3}H]myoinositol to proteins. We therefore tested the addition of phospholipid moieties in *sec53-6*, a mutant which is deficient in the biosynthesis of GDP-mannose due to a mutation in phosphomannomutase which renders this enzyme inactive at 37°C (Kepes and Schekman, 1988a). This mutant was isolated as an ER-fragmenting secretion mutant which becomes dense at the restrictive temperature (37°C) (Ferro-Novick *et al.*, 1984a,b). *sec53* is allelic to *alg4* mutants which were obtained independently by selecting for a deficiency in the incorporation of [^{3}H]mannose into cells (Huffaker and Robbins, 1983). The mutants are therefore deficient in the biosynthesis of *N*- and *O*-linked oligosaccharides. *sec53* cells did not incorporate any [^{3}H]myoinositol into proteins at the restrictive temperature although the incorporation into lipids was not affected by the temperature (Figure 7a, lanes 1 and 2). *sec59* cells showed a defect similar to the one of *sec53* although the defect seemed to be less tight than the one of *sec53* (Figure 7a, lanes 3 and 4). This result is entirely compatible with the view that mannoses are necessary for the attachment of phosphoinositide moieties to proteins. However, since the defect of *sec53* abolishes *N*- and *O*-glycosylation we considered the possibility that the failure to detect [^{3}H]myoinositol-labeled proteins at 37°C in *sec53* was due to proteolytic degradation of unglycosylated but phospholipid-decorated proteins during the 2 h labeling procedure. However, this possibility seems unlikely since no incorporation of [^{3}H]myoinositol into proteins was seen even during a short labeling at 37°C (Figure 7a, lanes 5 and 6) whereas the incorporation into lipids was again the same at both temperatures. Tunicamycin, a drug which interferes with the addition of *N*-glycosides, did not interfere with the incorporation of [^{3}H]myoinositol into proteins (Figure 7a, lanes 7–11). Although tunicamycin does not mimic the glycosylation defect of *sec53* completely, inasmuch as it does not interefere with *O*-glycosylation, this experiment also presents evidence against proteolytic degradation as the primary cause of the absence in *sec53* of [^{3}H]myoinositol incorporation into proteins at 37°C.

When labeling *sec53* and *sec59* cells with [^{3}H]palmitic acid we again observed a dramatic reduction of incorporation into proteins when the labeling was done at 37°C (Figure 7b, lanes 12–15). The weak incorporation of [^{3}H]palmitic acid at 37°C may be due to labeling of membrane-associated acylated proteins since we did not want to purify glycoproteins by Con A–Sepharose in this experiment. Alternatively this residual incorporation might reflect a slow turnover of acyl chains on the phosphoinositide moieties of the membrane glycoproteins.

The membrane proteins of the TX-114 detergent phase of $^{35}SO_4$-labeled *sec53* cells did not contain PI-PLC-sensitive proteins if the labeling was performed at the restrictive temperature (Figure 8, lanes 5–8) whereas a large 110–118 kd protein was released by PLC from the TX-114 detergent phase extracted from cells labeled at 24°C (Figure 8, lanes 1–4). It is likely that this protein represents a slightly underglycosylated form of the corresponding PLC-treated 121 kd protein observed in wild-type cells (Conzelmann *et al.*, 1988a). The absence of a PLC-sensitive 121 kd protein is expected if phospholipid anchors are not added to precursor proteins but might also be due to proteolytic degradation of the non-glycosylated protein precursor which is expected to be generated at 37°C.

sec53 cells were used to see whether myoinositol-containing phospholipids remained available for incorporation into glycoproteins for some time after their biosynthesis as shown in Figure 9. Myoinositol is rapidly incorporated into phospholipids (Figure 1b) even in *sec53* (not shown). Yet, after 90 min of incubation of *sec53* cells at 37°C with

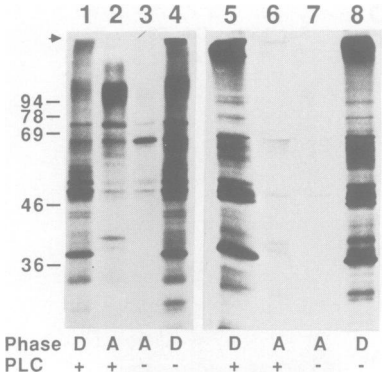

Fig. 8. *sec53* cells were pre-incubated for 10 min, labeled with $^{35}SO_4$ at 24°C (**lanes 1–4**) or 37°C (**lanes 5–8**) for 30 min and chased for 20 min. Membrane proteins were solubilized by TX-114 (procedure A) and incubated with (+) or without (−) PI-PLC from *B.cereus*. After treatment, proteins were partitioned by TX-114 phase separation and the aqueous (A) and 1/6 of the detergent (D) phases were analysed by SDS–PAGE followed by fluorography.

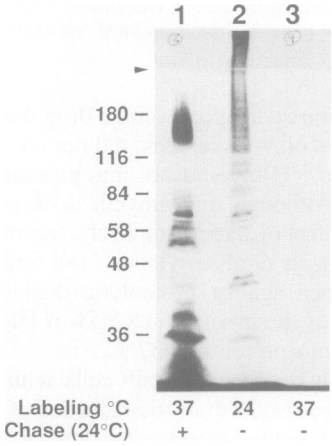

Fig. 9. Two OD units of *sec53* cells per tube were pre-incubated and then labeled with 30 μCi of [^{3}H]myoinositol for 90 min at 24 or 37°C. One tube (**lane 1**) was further incubated after addition of cold myoinositol (10 mM final) at 24°C for 90 min. Cells were lysed (procedure C) and proteins analysed by SDS–PAGE/fluorography.

[^{3}H]myoinositol, the label could still be incorporated efficiently into proteins upon return to 24°C (Figure 9, lane 1). This further demonstrates that myoinositol is not directly attached to proteins but transferred from a lipid intermediate. The fact that less high mol. wt material was made during this chase (lane 1) than in a continuous labeling at 24°C (lane 2) might be due to the slow reactivation of the secretory pathway and a consequently delayed maturation of *N*-glycans on the [^{3}H]myoinositol-labeled proteins.

Other glycosylation and secretion mutants add phospholipid moieties normally

alg2 and *alg3* cells have defects in the biosynthesis of the dolichol-linked oligosaccharide precursor of *N*-glycosides (Huffaker and Robbins, 1983). Although the exact biochemical defect in these mutants is not established, it is likely that they affect mannosyltransferases operative at the cytoplasmic (*alg2*) or luminal (*alg3*) side of the ER (Kukuruzinska *et al.*, 1987). Both of these mutants incorporated normal amounts of [^{3}H]myoinositol both at 24°C and 37°C. This indicates that the *ALG2* and *ALG3* gene products are not required for the biosynthesis of the core of the yeast phospholipid moiety.

We also analysed the secretion mutants in which the vesicular traffic is blocked either at the exit from the Golgi apparatus (*sec7* and *sec14*) or at the stage of the fusion of secretory vesicles with the plasma membrane (*sec1,2,3, 4,5,6,8,9,10,15*) or at multiple stages (*sec19*) (10,32,33) and found that all of these mutants incorporated normal amounts of [^{3}H]myoinositol into glycoproteins (data not shown). The profile of proteins that were labeled by [^{3}H]myoinositol at 37°C in these mutants was similar to the one of wild-type cells. Moreover, *sec11*, a mutant which fails to remove signal peptides at 37°C (Böhni *et al.*, 1988) was found to incorporate normal amounts of [^{3}H]myoinositol into proteins at 37°C but did not show the pattern of distinct bands typical of mutants blocked between the ER and the Golgi (Figure 1a).

Discussion

Most if not all protein-linked myoinositol of *S.cerevisiae* is part of a covalently attached phospholipid. The evidence for this statement is based on the fact that the profiles of [^{3}H]myoinositol-labeled and [^{3}H]palmitic acid-labeled proteins are virtually identical in this organism and that most labeled peptides generated from [^{3}H]myoinositol-labeled proteins contain a hydrophobic component which can be released by PI-PLC. The concerted disappearance of myoinositol and palmitic acid incorporation into proteins in *sec53* corroborates the notion that all [^{3}H]myoinositol is incorporated into a similar phospholipid moiety on different proteins. Using [^{3}H]myoinositol incorporation as the criterion, we find that *S.cerevisiae* contains at least four major and many minor glycoproteins with covalently attached phosphoinositides. In our hands the profile of [^{3}H]myoinositol-labeled proteins is most reproducible if labeled cells are boiled rapidly in SDS at the end of the labeling (procedure C, Figure 4) or are dissolved in NaOH (procedure F, Figure 3). Thus, [^{3}H]myoinositol labeling allowed the identification of many more phospholipid-containing membrane proteins than could be detected by screening for $^{35}SO_4$-labeled proteins that lost their detergent-binding moiety upon PI-PLC treatment (Conzelmann *et al.*, 1988a). In these previous experiments only a major 121 kd band and minor bands at 105, 81 and 30 kd were observed to be released from the TX-114 detergent phase into the aqueous phase by PI-PLC treatment, whereas here we find large amounts of [^{3}H]myoinositol-labeled material with mol. wts >125 kd (Figures 1a, 4). Why was this material not released from the TX-114 to the aqueous phase by PI-PLC in these earlier experiments? It cannot be argued that the phosphoinositide moieties of these proteins are PI-PLC resistant since PI-PLC does remove the [^{3}H]palmitic acid label from these proteins (Figure 5). It seems more likely that these proteins have other detergent-binding domains in addition to the phospholipid moiety (e.g. stretches of hydrophobic amino acids) or that they are complexed to other proteins which bind detergent via classical stop–transfer sequences. Some of them might not be extracted by or not be stable in TX-114 (extraction procedure A) which was used in these earlier experiments. Interestingly, immunoprecipitation of the 125 kd protein

from [^{3}H]myoinositol-labeled cells repeatedly indicated that this protein does not belong to the major [^{3}H]myoinositol-labeled proteins (data not shown).

The phosphoinositide moieties of [^{3}H]myoinositol-labeled proteins bear a resemblance to GPI anchors of trypanosomes and mammalian organisms not only because of their composition and the sensitivity to PI-PLC and nitrous acid but also since (i) they are found exclusively on membrane glycoproteins which travel by the regular secretory pathway from the ER to the Golgi (Duszenko *et al.*, 1988), (ii) the addition of phosphoinositide moieties can occur rapidly after completion of translation in the ER and (iii) addition is not abolished by tunicamycin (Bangs *et al.*, 1985; Ferguson *et al.*, 1986). However, more work is required to allow comparison at the structural level of these phosphoinositide moieties with GPI anchors of other organisms and to identify the site(s) of attachment of these moieties on the proteins.

Incorporation of [^{3}H]palmitic acid into yeast proteins has been described before. Wen and Schlesinger (1984) observed [^{3}H]palmitic acid-labeled proteins of 120, 50, 30 and 20 kd in *sec18*, but not in *sec53* or wild-type cells when labeling at 37°C. They characterized these proteins as endoglycosidase H-sensitive glycoproteins which could be sedimented with a membrane fraction. They also found that tunicamycin does not block the incorporation of [^{3}H]palmitic acid into these proteins and that the label could be removed by mild alkaline methanolysis but was resistant to boiling in 5% 2-mercaptoethanol. It is thus very likely that these proteins correspond to the proteins of 140, 54, 22 and 17 kd observed by us (Figure 4) which have the same properties, the difference in mol. wts being attributable to the use of polyacrylamide gels of different concentrations (15% versus 10%) and of different mol. wt standards. Although the existence of GPI anchors was not firmly established at the time of their investigation, Wen and Schlesinger did consider the possibility that these proteins contain covalently bound phospholipid. However, finally they did not opt for this idea and assumed that the acyl chains were directly linked to the proteins via an ester linkage since they were unable to label these proteins with $^{32}PO_4$ or [^{3}H]glycerol. After our studies however we are convinced that these [^{3}H]palmitic acid-labeled proteins of *sec18* have phosphoinositide moieties since we could remove the label from the proteins by treatment with PI-PLC (Figure 5). Moreover, all the labeled proteins were also labeled by [^{3}H]myoinositol (Figure 4). Therefore we would like to conclude that most of [^{3}H]palmitic acid incorporated into glycoproteins in *S.cerevisiae* at any temperature is associated with phosphoinositide moieties. It seems likely to us that the accumulation of 120, 50, 30 and 20 kd proteins in *sec18* cells described by Wen and Schlesinger represents the accumulation of biosynthetic intermediates for the mol. wt material which we observe at 24°C. The reason why these authors could not find [^{3}H]palmitic acid-labeled proteins in wild-type cells as we do might be partly explained by the fact that they used 15% polyacrylamide gels which most likely did not let all of this material enter the gel. However, it is conceivable that other factors such as the use of different labeling medium also played a role. While biosynthetic studies by Schlesinger's group in mammalian cells had suggested that direct acylation of membrane glycoproteins occurred only upon entry of these proteins into the *cis*-Golgi (Schmidt and Schlesinger, 1980), the findings of Wen and Schlesinger in yeast were taken as evidence for the ER localization of the corresponding acyltransferase (Rose and Doms, 1988). This argument clearly has to be revised.

Direct acylation of proteins such as ras1 and ras2 and ypt1 by [^{3}H]palmitic acid has also been demonstrated in *S. cerevisiae* (Tamanoi *et al.*, 1984; Fujiyama and Tamanoi, 1986; Deschenes and Broach, 1987; Molenaar *et al.*, 1988). However since these proteins cannot be detected unless overproduced, they probably only acount for a relatively small proportion of [^{3}H]palmitic acid incorporation into proteins. This would explain why we found almost no incorporation of [^{3}H]palmitic acid into proteins other than the ones which have phospholipid moieties.

sec53 and *sec59* were the only mutants that did not incorporate [^{3}H]myoinositol and [^{3}H]palmitic acid at the restrictive temperature. In principle, this finding might be explained in various ways. (i) The [^{3}H]myoinositol-labeled proteins might be proteolytically degraded in *sec53* because of their deficiency in *N*- and *O*-linked glycans. However, it appears unlikely to us that the non-glycosylated forms of the glycoproteins which normally incorporate [^{3}H]myoinositol would all be unstable during a short labeling of 15 min (Figure 7) whereas they are stable for 2 h when labeled in the presence of tunicamycin. (ii) The absence of [^{3}H]myoinositol-labeled proteins in *sec53* might be due to the failure of this mutant to transport the precursor proteins to the intracellular compartment in which phosphoinositide moieties are added. However, this explanation is highly unlikely since the phosphoinositide moieties are added to proteins in all other secretion mutants in which the proteins do not get out of the ER. (iii) There might be enzymes required for the phospholipid moiety biosynthesis which are not stable or enzymatically inactive when not properly glycosylated. However, the fully glycosylated forms of such enzymes synthesized during the preculture at 24°C should be sufficient to support biosynthesis of phospholipid moieties for as short a period as 15 min. It was reported that *sec53* cells incorporate [^{3}H]palmitic acid into ras1 and ras2 proteins normally (Fujiyama and Tamanoi, 1986) indicating that their defect is restricted to the incorporation of [^{3}H]palmitic acid in the context of phospholipid moieties. (iv) The only good explanation that we can think of for the deficiency in [^{3}H]myoinositol incorporation of *sec53* is that GDP-mannose is required for the biosynthesis of phosphoinositide moieties. This interpretation is likely since mannose is present in the [^{3}H]myoinositol-labeled phosphoinositide moieties of *S.cerevisiae* (unpublished) as well as the core glycan of both mammalian and protozoan GPI anchors (Ferguson *et al.*, 1988; Homans *et al.*, 1988).

The phenotype of *sec59* is similar to that of *sec53* in that it also has a severe glycosylation defect and recent evidence suggests that it might affect mannose transfer to dolichol-linked oligosaccharides (Kepes and Schekman, 1988b). We could not restore growth of this mutant at 37°C by adding mannose to the culture medium. *sec59* might also be deficient in the incorporation of [^{3}H]myoinositol because of the inability to add mannose to the phosphoinositide moieties.

Our results raise the formal possibility that the secretion block of *sec53* is a consequence of the failure to add phosphoinositide moieties to proteins. However, this possibility seems not to be supported by the finding that all 23 known secretion mutants isolated so far which do not affect glycosylation (class A mutants) do incorporate normal

amounts of [^{3}H]myoinositol into proteins, since it might be expected that mutants deficient in the biosynthesis of phosphoinositide moieties would have been picked up as secretion mutants, if their biosynthesis were essential for secretion. Additionally, six mammalian mutants deficient in GPI-anchor biosynthesis are not deficient in the export of plasma membrane proteins (Fatemi and Tartakoff, 1986, 1988; Conzelmann *et al.*, 1986, 1988b; Trowbridge *et al.*, 1978a,b). The mere elimination of *N*-glycosylation by tunicamycin does not interfere with secretion in *S.cerevisiae* (Ferro-Novick *et al.*, 1984a) and the importance of *O*-glycosylation for secretion is not known. [Interference with the addition of *O*-glycosides in mammalian cells results in the loss of certain surface glycoproteins due to proteolytic cleavage or to missorting but not to an immediate block of the secretory pathway as is observed in *sec53* (Kingsley *et al.*, 1986; Kozarsky *et al.*, 1988a,b; Reddy *et al.*, 1989).] It seems most likely that the secretion block of *sec53* is due either to a failure in *O*-glycosylation or to the combined effect of deficiencies in several GDP-mannose requiring processes which may include the addition of phosphoinositide moieties.

Materials and methods

Strains, growth conditions and materials

Haploid *S.cerevisiae* strains were used: 143-5C-1, α *sec18-1 pep4-3 ura3 his4* and 82-2, a *pep4-3 bar1-1* were kindly provided by Howard Riezman (Biozentrum, University of Basel, Switzerland) who also provided us with the following glycosylation (*alg*) and secretion (*sec*) mutants which he had obtained from Tim Huffaker and Randy Schekman (Department of Biochemistry, University of California, Berkeley, CA 94720) respectively; DBY1790, a *alg2-1 ade2-101 ura3-52*; DBY1791, α *alg3-1 ade2-101 ura3*; HMSF1, a *sel-1*; HMSF106, a *sec2-56*; HMSF68, α *sec3-2*; HMSF13, a *sec4-2*; HMSF134, a *sec5-24*; HMSF136, a *sec6-4*; SF294-2B, a *sec7-1*; HSMF95, α *sec8-6*; HMSF143, a *sec9-4*; SF239-2C, a *sec10-2*; HMSF154, a *sec11-7*; SF226-1C, a *sec12-4*; HSMF163, a *sec13-1*; HMSF169, a *sec14-3*; HMSF171, a *sec15-1*; HMSF174, a *sec16-2*; HMSF175, a *sec17-1*; HMSF176, a *sec18-1*; HMSF178, a *sec19-1*; HMSF179, a *sec20-1*; HMSF180, a *sec21-1*; HMSF183, a *sec22-3*; HMSF190, a *sec23-1*; HMSF331, a *sec53-6*; SF402-4D, a *sec59-1*; and the corresponding wild-type strains X2180-1A, a *SUC2 mal gal2 CUP1* and X2180-1B, α *SUC2 mal gal2 CUP1*. Cells were kept on YPD plates containing 1% Bacto yeast extract (Difco, Detroit, MI), 2% casein hydrolysate (peptone 140) (Gibco, Paisley, UK), 2% Bacto-Agar (Difco) and 2% glucose. To grow the cells we used Wickerham's minimal medium (Wickerham, 1946) with 2% glucose as a carbon source but omitting myoinositol. The optical density (OD) of dilute cell suspensions was measured in a 1 cm cuvette at 600 nm. 1 OD_{600} unit of cells corresponds to $1-2.5 \times 10^7$ cells depending on the strain.

Other reagents were obtained from the following sources: $^{35}SO_4$ (30 Ci/mg), myo-[2-^{3}H]inositol (14 Ci/mmol) and [9,10-^{3}H]palmitic acid (55 Ci/mmol) were from Amersham Corp. (Buckinghamshire, UK); EN^{3}HANCE and Aquasol were from New England Nuclear Corp.; TX-114 was from Serva (Heidelberg, FRG); pronase was from Calbiochem AG (Luzern, Switzerland); proteinase K, phenylmethylsulfonyl fluoride (PMSF) and benzamidine from Merck (FRG); endoglycosidase H from Genzyme (Boston, MA); Concanavalin A Sepharose 4B (Con A–Sepharose) from Pharmacia (Uppsala, Sweden); leupeptin, pepstatin, antipain and Triton X-100 (TX-100) from Fluka (Buchs, Switzerland); pre-distilled 6 N HCl from Pierce (BA OUD, Holland); phospholipase C (PLC) from *Bacillus cereus* (type III), Bis Tris Propane and a prestained mol. wt standard mixture for gel electrophoresis (SDS-7B) were from Sigma (St Louis, MO). Pure PI-PLC from *Bacillus thuringiensis* prepared by Sapporo Beer was obtained through Pharmacare (Lucerne, Switzerland). Highly purified phosphatidylinositolglycan-specific phospholipase C from *Trypanosoma brucei* was a generous gift from Roland Bülow and Peter Overath (Max Planck Institut für Biologie, Tübingen, FRG). [^{3}H]Myrisitic acid-labeled p63 protease from *Leishmania major* (Bouvier *et al.*, 1985) was kindly donated by Pascal Schneider (this institute).

Radiolabeling of cells

Cells were grown in minimal medium. Exponentially growing cells were centrifuged and resuspended in the same medium for labeling. Temperature-sensitive mutants were pre-incubated at the labeling temperature for 5–30 min before the addition of radiolabel. Labeling of cells with $^{35}SO_4$ and [^{3}H]myoinositol was done as described previously (Conzelmann *et al.*, 1988a) at a density of 10 OD_{600} units/ml except that here we diluted cells 4-fold with a minimal medium 40 min after the addition of [^{3}H]myoinositol. Labeling with [^{3}H]palmitic acid was done for 2 h using 40 μCi/ml and 1 OD_{600} unit of cells per ml. In all labelings we periodically checked the glucose levels in the medium using Clinistix (Miles SA, Lausanne, Switzerland). Labeled cells were processed using several different procedures (A–G).

Procedure A. Cells were broken up by vortexing with glass beads in minimal medium and were extracted with TX-114 as described (Conzelmann *et al.*, 1988a) except that cell debris was removed only after solubilization with TX-114.

Procedure B. Cells were broken in minimal medium as in procedure A but subsequently trichloroacetic acid (TCA) was added (10% final concentration) and the precipitate was delipidated by repeated extractions with chloroform/methanol/water (10:10:3).

Procedure C. This is a modification of a described procedure (Wen and Schlesinger, 1984): cells were centrifuged at 4°C to remove the labeling medium. Then we added Laemmli 'final sample buffer' (Laemmli, 1970) containing 2% SDDS and 5% 2-mercaptoethanol (150 μl per 10 OD_{600} units of cells) plus 10 mM PMSF (from a 200 mM stock in ethanol) and broke them by vortexing with glass beads (4 × 1 min).

Procedure D. Cells were broken as in procedure A, debris was removed by centrifugation (300 *g* for 5 min, 4°C) and the supernatant was ultracentrifuged (80 000 *g* for 60 min, 4°C) either at neutral pH or after addition of Na_2CO_3, pH 10.5.

Procedure E. This procedure was identical to D but cells were broken in 100 mM Tris–HCl, pH 8.3, 200 mM NaCl, 10 mM benzamidine, 5 mM $FeCl_3$ and 5 mM 2-mercaptoethanol.

Procedure F. Cells were lysed using NaOH, 2-mercaptoethanol and SDS as described (Dulic and Riezman, 1989), followed by neutralization and addition of sample buffer.

Procedure G. Cells were broken by vortexing with glass beads in chloroform/methanol/water (10:10:3).

Adsorption on Con A–Sepharose

For adsorption of native proteins onto Con A–Sepharose, the TX-114 extract from labeled cells (procedure A) was phase separated and the TX-114 detergent phase was cleared of water-soluble proteins by two more phase separations as described (Conzelmann *et al.*, 1986). The resulting detergent phase was diluted wth 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and protease inhibitors and was then batch adsorbed onto Con A–Sepharose for 2 h at 7°C or at room temperature. 100 μl of Con A–Sepharose were sufficient to completely adsorb the [^{3}H]myo-inositol-labeled glycoproteins from 10 OD_{600} units of cells. Beads were washed with the same buffer supplemented with TX-100 (0.1%) and bound proteins eluted either by boiling the beads in Laemmli 'final sample buffer' (Laemmli, 1970) or by incubating twice with α-methylmannoside (0.25 M final concentration) for 10 min at 37°C. For adsorption of denatured proteins onto Con A–Sepharose the lysate was prepared by procedure C and diluted with 20 vol of 50 mM Tris–HCl, pH 7.4, 1% TX-100, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM benzamidine. Then cell walls were removed by centrifugation for 5 min at 10 000 *g*.

Preparation and phospholipase C treatment of [^{3}H]myoinositol-labeled peptides

40 OD_{600} units of cells were labeled with 400 μCi of [^{3}H]myoinositol for 1 or 2 h and membranes were prepared by ultracentrifugation at pH 10.5 (procedure D). After extensive delipidation the glycoproteins were separated by preparative SDS–PAGE together with pre-colored standard proteins. The gel was cut into slices and regions containing radioactive bands were electroeluted as described (Bhown *et al.*, 1980). The eluted proteins were then treated with proteinase K (1 mg/ml) in 25 mM Tris–HCl, pH 8.0, 1 mM $CaCl_2$ for 4 h at 37°C. After boiling for 15 min and centrifugation at 1500 *g* for 10 min the peptides in the supernatant were used for various analytical procedures. For PLC treatments, the peptides were resuspended in 0.2% TX-100, 100 mM HEPES-KOH, pH 7.0, 5 mM EDTA and 1 mM dithiothreitol. Aliquots containing 500–1000 c.p.m. were incubated in a

final volume of 100 μl with or without 5 U of PLC from *B.cereus* or 0.05 U of PI-PLC from *B.thuringiensis* for 90 min at 30°C. After addition of 500 μl of 1% TX-114 in Tris–HCl buffer and phase separation, the counts in the aqueous and detergent phases were determined by liquid scintillation counting. The active principle in the PLC from *B.cereus* is a PI-PLC, whereas the phosphatidylcholine-specific PLC in this preparation is blocked by the presence of EDTA as discussed previously (Conzelmann *et al.*, 1987, 1988a).

Other methods

Treatment of proteins with endoglycosidase H and with PI-PLC as well as strong acid hydrolysis of [^{3}H]myoinositol-labeled peptides and analysis of the products by paper chromatography was done as described (Conzelmann *et al.*, 1988a). All proteins were boiled in Laemmli 'final sample buffer' containing 5% 2-mercaptoethanol before being separated by SDS–PAGE using polyacrylamide concentrations of 5% for stacking and 8–10% for running gels (Laemmli, 1970).

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