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Lipid remodeling leads to the introduction and exchange of defined ceramides on GPI proteins in the ER and Golgi of *Saccharomyces cerevisiae*

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Previous experiments with *Saccharomyces cerevisiae* had suggested that diacylglycerol-containing glycosylphosphatidylinositols (GPIs) are added to newly synthesized proteins in the endoplasmic reticulum (ER) and that ceramides subsequently are incorporated into GPI proteins by lipid remodeling. Here we prove this hypothesis by labeling yeast cells with [^{3}H]dihydrosphingosine ([^{3}H]DHS) and showing that this tracer is incorporated into many GPI proteins even when protein synthesis and, hence, anchor addition, is blocked by cycloheximide. [^{3}H]DHS incorporation is greatly enhanced if endogenous synthesis of DHS is inhibited by myriocin. Labeled GPI anchors contain three types of ceramides which, based on previous and present results, are identified as DHS-C26:0, phytosphingosine-C26:0 and phytosphingosine-C26:0-OH, the latter being found only on proteins which have reached the Golgi. Lipid remodeling can occur both in the ER and in a later secretory compartment. In addition, ceramide is incorporated into GPI proteins a long time after their initial synthesis by a process in which one ceramide gets replaced by another ceramide. Remodeling outside the ER requires vesicular flow from the ER to the Golgi, possibly to supply the remodeling enzymes with ceramides.

Keywords: ceramides/endoplasmic reticulum/glycosylphosphatidylinositol/Golgi/*Saccharomyces cerevisiae*

Introduction

The biosynthesis of glycosylphosphatidylinositol (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 and, in most organisms, the lipid moieties of GPI anchors do not reflect the lipid moieties present in the phosphatidylinositol (PI) which would appear as a natural starting point for the GPI biosynthesis in the endoplasmic reticulum (ER) (Thomas *et al.*, 1990; McConville and Ferguson, 1993). The situation in yeast is peculiar since two very different types of lipid moieties can be found: ceramide (Cer) and diacylglycerol (DAG). The Cers are found on the majority of yeast anchors; they consist mainly of C18:0 phytosphingosine (PHS) and a C26:0 fatty acid. Curiously, they are different from the main Cer found in the abundant inositolphosphoceramides (IPCs) (Smith and Lester, 1974; Conzelmann *et al.*, 1992; Lester and Dickson, 1993). On the other hand, Gas1p, a well characterized GPI protein of yeast, is made with a C26:0 fatty acid-containing mild base-sensitive lipid (Fankhauser *et al.*, 1993). In both types of lipid moieties, the C26:0 may be hydroxylated on C2.

Complete GPI precursor lipids (CPs) ready to be transferred to proteins are normally present only in very low amounts in wild-type cells, but can be detected in lipid extracts of [2-^{3}H]mannose-labeled *pmi40*, a strain with a conditional defect in mannose biosynthesis which incorporates exogenously added [^{3}H]mannose at 37°C with very high efficiency (Sipos *et al.*, 1994). CPs also become detectable in extracts from [^{3}H]inositol ([^{3}H]Ins)-labeled *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 Cer moieties have to be introduced at a later stage by lipid remodeling of protein anchors. However, it cannot be excluded that the CPs one observes in these mutants are not representative of the true precursor lipids but are further processed forms thereof, forms which arise because the mutation prevented their normal rapid utilization as anchoring devices. Thus, the data so far still cannot exclude models by which the Cer-anchored proteins would arise through the transfer of Cer-based CPs, although Cer-based CPs have never been observed. Previous attempts to show incorporation of palmitic acid into GPI proteins directly in the absence of protein synthesis have failed (our unpublished data). Here we unambiguously demonstrate the incorporation of [^{3}H]dihydrosphingosine ([^{3}H]DHS) into GPI anchors occurring in the absence of protein synthesis, and thereby firmly establish the remodeling of lipid moieties in GPI anchors. To avoid confusion with other types of lipid exchange, the terms remodeling/remodelase will be restricted herein to designate events/enzymes by which [^{3}H]DHS or a derivative thereof is introduced into GPI anchors.

Results

Yeast cells incorporate exogenously added [^{3}H]DHS into numerous proteins

Yeast cells were incubated for 2 h with [^{3}H]DHS and proteins were analyzed by SDS–PAGE followed by fluorography. As shown in Figure 1A, lane 1, a diffuse array of high molecular weight proteins was labeled. The labeled proteins will be shown below to be GPI proteins.

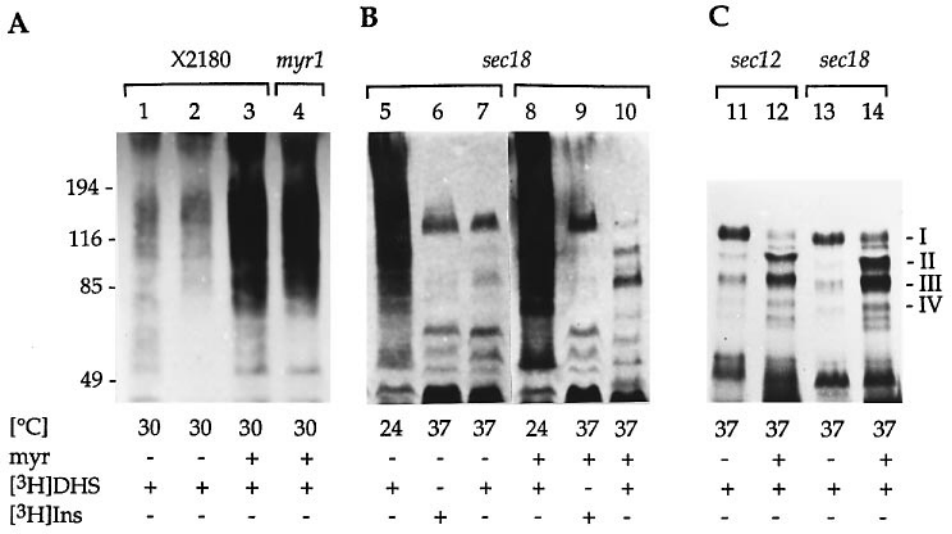


Fig. 1. Incorporation of [^{3}H]DHS into proteins in the presence or absence of myriocin. (**A**) X2180 (lanes 1–3) and *myr1* (lane 4) cells were radiolabeled at 30°C with 25 μCi of [^{3}H]DHS and proteins were analyzed by SDS–PAGE/fluorography. Cells were pre-incubated for 10 min and radiolabeled in the presence of 40 μg/ml of myriocin (myr, lanes 3 and 4) or without inhibitors (lanes 1 and 2). The overnight pre-culture of *myr1* also contained myriocin in order to deplete cells of endogenous sphingolipids. In lane 2, the [^{3}H]DHS was added in tergitol NP-40 (final concentration = 0.01%). (**B** and **C**) *sec12* and *sec18* cells were pre-incubated for 20 min and labeled at 24 or 37°C with [^{3}H]DHS or [^{3}H]Ins in the presence or absence of myriocin as indicated. Myriocin was always added at the beginning of the pre-incubation. [°C] = labeling temperature. I–IV indicate some major protein bands labeled at 37°C and having apparent mol. wts of 155, 135, 115 and 75 kDa.

Incorporation of [^{3}H]DHS into proteins was not improved by prior solubilization of [^{3}H]DHS in detergent (lane 2), but was enhanced significantly when the endogenous biosynthesis of DHS was blocked by myriocin, an inhibitor of the serine palmitoyl transferase (lane 3) (Miyake *et al.*, 1995). This was not unexpected since, by labeling with [^{3}H]Ins, Horvath *et al.* had shown previously that myriocin increases the relative abundance of non-remodeled GPI proteins and concomitantly decreases the amount of endogenous IPCs (Horvath *et al.*, 1994). Incorporation of [^{3}H]DHS was no better when *myr1*, a myriocin-resistant mutant was labeled in the presence of myriocin (lane 4) (Horvath *et al.*, 1994).

The SDS–PAGE profile of [^{3}H]DHS-labeled proteins was very reminiscent of that of GPI proteins labeled with [^{3}H]Ins, but the elongation of *N*-glycans in the Golgi renders most GPI proteins very heterogeneous and makes their identification by sizing on SDS–PAGE impossible. To compare the core-glycosylated ER forms of GPI proteins, we tried to label *sec12* and *sec18*, thermosensitive mutants in which secretory vesicles either cannot bud off the ER or cannot fuse with the subsequent *cis*-Golgi compartment (Novick *et al.*, 1980; Kaiser and Schekman, 1990). As shown in Figure 1B, the pattern of [^{3}H]Ins-labeled, core-glycosylated GPI proteins of *sec18* is very similar to the one of [^{3}H]DHS-labeled proteins (lanes 6 and 7). If the same experiment is carried out in the presence of myriocin, the pattern of [^{3}H]Ins-labeled proteins remains unchanged (lane 9) whereas [^{3}H]DHS is more strongly taken into protein bands labeled II and III and is incorporated less into protein bands I and IV (lanes 10, 12 and 14). The co-migration of the [^{3}H]DHS-labeled proteins with the core-glycosylated [^{3}H]Ins-labeled GPI proteins suggests that [^{3}H]DHS is labeling GPI proteins specifically and that most GPI proteins are modified by the addition of a Cer.

When added to cells, [^{3}H]DHS is taken up very rapidly into the cells (not shown), but the incorporation into proteins is a slower process. As seen in Figure 2, labeled proteins appear already after 5 min of pulse, and incorporation reaches a plateau at ~30 min (Figure 2B). The plateau might represent a state where the continuing incorporation of [^{3}H]DHS into newly synthesized proteins is counterbalanced by degradation of GPI proteins or by removal of their anchor components at the cell surface, a process which has been reported to be a prerequisite for the incorporation of GPI proteins into the cell wall (Lu *et al.*, 1994, 1995). Alternatively, the plateau might indicate that all of [^{3}H]DHS has been incorporated into proteins or lipids. To distinguish between these possibilities, we analyzed the lipid extracts after labeling for 2 h.

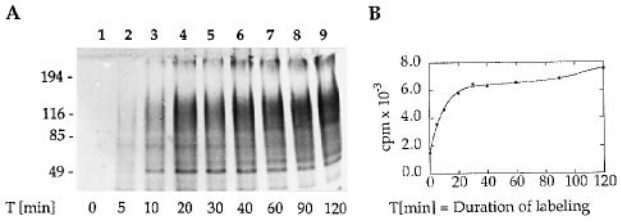


Fig. 2. Kinetics of the appearance of [^{3}H]DHS in proteins. (**A**) X2180 cells were radiolabeled at 30°C with [^{3}H]DHS for 0–120 min and proteins were visualized by SDS–PAGE/fluorography. T [min] = duration of labeling. (**B**) The amount of radiolabeled proteins at each time point shown in (A) was determined by cutting up each gel lane and measuring its radioactivity by scintillation counting.

Yeast cells incorporate exogenously added [^{3}H]DHS into all known sphingolipids

The most prominent sphingolipids of *Saccharomyces cerevisiae* are IPC/C and IPC/D (two forms of IPC differing slightly in their lipid moiety), mannosyl-IPC (MIPC) and inositolphospho-MIPC [M(IP)$_2$C] (Smith and Lester, 1974; Puoti *et al.*, 1991; Lester and Dickson, 1993). Lipid

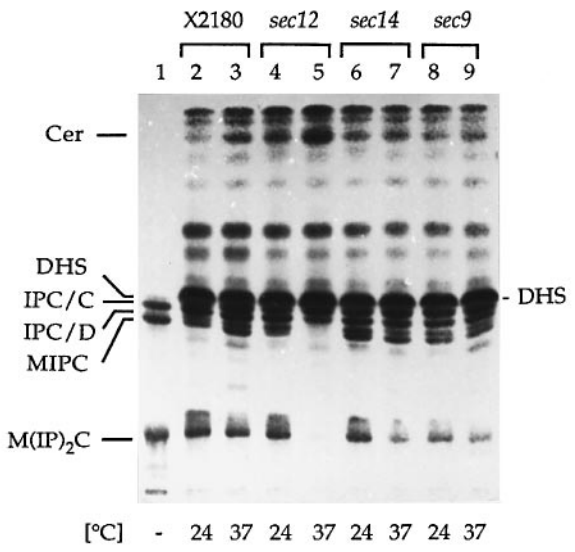


Fig. 3. [^{3}H]DHS becomes incorporated into all known sphingolipids. X2180, *sec12*, *sec14* and *sec9* cells were pre-incubated (20 min) and labeled (120 min) at 24 or 37°C with [^{3}H]DHS. The lipids were extracted and analyzed by TLC developed in solvent system 2, using as standards [^{3}H]DHS- and [^{3}H]Ins-radiolabeled sphingolipids (lane 1). The latter were obtained by mild base treatment of the lipid extract of [^{3}H]Ins-labeled X2180 cells. Mild base hydrolyzes DAG but not Cer, and was used to destroy glycerophospholipids selectively.

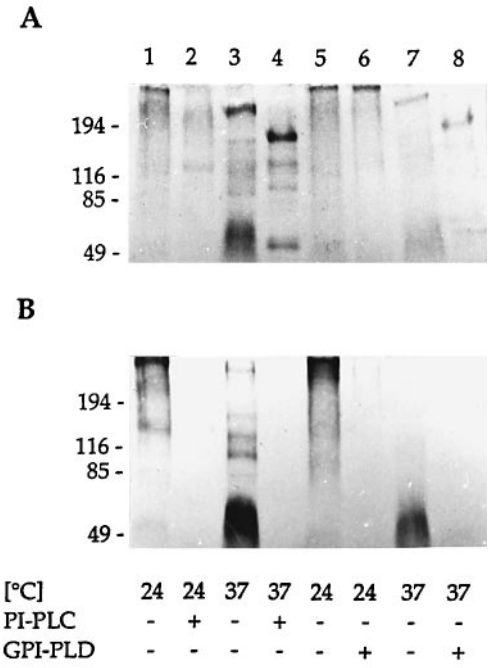


Fig. 4. Phospholipase treatments of [^{3}H]DHS-labeled proteins. *sec18* cells were pre-incubated for 20 min and radiolabeled with [^{3}H]Ins (**A**) or [^{3}H]DHS (**B**) at 24 or 37°C, as indicated. The protein extracts were treated with PI-PLC or GPI-PLD and subsequently analyzed by SDS–PAGE/fluorography.

extracts of cells labeled with [^{3}H]DHS for 2 h were analyzed by TLC and fluorography as shown in Figure 3. Cells incorporate [^{3}H]DHS into all major classes of sphingolipids, namely IPC/C and IPC/D, MIPC and $M(IP)_2C$ (Figure 3, lanes 2 and 3). When labeling secretion mutants blocking the budding of vesicles from the ER (*sec12*) (Kaiser and Schekman, 1990), from the Golgi (*sec14*) (Ohashi *et al.*, 1995) or the fusion of secretory vesicles with the plasma membrane (*sec9*) (Brennwald *et al.*, 1994), we find that no lipids co-migrating with IPC/D, MIPC and $M(IP)_2C$ are present in lipid extracts of *sec12* and *sec18* cells labeled at 37°C (Figure 3, lane 5; data not shown). This confirms and extends a previous study reporting that IPC/D, MIPC and $M(IP)_2C$ do not become labeled by [^{3}H]Ins if secretion mutants blocking the transport of secretory vesicles from the ER to the Golgi are labeled at the restrictive temperature (Puoti *et al.*, 1991). The rapidly migrating, very hydrophobic lipids in Figure 3 run in the region of animal Cer standards and will be shown below to be Cers.

Our data indicate that ~95% of [^{3}H]DHS added to cells get taken up rapidly into cells, and 75% of it gets incorporated into sphingolipids within 30 min (not shown). The quantitation of the results in Figure 3 shows that after a 2 h labeling, 12–19% of the label is still present as free [^{3}H]DHS. We thus calculate that each cell incorporated 1.7×10^{-17} mol of [^{3}H]DHS into sphingolipids during the 2 h labeling period. On the basis of literature data (Hunter and Rose, 1972; Patton and Lester, 1991; Sherman, 1991), we estimate that ~2.8×10^{-16} mol of sphingolipids have to be made per cell during 2 h in order to account for the increase in biomass during exponential growth in SDC medium at 30°C. Assuming a complete block of the endogenous DHS biosynthesis by myriocin, we thus estimate that under standard labeling conditions the added [^{3}H]DHS satisfies the cell's need for long chain bases for only ~6% of the labeling time. This favors the assumption that the plateau of protein labeling in Figure 2B is explained by the fact that all [^{3}H]DHS has been used up for biosynthesis of IPCs. The residual [^{3}H]DHS may be trapped in some unphysiological compartment, e.g. in the cell wall.

Proteins labeled by [^{3}H]DHS are GPI anchored

To confirm further the identity of the [^{3}H]DHS-labeled proteins, we treated them with highly purified phospholipases C and D (PLC and PLD), which remove the lipid moiety but not the Ins from GPI anchors. Indeed, as shown in Figure 4, PI-specific PLC (PI-PLC) and GPI-specific PLD (GPI-PLD) removed the label from all [^{3}H]DHS-labeled, but not from the [^{3}H]Ins-labeled proteins. This result is expected if [^{3}H]DHS selectively labels the Cer-containing GPI proteins. The apparent molecular mass changes of [^{3}H]Ins-labeled proteins caused by the PI-PLC and GPI-PLD treatments (Figure 4A, lanes 3, 4, 7 and 8) cannot be explained by the mere loss of a lipid moiety but rather may be due to the action of some protease or glycosidase which would act during incubation with phospholipases. [If present in the yeast extract, this activity would have to act only on free but not on micelle-bound GPI proteins, since no corresponding degradation was observed in control incubations (lanes 3 and 7).]

The form in which [^{3}H]DHS was incorporated into GPI anchors was studied through the preparation of labeled anchor peptides from the total mixture of cellular proteins and subsequent analysis of their lipid component. As shown in Figure 5, lanes 1 and 4, the purified anchor peptides did not migrate on TLC and hence were free of contaminating lipids. Strong acid hydrolysis of these

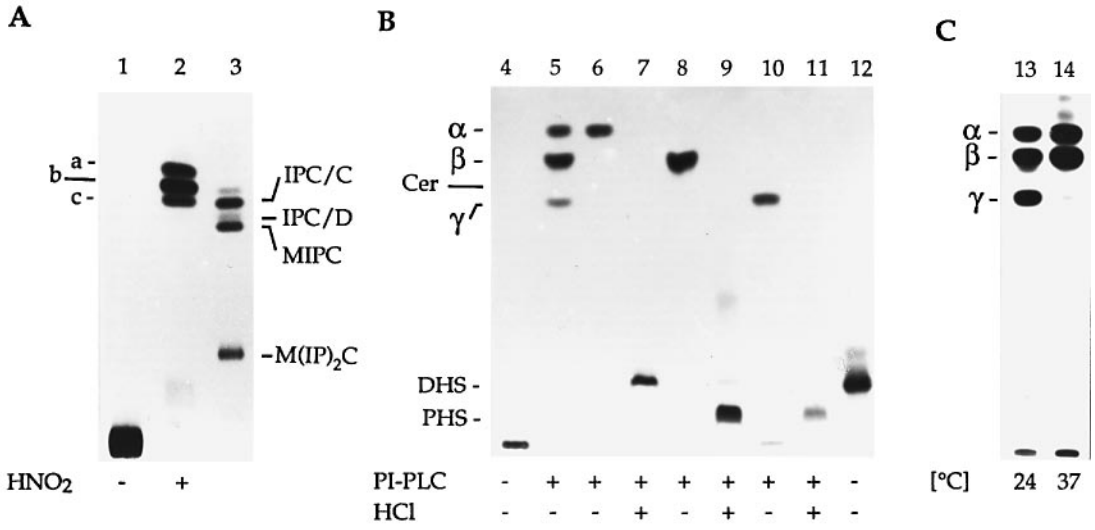


Fig. 5. [^{3}H]DHS-labeled anchor peptides contain three different lipid components. (**A** and **B**) Twenty OD_{600} of X2180 were radiolabeled with [^{3}H]DHS, and GPI peptides free of contaminating radiolabeled lipids were prepared. Aliquots were either control incubated (lane 1), treated with nitrous acid (lane 2) or with PI-PLC (lane 5) or left untreated (lane 4). Lipids α, β and γ visible in lane 5 were purified by two consecutive rounds of preparative TLC. One sample of each purified lipid was hydrolyzed by strong HCl hydrolysis. An untreated and a hydrolyzed sample of each of the three lipids α, β and γ are shown in lanes 6 and 7, 8 and 9 and 10 and 11, respectively. TLC was performed using solvent system 2 (A) or 1 (B). Standards are: PHS, bovine brain Cer, [^{3}H]DHS used for labeling (lane 12), [^{3}H]Ins-radiolabeled sphingolipids (lane 3). (**C**) *sec12* cells were radiolabeled at 24 and 37°C with [^{3}H]DHS in the presence of myriocin, the GPI peptides were purified and subjected to PI-PLC. The resulting Cers were analyzed on TLC using solvent system 1.

anchor peptides under conditions which hydrolyze the amide bond of the presumed Cers yielded two labeled products co-migrating in TLC with [^{3}H]DHS and PHS, respectively (not shown). Anchor peptides were subjected further to nitrous acid deamination, a procedure which selectively cleaves the bond between the non-acetylated glucosamine and Ins of GPIs (Ferguson *et al.*, 1988). Based on the known structure of the yeast GPI anchor (Fankhauser *et al.*, 1993), this treatment is expected to yield a labeled IPC. Nitrous acid treatment produced three different lipids a, b and c (Figure 5A, lanes 2 and 3). Lipid c was the least abundant species and co-migrated with IPC/C. Lipids a and b migrated with a higher R_f and therefore seem to be more hydrophobic than IPC/C.

PI-PLC treatment liberated three lipids, α, β and γ, from the anchor peptides (Figure 5, lane 5), and no more than three components could be resolved when several other TLC systems were tried (not shown). As expected, α, β and γ were more hydrophobic than lipids a, b and c and migrated in the region of animal Cer standards. Although not shown directly, it seems likely that α, β and γ are the Cer moieties of the IPCs a, b and c. The presumed Cers α, β and γ were purified by two consecutive rounds of preparative TLC and were hydrolyzed individually with strong HCl. As shown in Figure 5, lane 7, α contains [^{3}H]DHS whereas β and γ contain [^{3}H]PHS (lanes 9 and 11). Based on this analysis and on the previous compositional analysis of Cer-containing anchor peptides which showed the presence of PHS, C26:0 and mono-hydroxylated C26:0 as the main components (Fankhauser *et al.*, 1993), we propose the following structures for the Cer moieties of GPI anchors: a/α = DHS-C26:0, b/β = PHS-C26:0 and c/γ = PHS-C26:0-OH. This last assignment is in agreement with the fact that lipid c co-migrates with IPC/C which also contains PHS-C26:0-OH (Smith and Lester, 1974; Lester and Dickson, 1993). Lipid b/β might be contaminated with a trace of DHS-C26:0-OH, since the acid hydrolysis of β also yielded a trace of [^{3}H]DHS (Figure 5, lane 9).

The fact that nitrous acid deamination or PI-PLC treatment quantitatively released the lipid moieties from the labeled peptides further illustrates that [^{3}H]DHS is incorporated only into GPI proteins and into no other type of lipoprotein.

Incorporation of [^{3}H]DHS can occur in the absence of protein synthesis

Previous experiments had suggested that Cers are not incorporated into GPI proteins as part of the pre-formed GPI anchor lipid but at a later stage by lipid remodeling of protein anchors. We attempted to prove this notion formally by trying to label GPI proteins with [^{3}H]DHS after pre-incubation of cells with cycloheximide (Chx), a drug which blocks cytoplasmic protein synthesis. Chx has been shown to block rapidly incorporation of [^{3}H]Ins into proteins (Conzelmann *et al.*, 1990), implying that GPI anchors are added rapidly after completion of protein synthesis. As shown in Figure 6A, Chx not only abolishes the incorporation of [^{3}H]Ins into proteins but also reduces the incorporation of [^{3}H]DHS. Yet, the incorporation of [^{3}H]DHS in the presence of Chx is stimulated greatly by pre-incubation of the cells with myriocin (Figure 6B). It is interesting to note that myriocin, although in all cases present throughout the 2 h labeling period, only had an enhancing effect if it was added some time before the addition of [^{3}H]DHS. This observation might be explained if we assume that the depletion of endogenously synthesized sphingolipids takes some time and that most of the observed incorporation into proteins occurs rapidly after the addition of [^{3}H]DHS. The incorporation of [^{3}H]DHS under Chx represents convincing evidence for the hypo-

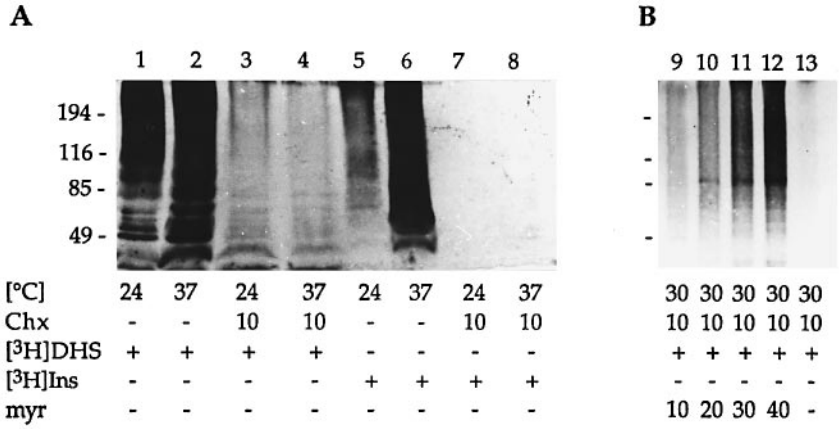


Fig. 6. The incorporation of [^{3}H]DHS only takes place *after* the attachment of GPIs to proteins. (**A**) X2180 cells were grown at 24°C and pre-incubated and radiolabeled at 24 or 37°C with [^{3}H]DHS or [^{3}H]Ins as indicated. All samples were pre-incubated for 20 min, some in the presence of 200 μg/ml of Chx. (**B**) X2180 cells were pre-incubated at 30°C for different times in the absence (lane 13) or presence of myriocin and of Chx. The numbers below each lane indicate at how many minutes before the addition of the radiotracer the various drugs (Chx, myr) had been added or cells had been shifted to 37°C for further pre-incubation.

thesis that Cers are integrated into GPI proteins by lipid remodeling of the primary DAG-containing GPI anchor.

Anchor remodeling can occur in the Golgi or a later secretory compartment

Proteins labeled by a 5 min pulse of [^{3}H]DHS were of mature size (Figure 2, lane 2), suggesting that [^{3}H]DHS may mostly be incorporated directly into mature Golgi forms of proteins. On the other hand, in *sec18* cells, [^{3}H]DHS was incorporated into immature ER forms of proteins and no labeling of mature proteins occurred (Figure 1, lanes 7 and 10–14), although these cells, when blocked at 37°C, contain GPI proteins in many secretory compartments including the Golgi (Graham and Emr, 1991). Our difficulties in interpretation were due mainly to the fact that we did not know in which compartment the remodelase activity is localized (Golgi, ER or both?). To approach this problem, we asked whether the Cers incorporated into the ER forms of GPI proteins were the same as those present in mature sized proteins. We therefore labeled wild-type cells or various secretion mutants with [^{3}H]DHS. As shown in Table I, all strains tested made the same three Cers, with PHS-C26:0 being the most abundant species. However, secretion mutants blocking the ER to Golgi transport of secretory proteins (*sec12*, *sec18*) were unable to make PHS-C26-OH-containing anchors at 37°C (Figure 5C, Table I). These data show that PHS-C26-OH-type anchors are only made when proteins reach the Golgi. One of the following hypothetical reactions, if limited to the Golgi, may explain this finding: (i) replacement of DAG by a PHS-C26-OH (DAG–Cer remodeling); (ii) replacement of a PHS-C26 or a DHS-C26 by a PHS-C26-OH (Cer–Cer remodeling); (iii) replacement of the C26 of PHS-C26 by a C26-OH; or (iv) the hydroxylation of the C26 of PHS-C26 while the Cer remains attached to the GPI protein. The absence of lipid γ in blocked *sec12* and *sec18* can therefore not be taken as evidence for a Golgi remodelase introducing an entire Cer moiety, since we cannot exclude that γ is made by a modification reaction of type (iii) or (iv) on a Cer, which became introduced into the GPI protein by a remodelase in the ER. We repeatedly tried to localize

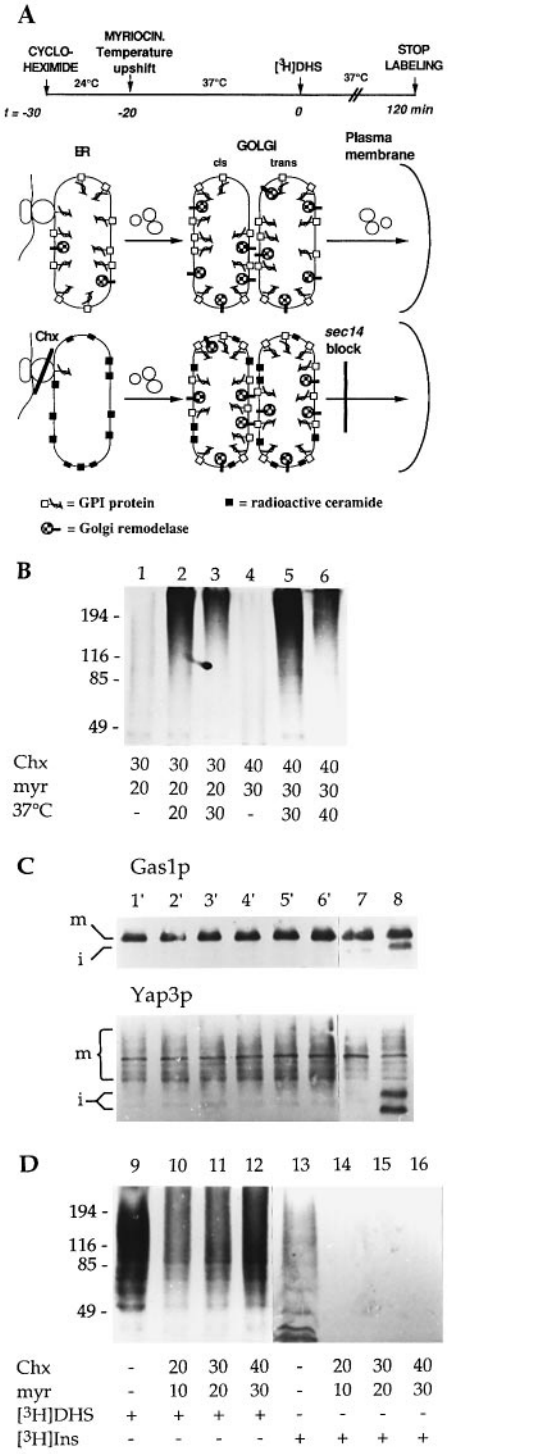
Table I. Relative amounts of ceramides α, β and γ in anchor peptides[a]

Strains and labeling conditions	α = DHS-C26 (%)	β = PHS-C26 (%)	γ = PHS-C26-OH (%)
Wild-type (24°C)	18	55	27
Wild-type (37°C)	11	64	25
Wild-type + myriocin (24°C)	18	59	23
Wild-type + myriocin (37°C)	27	61	12
sec12 (24°C)	22	55	23
sec12 (37°C)	26	74	0
sec12 + myriocin (24°C)	21	50	29
sec12 + myriocin (37°C)	48	51	1
sec18 (24°C)	25	67	8
sec18 (37°C)	30	70	0
sec18 + myriocin (24°C)	28	66	6
sec18 + myriocin (37°C)	47	53	0
sec18, Figure 8, lane 16	51	49	0
sec14 (24°C)	13	53	34
sec14 (37°C)	24	69	7
sec14, Figure 7, lane 5	42	43	15
sec9 (24°C)	8	64	28
sec9 (37°C)	16	62	22

[a]Ten OD_{600} of wild-type and *sec* mutant cells were pre-incubated and labeled with [^{3}H]DHS at 24 or 37°C in the presence or absence of myriocin as indicated. Proteins were delipidated, anchor peptides were prepared and treated with PI-PLC. The released Cers were separated by TLC and quantitated by radioscanning.

remodelases by adding [^{3}H]DHS to subcellular fractions *in vitro*, but these attempts were rather unsuccessful. Therefore, alternative ways were explored. The following type of experiment was done to demonstrate an autochthonous remodelase activity in the Golgi. As shown in Figure 7A, the strategy was to deplete the ER of GPI proteins by a pre-incubation in the presence of Chx and to see if [^{3}H]DHS was still incorporated into GPI proteins. Also, we used *sec14* cells in order to be able to retain GPI proteins in the Golgi. As can be seen in Figure 7B, even after pre-incubations of 30 or 40 min in the presence of Chx, vigorous incorporation of [^{3}H]DHS into mature sized proteins could be observed (lanes 2, 3, 5 and 6). Incorporation of [^{3}H]DHS under these conditions was only efficient in the presence of myriocin. This drug has been reported to slow drastically the kinetics of export of

the GPI-anchored Gas1p from the ER to the Golgi (Horvath *et al.*, 1994). Therefore, myriocin only was added 10 min *after* Chx in order to allow cells to export GPI proteins from the ER with normal kinetics during the first 10 min of pre-incubation. Indeed, control experiments showed that immature ER forms of the GPI proteins Gas1p and Yap3p (Ash *et al.*, 1995) had disappeared completely at the moment when [^{3}H]DHS was added (Figure 7C, lanes 1′–6′). In contrast, a secretion block in *sec18* cells led to the accumulation of the immature 105 kDa ER form of Gas1p and two immature forms of Yap3p of 115 and 85 kDa (Figure 7C, lanes 7 and 8). From these results, we conclude that [^{3}H]DHS can become incorporated into mature sized proteins under conditions in which all GPI proteins have undergone glycan maturation in the Golgi, as depicted in the bottom diagram of Figure 7A. The incorporation of [^{3}H]DHS was stronger when secretion was blocked (lanes 2 and 5) than when it was not (lanes 1 and 4). This may suggest that remodeling occurs in the Golgi. However, the same experiment as done with *sec14* in Figure 7B was repeated using wild-type cells, and we again obtained vigorous incorporation of [^{3}H]DHS into mature sized proteins (Figure 7D). This indicates that proteins remain available for DAG–Cer or Cer–Cer remodeling for prolonged periods even if they are not retained in the Golgi by a secretion block. The data also raise the possibility that the higher efficiency of [^{3}H]DHS incorporation in blocked *sec14* cells (lanes 1, 2, 4 and 5) may in fact be due to the higher labeling temperature rather than the secretion block, and that the remodeling of mature proteins in Figure 7 may occur not in the Golgi but at an even later stage of the secretory pathway. To see if the Cers added to proteins after prolonged Chx pre-incubations were the same as those added in the absence of the inhibitor, we prepared anchor peptides from the material shown in Figure 7B, lane 5, and analyzed the Cer moieties on TLC. Results are shown in Table I: PI-PLC released three anchor lipids which co-migrated exactly with the lipids α, β and γ identified as DHS-C26, PHS-C26 and PHS-C26-OH.



Anchor remodeling can also occur in the ER

The labeling of immature but not mature forms of GPI proteins in the secretion mutants *sec12* and *sec18* shown in Figure 1 (lanes 7 and 10–14) clearly indicates that the introduction of Cer anchors occurs in the ER. Thus, it is evident that the ER of *sec12* and *sec18* mutants contains a remodelase and the necessary Cer substrate if the vesicular traffic is blocked. The question arises as to whether this activity represents newly synthesized Golgi remodelase which is trapped in the ER because of the secretion block or if this remodelase is also present in the

Fig. 7. Anchor remodeling takes place in the Golgi apparatus. (**A**) The top diagram outlines the labeling procedure. Time points are chosen to match the sample shown in (B), lane 2. The diagram in the middle shows the distribution of Golgi remodelase and GPI proteins before the start of the pre-incubation; the bottom diagram shows the presumed situation during labeling with [^{3}H]DHS. Protein labeling can only occur if GPI proteins, labeled Cer and remodelase are present concomitantly in the same compartment. (**B**) *sec14* cells were pre-incubated at 24°C for 40 min. The numbers below each lane indicate at how many minutes before the addition of [^{3}H]DHS the various drugs (Chx, myriocin) had been added or cells had been shifted to 37°C for further pre-incubation. After pre-incubation, cells were labeled with [^{3}H]DHS at 24 (lanes 1 and 4) or 37°C (other lanes). Proteins were extracted and analyzed by SDS–PAGE/fluorography. (**C**) *sec14* cells were pre-incubated under conditions identical to the cells used to produce lanes 1–6 of (B); at the end of the pre-incubation, no radiotracer was added but secretion was arrested with NaF/NaN_3 and proteins were immediately extracted, separated on SDS–PAGE and mature (m) and immature (i) forms of Gas1p and Yap3p were detected by Western blotting. As a control, *sec18* cells were incubated at 24 (lane 7) and 37°C (lane 8) for 20 min and proteins were analyzed in the same way. (**D**) X2180 cells were pre-incubated with Chx and myriocin and shifted to 37°C as indicated. Subsequently, cells were radiolabeled with [^{3}H]DHS (lanes 9–12) or [^{3}H]Ins (lanes 13–16). Proteins were extracted and analyzed by SDS–PAGE/fluorography.

ER of wild-type cells. Hoping that some GPI proteins would linger in the ER for some time, we tried to demonstrate the presence of an autochthonous ER remodelase using the following scheme: *sec18* cells were pre-incubated with Chx for 10 min at 24°C to empty the ER of all newly synthesized Golgi remodelase. Thereupon, the protein export from the ER was blocked by a temperature shift to 37°C and myriocin was added. After a further 30 min incubation to deplete cells of sphingolipids, we tried to label the residual GPI proteins of the ER by adding [^{3}H]DHS. As shown in Figure 8B, lanes 5–8, incorporation of [^{3}H]DHS was minimal. Controls showed that a prolonged pre-incubation of blocked *sec18* with myriocin in the absence of Chx did not compromise the incorporation of the label (Figure 8B, lanes 2–4). Relying on the reported ability of myriocin to retain Gas1p in the ER (Horvath *et al.*, 1994), we repeated these experiments but added myriocin already some time before Chx in an attempt to accumulate specifically GPI proteins in the ER as depicted in Figure 8A. A control experiment confirmed that the pre-incubation of cells in the absence of Chx and in the presence of myriocin indeed caused the accumulation of the immature ER forms of Gas1p and, to a lesser extent, of Yap3p (Figure 8D, lanes 11′–16′). (This accumulation was not due to the secretion block since this block was only installed 10 min after protein synthesis had been arrested.) As shown in Figure 8C, vigorous incorporation of [^{3}H]DHS into ER forms of proteins II and III was observed under these conditions. In contrast, band I was not labeled at all and the identity of the labeled proteins in the region of band IV was uncertain although bands I and IV were strongly labeled in the absence of inhibitors (lane 10). This experiment appears to provide a strong argument for the presence of an autochthonous remodelase in the ER, although it cannot formally be excluded that the Golgi remodelase is somehow physically associated with some GPI protein and therefore would accumulate in the ER under myriocin. Labeling was stronger after 60 min (lanes 15 and 16) than after 20 min of pre-incubation with myriocin alone (lanes 11 and 12). This may be attributed either to an increased amount of accumulated GPI proteins or to a more complete depletion of sphingolipids after 60 min. As possible explanations for the fact that we mainly labeled bands II and III but not I and IV, we considered the following possibilities: (i) only bands II and III are retained in the ER under myriocin; (ii) only bands II and III are stable in the ER; (iii) only on proteins II and III are the Cers continuously exchanged whereas band I and IV are remodeled immediately after the attachment of the GPI but, thereafter, the lipid moieties are no longer exchanged; (iv) bands I and IV are remodeled by a myriocin-sensitive ER remodelase whereas bands II and III are remodeled by a different ER remodelase which is not inhibited by myriocin. We obtained experimental evidence against three of these possibilities: Possibility (i) seemed unlikely since a similar increase of labeling of bands II and III was also seen when myriocin was only added at the moment of the temperature upshift (Figure 1, lanes 7 and 10–14) in which case *all* GPI proteins are retained in the ER because of the secretion block. As to possibility (ii), a control experiment using a 20 min pulse of blocked *sec18* cells with [^{3}H]Ins and a subsequent chase in the presence of Chx showed that bands I, II, III and IV were all completely stable throughout a 2 h chase period whether or not myriocin was present (data not shown). Possibility (iii) was excluded by the following

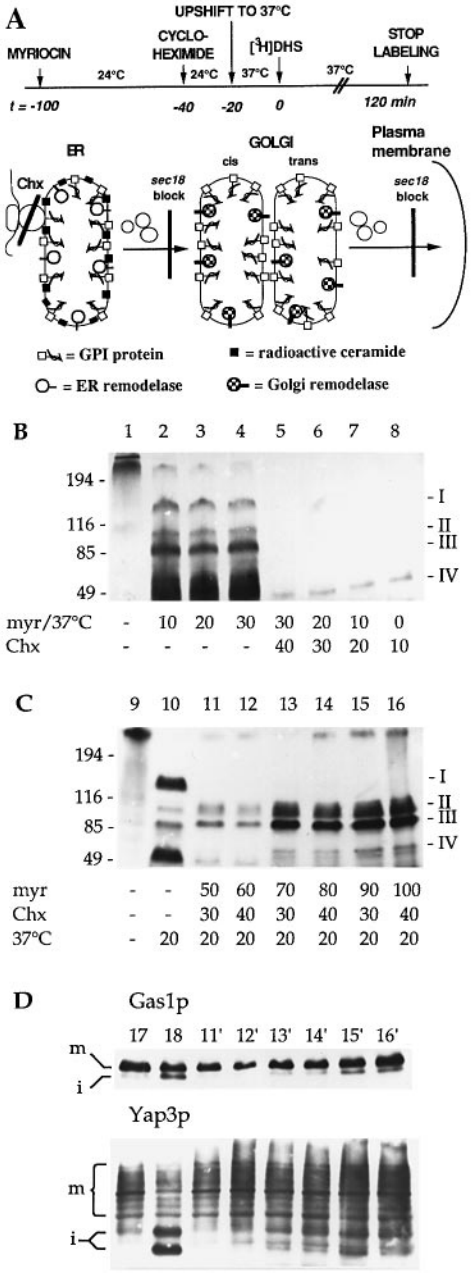


Fig. 8. Anchor remodeling in the ER. (**A**) The top diagram outlines the labeling procedure. Time points are chosen to match the sample shown in (C), lane 16. The diagram at the bottom shows the distribution of Golgi remodelase, GPI proteins and labeled Cer during labeling for the same sample. (**B** and **C**) *sec18* cells were first pre-incubated at 24°C. The numbers below each lane indicate at how many minutes before the addition of [^{3}H]DHS the various drugs (Chx, myriocin) had been added or cells had been shifted to 37°C for further pre-incubation. For control, cells were radiolabeled in the absence of the two toxins (lanes 1, 9 and 10). (**D**) *sec18* cells were subjected to the same pre-incubations as in lanes 11–16 of (C) but, at the time corresponding to the addition of the radiolabel, their metabolism was arrested with NaF/NaN_3. Proteins were extracted, separated by 7.5% SDS–PAGE and the maturation status of Gas1p and Yap3p was analyzed by Western blotting. As a control, *sec18* cells were incubated at 24 and 37°C for 20 min and proteins analyzed in the same way (lanes 17 and 18).

Table II. Ceramides in individual GPI proteins of *sec18* labeled at 37°C[a]

Protein bands and labeling conditions	α = DHS-C26 (%)	β = PHS-C26 (%)	γ = PHS-C26-OH (%)
I	18	82	0
I + myriocin	80	20	0
II	15	85	0
III	14	86	0
III + myriocin	51	49	0
IV	16	84	0
IV + myriocin	54	46	0

[a]Ten OD_{600} of *sec18* cells were pre-incubated and labeled with [^{3}H]DHS at 37°C in the presence or absence of myriocin as indicated. Proteins were extracted and separated by SDS–PAGE. After fluorography, individual bands of the gel were excised and treated in order to obtain anchor peptides. The peptides were treated with PI-PLC, the released Cers were separated by TLC and quantitated by radioscanning.

experiment (performed without myriocin): *sec18* cells were shifted to 37°C and incubated for 20 min in order to accumulate GPI proteins in the ER. Chx was added and cells further incubated for 10–60 min at 37°C. Finally, [^{3}H]DHS was added. Proteins I, II, III and IV were all strongly labeled and were as strongly labeled after 60 min of pre-incubation with Chx as after 10 min (data not shown). This shows that bands I and IV can be remodeled a long time after their synthesis. We presently do not have any experimental data against hypothesis (iv), i.e. the existence of two different ER remodelases, but this possibility remains highly conjectural. In view of the differential labeling efficiencies of different proteins, we wanted to see if bands I and IV receive the same type of anchor lipids as bands II and III. To this end, we analyzed lipid moieties of individual protein bands of *sec18* cells labeled with [^{3}H]DHS at 37°C in the presence or absence of myriocin as summarized in Table II. It is apparent that all of these proteins receive both DHS-C26 and PHS-C26 but no PHS-C26-OH as expected. Myriocin consistently increased the incorporation of DHS-C26. We also verified that the Cer added to proteins after prolonged myriocin pre-incubations were the same as those added in the absence of the inhibitor. For this, we prepared anchor peptides from the material shown in Figure 8C, lane 16 and analyzed the Cer moieties on TLC. Results are shown in Table I: PI-PLC released two anchor lipids which co-migrated exactly with the lipids α and β identified as DHS-C26 and PHS-C26.

The major anchor ceramide added in the ER is not detectable within the pool of free ceramides

Theoretically, the event by which sphingolipids are introduced into GPI anchors may consist of the replacement of either DAG, phosphatidic acid or PI moieties of the primordial GPI anchor. The latter is unlikely since, in contrast to [^{3}H]DHS, [^{3}H]Ins was never observed to become incorporated into GPI anchors under Chx (Conzelmann *et al.*, 1990) (Figure 6, lanes 7 and 8). It is most likely that the anchor remodeling proceeds in a manner analogous to IPC biosynthesis, i.e. that the DAG moiety of PI is replaced by a Cer (Becker and Lester, 1980). Regardless of whether Cer or Cer-phosphate is introduced, remodeling will draw on the free Cer pool. We therefore tried to evaluate whether the Cers appearing in the GPI anchors are representative of the free Cers of the cell. For this, *sec18* cells were labeled at 24 and 37°C with [^{3}H]DHS and lipid extracts were analyzed in a solvent system allowing the separation of Cers, as shown in Figure 9, lanes 2–5. It appeared that the relative amount of Cers was increased at 37°C, as already observed in Figure 3, lane 5, possibly because at 37°C the Cers cannot be used for the synthesis of IPC/D, MIPC and $M(IP)_2C$. The very hydrophobic lipids 1–7 running in the region of mammalian Cer standards were purified by preparative TLC. Further experiments showed that bands 3, 4 and 5 exactly co-migrate with Cers α, β, and γ which are obtained by PI-PLC treatment of GPI anchor peptides (Figure 5, lane 5). To confirm the identity of lipids 3–5, they were subjected to mild alkaline and strong acid hydrolysis conditions. As shown in Figure 9, lanes 7, 11, 14, lipids 3, 4 and 5 resisted mild base treatment. Strong acid hydrolysis liberated [^{3}H]DHS from lipids 3 and 4 but [^{3}H]PHS from lipid 5. These data confirm the Cer nature of lipids 3–5. However, the exclusive presence of [^{3}H]DHS in lipid 4 was quite unexpected in as much as lipid 4 exactly co-migrates with anchor-derived lipid β which contains [^{3}H]PHS. This leads us to assume that lipid 4 either is [^{3}H]DHS-C26-OH or that it contains a fatty acid which is shorter than C26. Further analysis showed that lipids 1, 2 and 6 also only contained [^{3}H]DHS but no [^{3}H]PHS, whereas lipid 7 resisted strong acid hydrolysis and therefore is not a Cer. This analysis revealed that after 2 h of labeling at 37°C, *sec18* cells do not contain a free Cer corresponding to the main β Cer (i.e. PHS-C26) of GPI anchors. *sec18* cells were also labeled as in Figure 9, lane 4, but for only 15 or 30 min. The profile of free Cers was identical to that obtained after 2 h of labeling (Figure 9, lane 4) and lipid 4 only contained [^{3}H]DHS but no [^{3}H]PHS (not shown). Thus, free Cers seem to be different from the anchor Cers even at this early stage when most of the [^{3}H]DHS incorporation into GPI proteins occurs (Figure 2B). Since lipid 4 had been isolated from *sec18* cells labeled at 37°C, we undertook to verify the nature of lipid β from GPI anchor peptides generated from the same *sec18* strain labeled under identical conditions. As shown in Figure 10, lanes 4 and 6, lipids α and β contain DHS and PHS, respectively. It follows that, at least in *sec18* at 37°C, the Cer addition in the ER proceeds with an unsuspected specificity: the main free Cer, namely PHS-C26-OH, does not seem to be utilized for remodeling although it is used for the biosynthesis of IPC/C. Rather, the anchors contain PHS-C26, a Cer species which is not present as a major species in the pool of free Cers. Further experimentation will be required to decide whether PHS-C26-OH is not available in the ER of *sec18* cells at 37°C, or if the remodelase activity observed under these conditions cannot utilize PHS-C26-OH.

Discussion

Several protozoan organisms contain Cer in their GPI anchors (Stadler *et al.*, 1989; Güther *et al.*, 1992; Serrano *et al.*, 1995; Heise *et al.*, 1996), and the absence of Cer-containing CP lipids in *Trypanosoma cruzi*, the causative

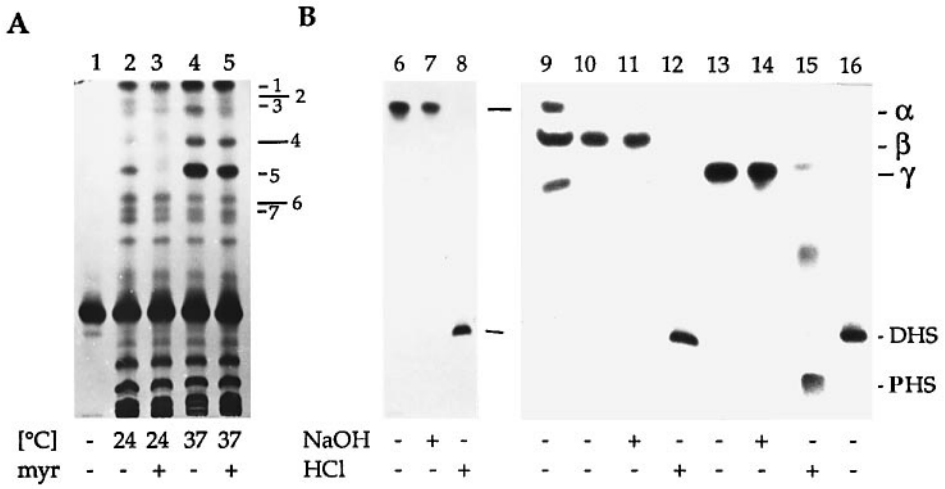


Fig. 9. Comparison of free ceramides with ceramides of GPI anchors. (**A**) *sec18* cells were radiolabeled at 24 and 37°C with [^{3}H]DHS in the presence or absence of myriocin. The radiolabeled lipids were extracted and analyzed on TLC using solvent system 1. [^{3}H]DHS was used as a standard (lane 1). By radioscanning, the lipid extracts in lanes 2–5 contained 21–25% of [^{3}H]DHS. (**B**) Ten OD_{600} of *sec18* cells were radiolabeled at 37°C with [^{3}H]DHS without myriocin, the extracted lipids were separated by preparative TLC, lipids 1–7 (see A) were scraped off and purified by two consecutive rounds of preparative TLC. Purified lipids 3 (lane 6), 4 (lane 10) and 5 (lane 13) were subjected to mild base treatment (NaOH, lanes 7, 11 and 14) or to strong acid hydrolysis (HCl, lanes 8, 12 and 15). The products were analyzed by TLC using solvent system 1 and fluorography. Standards were PHS, [^{3}H]DHS (lane 16) and [^{3}H]DHS-radiolabeled GPI Cers (lane 9). The latter were obtained from wild-type cells as described in Figure 5, lane 5.

agent of Chagas' disease, suggests that Cers are introduced into GPI anchors of this parasite in a similar way to that described here for yeast (Heise *et al.*, 1996). To study the incorporation of Cers, this report is pioneering the use of [^{3}H]DHS. Although not available commercially, [^{3}H]DHS is obtained easily through hydrogenation of mammalian sphingosine (Crossman and Hirschberg, 1977), whereas PHS, the prevalent long chain base of *S.cerevisiae*, cannot be made easily in radioactive form. [^{3}H]DHS is taken up readily into cells and utilized to make all known classes of sphingolipids and GPI anchors. This is not unexpected since DHS is believed to be an obligate precursor of all sphingolipids of *S.cerevisiae* (Pinto *et al.*, 1992). Moreover *lcb1*, a mutant deficient in serine palmitoyltransferase, is auxotrophic for exogenous sphingolipids (Wells and Lester, 1983; Buede *et al.*, 1991). It grows equally well with DHS as with PHS and makes the complete array of PHS-containing sphingolipids when fed with DHS (Wells and Lester, 1983).

The previous analysis of yeast GPI anchor lipids by GC-MS showed that only 5.5% of Cer anchors contain DHS whereas the remainder contain PHS (Fankhauser *et al.*, 1993). Moreover, DHS-C26 (Figure 9A, lipid 3) is not found among the free IPCs unless cells are grown anaerobically (Lester and Dickson, 1993). The comparison of the IPCs generated by nitrous acid treatment from [^{3}H]DHS- and [^{3}H]Ins-labeled GPI anchor peptides revealed the presence of only two [^{3}H]Ins-labeled IPCs which co-migrated exactly with lipids b and c (Figure 5A), whereas no [^{3}H]Ins-labeled homolog of lipid a could be observed (G.Sipos, F.Reggiori, C.Vionnet, E.Canivenc-Gansel and A.Conzelmann, unpublished). We therefore assume that labeling with [^{3}H]DHS produces a faithful picture of GPI anchor lipids, except that the DHS-containing type a/α is strongly overrepresented. Exogenous DHS may have less access to the monooxygenase which hydroxylates C4 of DHS than does endogenous DHS (Lester and Dickson, 1993). In summary, labeling with [^{3}H]DHS seems to be an excellent tool to study remodeling of GPI anchors and promises to be useful also for studies of IPC biosynthesis in many kinds of yeast (Wells *et al.*, 1996). Labeling with [^{3}H]DHS may also provide a sensitive assay of free Cer levels, since Cers recently have been proposed to act as second messengers operating a G_1 arrest in yeast (Nickels and Broach, 1996).

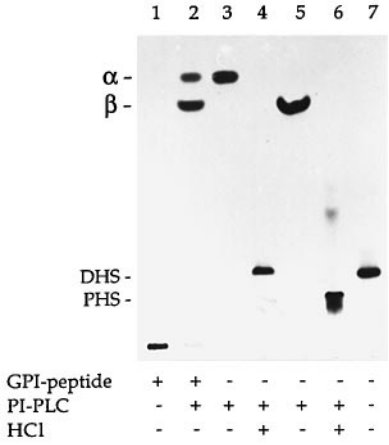


Fig. 10. Lipid β of GPI anchors made in the ER of *sec18* cells contains PHS. Twenty OD_{600} of *sec18* cells were radiolabeled at 37°C with [^{3}H]DHS and the GPI peptides purified (lane 1). The material was treated with PI-PLC to produce the GPI-Cers, and an aliquot was spotted to control the efficiency of the hydrolysis (lane 2). The Cers were purified by two consecutive rounds of preparative TLC (lanes 3 and 5) and were hydrolyzed individually with strong acid hydrolysis (lanes 4 and 6). The products were analyzed on TLC using solvent system 1. [^{3}H]DHS (lane 7) and PHS were used as standards.

By blocking the endogenous sphingolipid biosynthesis, myriocin greatly enhances the incorporation of [^{3}H]DHS into proteins. Since this drug strongly resembles PHS, it may not only inhibit serine palmitoyltransferase but also

other enzymes which use sphingolipid substrates. Although all IPCs were made normally in the presence of this drug and although [^{3}H]Cer became incorporated into GPI anchors, it should be borne in mind that remodelases may be influenced by myriocin. For instance, we have not excluded that myriocin may selectively inhibit a particular ER remodelase specifically acting on protein bands I and IV (Figure 1, lanes 10–14).

Labeling of cells with [^{3}H]DHS under Chx allowed us to demonstrate directly lipid remodeling of GPI proteins. It can safely be excluded that this labeling is due to the addition of [^{3}H]Cer-containing CPs onto GPI precursor proteins by the putative transamidase (Maxwell *et al.*, 1995). This can be said because CPs are added to newly synthesized GPI protein precursors immediately after translocation into the ER. This follows from the demonstration that the incorporation of [^{3}H]Ins into proteins ceases within <5 min after addition of Chx (Conzelmann *et al.*, 1990) although GPI precursor proteins seem to remain stable in the ER (Nuoffer *et al.*, 1993; Doering and Schekman, 1996). In contrast, in many experiments reported here, [^{3}H]DHS labeling was observed even 40 or 60 min after the addition of Chx. Moreover, we failed to obtain evidence for the appearance of Cer-containing GPI lipids by labeling with either [^{3}H]DHS or [^{3}H]Ins even in mutants which accumulate CPs, such as *gpi8* and *gaa1* (not shown) (Benghezal *et al.*, 1995; Hamburger *et al.*, 1995).

Data obtained in *sec14* cells by depleting the ER of GPI proteins through Chx pre-incubation (Figure 7) argue for a remodelase in the Golgi. Both Cer β and Cer γ were added in this experiment (Table I). Thus, the Golgi remodelase either can add both of these lipids or the Golgi contains some additional enzyme which transforms β (PHS-C26) into γ (PHS-C26-OH). It cannot formally be excluded that the proteins labeled after ER depletion would return transiently to the ER for anchor remodeling, but this type of retrotransport is highly unlikely since GPI proteins do not contain any known retrieval motif and all known GPI proteins are directed to the plasma membrane or to the cell wall (Stratford, 1994). The data also indicate the presence of at least one autochthonous ER remodelase. This remodelase only adds Cer β (PHS-C26) but no Cer γ (PHS-C26-OH) (Figure 8C, lane 16, and Table I).

The data obtained in this study have to be compared with that of an earlier study where GPI proteins were pulse labeled for 5–10 min with [^{3}H]Ins and chased with Chx, and where the presence of mild base-resistant anchor peptides was monitored (Conzelmann *et al.*, 1992). These experiments demonstrated that 20–40% of anchors were already resistant to mild base after the pulse and that the relative amount of mild base-resistant anchors stayed constant after 20 min of chase (early *sec* mutants at 37°C) or 60 min of chase (wild-type cells at 24 or 30°C). Here we observed vigorous incorporation of [^{3}H]DHS into GPI proteins after the cells had been exposed for 30 or 60 min to Chx at 37°C (Figure 7B, lanes 5 and 6; Figure 7D, lane 12; Figure 8C, lanes 11–16; data not shown). These data show that ER as well as Golgi remodelase activities can incorporate [^{3}H]DHS into GPI proteins at times when the relative and absolute amount of Cer-containing anchors is constant. This consideration strongly suggests that Cer moieties of GPI anchors are continuously exchanged for other Cers over a long period and that the Cers of the anchors may be in equilibrium with free Cers. This latter notion, however, seems not to be true since the Cers of GPI anchors are different from the free Cers. However, anchor Cers may be in equilibrium with the free Cer pool of the particular compartment in which this continuous exchange occurs. Continuous Cer exchange is reminiscent of the recently discovered myristate exchange on the variant surface glycoprotein of *Trypanosoma brucei* through which the fatty acids of the DAG moiety of GPI anchors are exchanged continuously (Buxbaum *et al.*, 1996). Our data would argue that this continuous exchange can occur both in the ER and in a later secretory compartment. It occurs on all of the major GPI proteins retained in the ER of secretion mutants (not shown). However, it is not clear whether it occurs on all mature GPI proteins or only on a subpopulation which may recirculate through the Golgi (Harris and Waters, 1996). The continuous exchange in a later secretory compartment seems to introduce all three types of anchor Cers, α, β and γ.

Two different mannosyltransferases which reside in the *cis*- and *trans*-Golgi and which can add a mannose side chain onto the GPI carbohydrate core have been shown to act preferentially on DAG- and Cer-containing GPI anchors, respectively (Sipos *et al.*, 1995). Further experiments will be required to see if these transferases also discriminate between Cer β- and Cer γ-containing GPI anchors.

In view of the continuous exchange of Cers on GPI anchors, it is puzzling that no mature sized GPI proteins become labeled in early secretion mutants (*sec12, sec18*) at 37°C in the absence of Chx (Figure 1, lanes 11–14) or in its presence (Figure 7, lanes 2, 5, 11 and 12 versus Figure 8A, lanes 5 and 6). The finding can be explained by assuming that [^{3}H]DHS is transformed into Cer in the ER and that [^{3}H]Cer, in order to label mature sized proteins, has to get into the Golgi by an obligatory vesicular transport mechanism. Indeed, when making the reasonable assumption that [^{3}H]DHS diffuses freely to all cellular compartments, and observing the complete absence of labeling of MIPC and M(IP)$_2$C in blocked *sec12* cells (Figure 3, lane 5), we find the notion that Cer is not made in the yeast Golgi confirmed. In mammalian cells, Cer biosynthesis has been shown to be located in the cytosolic leaflet of the ER (Mandon *et al.*, 1992), but no analogous data are available for yeast. Similarly, the transport of Cer from the ER to the Golgi has only been studied in the mammalian system. This transport has been claimed to proceed via a non-vesicular, temperature-dependent mechanism which requires neither cytosol nor ATP. This conclusion, however, was reached with a cell-free microsomal transport system which seems difficult to control (Moreau *et al.*, 1993). Cer transport to the Golgi also seems to continue to some extent during mitosis (Collins and Warren, 1992). On the other hand, transport of sphingolipids from the Golgi to the cell surface has been shown to depend strictly on vesicular transport in mammalian cells (Lipsky and Pagano, 1985; van Meer and Burger, 1992) and in yeast (Hechtberger *et al.*, 1994).

The precise role of lipid remodeling of GPI anchors is presently a matter of speculation. Recent evidence demonstrates that GPI anchor lipids can be exchanged in several different ways and that the primary GPI gets

remodeled in virtually all GPI proteins (Sipos *et al.*, 1997). In fact, in some proteins, the DAG moiety of the primary anchor is modified in such a way as to contain very long fatty acids whereas in others it is replaced by Cers β or γ. It is worth noting that all remodeling events produce anchors containing large lipid moieties with very long fatty acids. This may help GPI proteins to sort into thicker membrane subdomains and be a prerequisite for the packaging of GPI proteins into transport vesicles. Indeed, it has been shown that the packaging of Gas1p into COPII vesicles is dependent on the addition of a GPI anchor (Doering and Schekman, 1996) and that its transport rate to the Golgi is enhanced greatly by ongoing ceramide biosynthesis (Horvath *et al.*, 1994). Further experiments will be required to decide whether transport of GPI proteins is dependent on lipid remodeling.

Materials and methods

Yeast strains, media and materials

The *S.cerevisiae* strains used were X2180-1B (*MAT*α *SUC2 mal gal2 CUP1*), 140-3A (*MAT*α *sec18*), SF226-1C (*MAT***a** *sec12-1*), HMSF169 (*MAT***a** *sec14*), HMSF143 (*MAT***a** *sec9-1*) and RH310-1A (*MAT***a** *his3 leu2 ura3 trp1 myr1*). Maintenance and growth conditions as well as the photometric determination of cell density have been described (Benghezal *et al.*, 1996). One OD_{600} of cells corresponds to $1–2\times10^7$ cells, depending on the strain. Reagents were obtained from the following sources: [2-^{3}H]*myo*-inositol from Anawa Trading SA, Wangen, Switzerland; Chx from Fluka Chemie AG (Buchs, Switzerland); myriocin was a kind gift of Dr N.Rao Movva (Sandoz AG, Basel, Switzerland), DHS, PHS, bovine ceramides, D-C_{18}-sphingosine from bovine brain, tergitol NP-40 and anti-rabbit IgG–peroxidase conjugate were from Sigma Chemical Co. (St Louis, MO); PI-PLC was from Boehringer Mannheim Gmbh, Mannheim, Germany.

Synthesis of [^{3}H]dihydrosphingosine

Catalytic reduction of 25 mg of D-C_{18}-sphingosine from bovine brain with tritium gas was performed by NEN (Du Pont De Nemours, Les Ulis, France) (Crossman and Hirschberg, 1977). The reduction product was purified by preparative TLC using 0.2 mm thick silica gel plates (Merck, Darmstadt, Germany) developed with chloroform:methanol:2 M NH_4OH (40:10:1). Labeled DHS was eluted from the silica gel with methanol (Crossman and Hirschberg, 1977). Radiochemical purity was >97% as judged by TLC using a two-dimensional radioscanning (LB2842, Berthold AG, Regensdorf, Switzerland), and the specific activity was 32.3 Ci/mmol.

Labeling of cells

Unless stated otherwise, 2.5 OD_{600} of exponentially growing cells were harvested, cells were resuspended in 250 µl of SDC medium and labeled as follows. Thermosensitive strains were pre-incubated and labeled at 24 or 37°C in a shaking water bath by adding 25 µCi of [^{3}H]Ins or 25 µCi of [4,5-^{3}H]DHS. After 40 min, the samples were diluted with 750 µl of fresh SDC medium and were incubated for a further 80 min. Labeling was terminated by adding NaF and NaN_3 (10 mM final concentrations). To enhance the labeling with [^{3}H]DHS, we frequently added myriocin at a final concentration of 40 µg/ml. Chx was used at 200 µg/ml in order to prevent protein synthesis. When myriocin or Chx were added during pre-incubations, the drugs remained present throughout the 2 h labeling period and were also present in the medium used for dilution of cells after 40 min.

Protein extraction and treatments

Cells were washed twice in NaF and NaN_3 and resuspended in a mixture of chloroform:methanol (1:1). Cells were disrupted by vortexing with glass beads. Beads were removed and proteins and cell wall debris were sedimented at 10 000 *g* for 5 min. The protein pellet was delipidated twice more with chloroform:methanol:water (10:10:3) and was dried in a Speed-Vac evaporator. For SDS–PAGE, proteins were resuspended and boiled for 5 min in reducing sample buffer (Laemmli, 1970).

For PI-PLC treatment, the delipidated proteins were dissolved in 50 µl of PI-PLC buffer I (20 mM Tris–HCl pH 7.5, 0.2 mM EDTA, 0.1% Triton X-100) (Conzelmann *et al.*, 1992) and boiled for 5 min. The sample was centrifuged at 10 000 *g* for 5 min and the supernatant divided into two 25 µl aliquots. Then 0.01 U of PI-PLC was added to one aliquot and the two tubes were incubated at 37°C for 5 h. The reaction was stopped by adding 25 µl of twice concentrated reducing sample buffer (Laemmli, 1970) and boiling for 5 min.

The same procedure was used for GPI-PLD treatment, but proteins were dissolved in GPI-PLD buffer (50 mM Tris–HCl pH 4.5, 10 mM NaCl, 2.6 mM $CaCl_2$, 0.018% Triton X-100) (Davitz *et al.*, 1989), 5 U of GPI-PLD from bovine serum (a kind gift of Dr Urs Brodbeck, Institut für Biochemie und Molekularbiologie, University of Bern, Switzerland) were added to one of two aliquots and samples were incubated at 37°C overnight.

In all cases, the proteins were separated on an SDS–PAGE 6–10% gradient gel (Laemmli, 1970) followed by fluorography (Benghezal *et al.*, 1995).

Lipid extraction and thin layer chromatography

When preparing cell extracts for protein analysis, the organic solvent extracts were saved, pooled and dried. Where indicated, lipids were subjected to mild base treatment using NaOH (Puoti *et al.*, 1991). Lipids were desalted by partitioning between *n*-butanol and water followed by a back extraction of the butanol phase with water (Krakow *et al.*, 1986). The desalted lipids were analyzed by ascending TLC using 0.2 mm thick silica gel plates with solvent system 1 [chloroform:methanol:2 M NH_4OH (40:10:1)] or solvent system 2 [chloroform:methanol:0.25% KCl (55:45:10)]. Radioactivity was detected and quantitated by two-dimensional radioscanning and fluorography (Benghezal *et al.*, 1995). Cold standards (DHS and PHS) where visualized after fluorography: Plates were sprayed with ninhydrin (Merck) and heated to 100°C until red spots appeared. Cer was detected with orcinol and sulfuric acid (Jork *et al.*, 1990).

Purification and analysis of GPI peptides

Even when working on a preparative scale, we always labeled aliquots of 2.5 OD_{600} since small aliquots resulted in better lipid extraction. Cells were broken with glass beads as described above, proteins were delipidated four times with chloroform:methanol:water (10:10:3) and twice by a 15 min incubation in ethanol:water:diethylether:pyridine: ammonia (15:15:5:1:0.018) at 37°C (Hanson and Lester, 1980). Dried proteins were boiled in 50 µl of sample buffer (Laemmli, 1970) and glycoproteins were purified by affinity chromatography on concanavalin A–Sepharose followed by SDS–PAGE, and were eluted with pronase as described (Conzelmann *et al.*, 1992) (therein procedure A, then F). To analyze the GPI-Cers of individual protein bands, the bands were localized using the fluorogram, the corresponding zone of the gel was excised, the fluor was eliminated according to a published procedure (Sefton *et al.*, 1982) and labeled anchor peptides were obtained by pronase treatment as above. Pronase-treated gel slices were washed twice with 0.1% Triton X-114 and the washes were added to the pronase eluate containing the anchor peptides. The pool was run over octyl-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) as described (Sipos *et al.*, 1994). The column was eluted successively by one column volume of 20, 25, 30, 35, 40, 45 and 50% propanol in water. Fractions of 500 µl were collected, and the radioactivity of each fraction was measured by β-counting. Fractions containing GPI peptides were pooled and dried.

PI-PLC treatment was performed by resuspending the GPI peptides in 150 µl of PI-PLC buffer II (20 mM Tris–HCl pH 7.5, 0.2 mM EDTA, 20% propanol) and adding 0.01 U of PI-PLC (Puoti and Conzelmann, 1993). The samples were incubated at 37°C for 3 h. The reaction was stopped by adding 300 µl of *n*-butanol, phases were separated and the water phase was extracted once more with 300 µl of *n*-butanol. The two butanol phases were collected and dried.

For nitrous acid treatment, the GPI peptides were taken up in 25 µl of sodium acetate buffer (100 mM sodium acetate pH 3.5, 0.01% Zwittergent 3-16) and processed and desalted using *n*-butanol as described (Güther *et al.*, 1994).

Strong HCl hydrolysis of lipids to hydrolyze Cers was carried out as described (Conzelmann *et al.*, 1992). The remaining HCl was evaporated in a Speed-Vac evaporator with two flashes of 200 µl of dry methanol.

Protein extraction and Western blot

Mock labeling of 2.5 OD_{600} of cells was carried out as described in the legends to Figures 7 and 8, and incubations were stopped by adding 10 mM NaF and 10 mM NaN_3. The cells were washed twice and were resuspended in 50 µl of TEPI buffer [1 M Tris–HCl, 20 mM EDTA,

1% dimethylsulfoxide (DMSO), 2 mM phenylmethylsulfonyl fluoride (PMSF), 30 μg/ml pepstatin, 30 μg/ml leupeptin, 30 μg/ml antipain]. Glass beads were added and cells were vortexed five times for 1 min. Then 50 μl of twice concentrated reducing sample buffer was added and the sample was boiled for 5 min. Generally, 20 μl samples (0.5 OD_{600}) were loaded on an SDS–PAGE gel, GPI proteins were detected with antibodies raised in rabbits followed by secondary antibodies coupled to horseradish peroxidase, whose reaction product was detected by chemiluminescence using an ECL kit (Amersham, Buckinghamshire, UK).

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Notes added in proof

(i) A more detailed description of the methods used herein will be published in *Methods in Molecular Biology*, Protein Lipidation Protocols (ed. Gelb,M.H.) Humana Press.
(ii) [^{3}H]DHS has recently become commercially available from ARC Inc. (St Louis, MO).