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The N-glycosylation defect of *cwh8Δ* yeast cells causes a distinct defect in sphingolipid biosynthesis

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***CWH8*/YGR036c of *Saccharomyces cerevisiae* has been identified as a dolichylpyrophosphate (Dol-PP) phosphatase that removes a phosphate from the Dol-PP generated by the oligosaccharyltransferase (OST), while it adds *N*-glycans to nascent glycoproteins in the endoplasmic reticulum (ER). Lack of *CWH8* was proposed to interrupt the so called dolichol (Dol) cycle by trapping Dol in the form of Dol-PP in the ER lumen. Indeed, *cwh8Δ* mutants display a severe deficiency in N-glycosylation. We find that *cwh8Δ* mutants have strongly reduced levels of inositolphosphorylceramide (IPC), whereas its derivative, mannosyl-(inositol-P)$_2$-ceramide (M(IP)$_2$C) is not affected. Microsomes of *cwh8Δ* contain normal ceramide synthase and IPC synthesis activities. Within a large panel of mutants affecting Dol dependent pathways such as N- or O-glycosylation, or glycosylphosphatidyl inositol (GPI)-anchoring, only the mutants having a deficiency of *N*-glycan addition show the defect in IPC biosynthesis. By mutating genes required for the addition of *N*-glycans or by treating cells with tunicamycin (Tm) one can similarly reduce the steady state level of IPC and exactly reproduce the phenotype of *cwh8Δ* cells. Some potential mechanisms by which the lack of *N*-glycans could lead to the sphingolipid abnormality were further explored.**

Key words: AUR1/dolichol/Golgi/unfolded protein response

Introduction

Mutants in *CWH8*/YGR036c were obtained in a screen for calcofluor white hypersensitivity and were shown to have an altered cell wall composition (Ram *et al*., 1994). Lack of Cwh8p leads to a deficiency in protein N-glycosylation, whereby *cwh8Δ* cells contain only 20% of the normal amount of dolichylpyrophosphate-linked oligosaccharides so that many glycoproteins are severely underglycosylated. Thus, only a fraction of the normally glycosylated Asn-X-Ser/Thr acceptor sites in carboxypeptidase (CPY) or invertase are N-glycosylated. The structure of the transferred oligosaccharides is, however, normal (van Berkel *et al*., 1999). *Cwh8Δ* accumulates dolichylpyrophosphate (Dol-PP) and have recently been shown to lack the Dol-PP phosphatase activity that converts Dol-PP into dolichylphosphate (Dol-P) (Fernandez *et al*., 2001). Cwh8p is an integral membrane protein with four transmembrane domains having a lumenally oriented active site (Fernandez *et al*., 2001). Indeed, Dol-PP is generated in the endoplasmic reticulum (ER) lumen by oligosaccharyltransferase (OST) that transfers oligosaccharides from Dol-PP onto nascent and newly made glycoproteins in the ER. To explain the underglycosylation phenotype of the *cwh8Δ* mutants the Dol cycle model has been proposed. The Dol cycle model assumes that the lumenally generated Dol-PP has to be dephosphorylated to Dol-P before being back transported to the cytosolic leaflet, a process through which Dol-P would regain the ability to serve as an acceptor for the cytosolic glycosyltransferases. The cytosolic glycosyltransferases use Dol-P and sugar-nucleotides to generate Dol-P-Man, Dol-P-Glc as well as Dol-PP-GlcNAc$_2$-Man$_5$, which all are then flipped to the lumenal leaflet where they serve as donors for N- and O-glycosylation as well as glycosylphosphatidyl inositol (GPI) anchor biosynthesis. The flippase for Dol-PP-GlcNAc$_2$-Man$_5$ has been identified as Rft1p (Helenius *et al*., 2002). The Dol cycle model is also supported by the fact that overexpression of *RER2* rescues the growth defect of *cwh8Δ* (Sato *et al*., 1999; Fernandez *et al*., 2001). *RER2* encodes the major cis-prenyltransferase of yeast, the key enzyme for Dol biosynthesis (Sato *et al*., 1999). The Dol cycle model suggests that *cwh8* and *rer2* mutants are similar in that both suffer from a relative lack of Dol-P at the cytosolic face of the ER membrane to serve as acceptor for glycosyltransferases. Interestingly, *rer2* cells also show an aberrant proliferation of ER- and Golgi-membranes, and they fail to retain Sec12p in the ER. These phenomena were not observed in other mutants causing hypoglycosylation such as the dolicholkinase mutant *sec59*. This led to the speculation that Dol or one of its derivatives in addition to playing a role in protein glycosylation, may function in protein retention and organelle homeostasis (Sato *et al*., 1999).

While carrying out a brute force screen requiring the metabolic labeling of yeast cells with [^{3}H]inositol ([^{3}H]Ins) we incidentally observed that *cwh8Δ* cells display a strong reduction of inositolphosphorylceramides (IPCs) similar to what was recently found in *erg26-1* and *arv1Δ* mutants (Swain *et al*., 2002a,b). Here we investigate the reason for this abnormality and try to evaluate whether it is a consequence of defects in one or several glycosylation pathways or if it is a more direct consequence of the perturbation of the Dol cycle.

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Results

The cwh8Δ *mutants show an abnormal profile of [^{3}H]Ins-labeled lipids*

The biosynthetic pathway for the elaboration of yeast sphingolipids is shown in Figure 1. As can be seen in Figure 2A and as quantified in Figure 2B, the incorporation of [^{3}H]Ins into IPCs, particularly IPC/C is drastically decreased in *cwh8Δ*; the abnormality is observed in different genetic backgrounds, and transfection of the wild-type (wt) gene restores a normal sphingolipid profile (Figure 2A, lanes 3–5). The identity of IPCs was confirmed by mild base deacylation (data not shown). Interestingly, the level of inositolphosphoryl-MIPC ($M(IP)_2C$) is not generally affected, whereas the band comprising IPC/D and mannosyl-IPC (MIPC) usually is low but sometimes appears normal. (Our thin-layer chromatography [TLC] system resolves IPC/D and MIPC only poorly.)

As shown in Figure 3A, the relative lack of IPC/C can be observed not only at the end of a 2 h standard labeling period but is observed throughout the labeling. Even if [^{3}H]Ins-labeled cells are further incubated for 20 h allowing [^{3}H]Ins to be chased by endogenously produced cold Ins, a conspicuous absence of IPC/C, IPC/D, and MIPC in *cwh8Δ* mutants persists, but $M(IP)_2C$ is normal or increased (Figure 3B). [^{3}H]Ins was incorporated by *cwh8Δ* cells as efficiently as by wt cells although the doubling time of *cwh8Δ* cells is twice the one of wt.

In vitro *IPC synthase and ceramide synthase activities of* cwh8Δ

Phytosphingosine (PHS) is efficiently taken up by yeast cells and, when added to the culture medium, fully compensates for the lack of *LCB1*, the essential key enzyme for PHS biosynthesis (Figure 1) (Buede *et al.*, 1991). Yet, the addition of PHS or dihydrosphingosine (DHS) to the medium did not stimulate the incorporation of [^{3}H]Ins into IPC/C (Figure 4A) in *cwh8Δ* cells. This suggests that PHS production is not limiting for IPC/C biosynthesis in *cwh8Δ*. Moreover, as can be seen from Figure 4B, and as verified by quantitation of the various spots, microsomes from *cwh8Δ*, when incubated with [^{3}H]DHS and C26-CoA, make normal amounts of ceramide and at a normal rate. *Cwh8Δ* derived microsomes also make rather high amounts of IPCs when incubated with water soluble short chain [^{3}H]ceramides (Figure 4C). Finally, IPC synthesis of *cwh8Δ* cells was also found to be

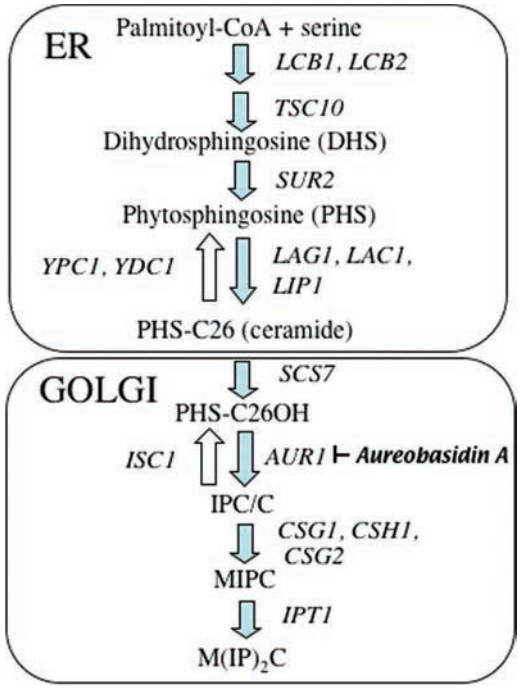

Fig. 1. Sphingolipid biosynthesis in yeast. DAG, diacylglycerol; MIPC, mannosyl-IPC; $M(IP)_2C$, inositolphosphoryl-MIPC; and PI, phosphatidyl inositol. Genes are in italics.

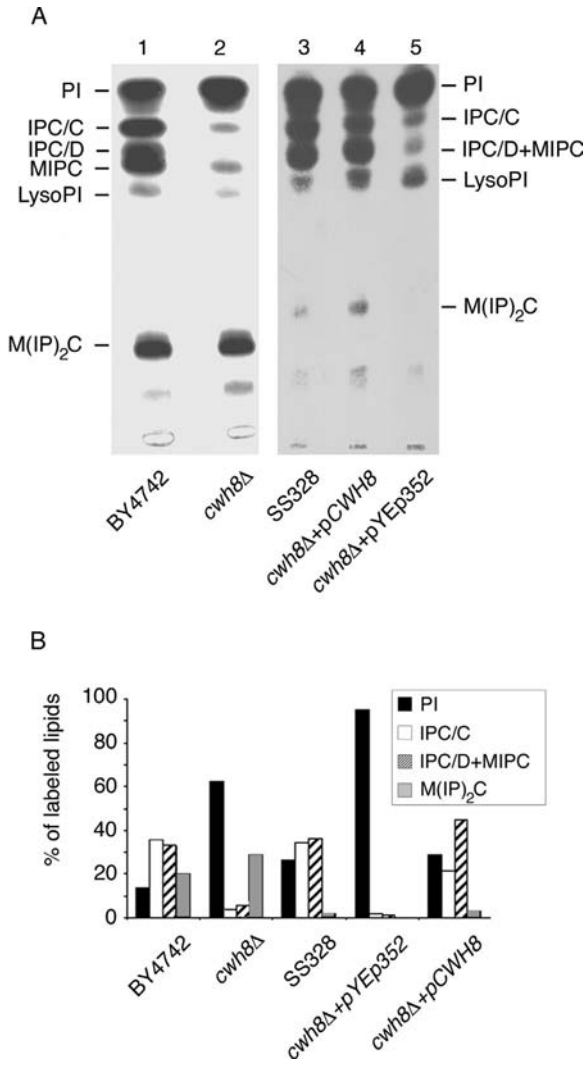

Fig. 2. Sphingolipid biosynthesis is defective in the absence of *CWH8*. (**A**) Strains with *cwh8Δ* deletions in the BY4742 or SS328 background were labeled with [^{3}H]Ins, lipids were extracted, and separated by TLC. Incorporation of [^{3}H]Ins into lipids was equally efficient in all strains. In lipid extracts stored in chloroform/methanol/water, $M(IP)_2C$ becomes insoluble over time, especially at –20°C (observation originally made by Jens Knudsen; personal communication). This explains the relative low abundance of this species in lanes 3–5. (**B**) Quantitation of results shown in panel **A** by Berthold radioscanning. The total of radioactivity in each lane is taken as 100%.

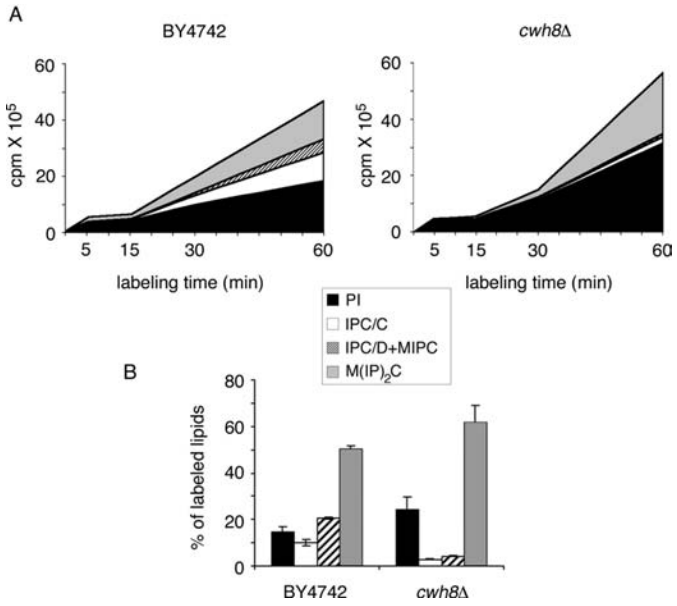

Fig. 3. Kinetics of [^{3}H]Ins incorporation in *cwh8Δ*. (**A**) Wt (BY4742) and *cwh8Δ* cells were labeled with [^{3}H]Ins for the indicated times. Lipids were extracted, separated on TLC, and the cpm in each lipid species was quantitated. (**B**) Cells were labeled with [^{3}H]Ins, and the incubation was continued for 20 h, allowing the radioactivity to be chased by the endogenously made, cold Ins. Lipids were extracted, separated on TLC, and quantified.

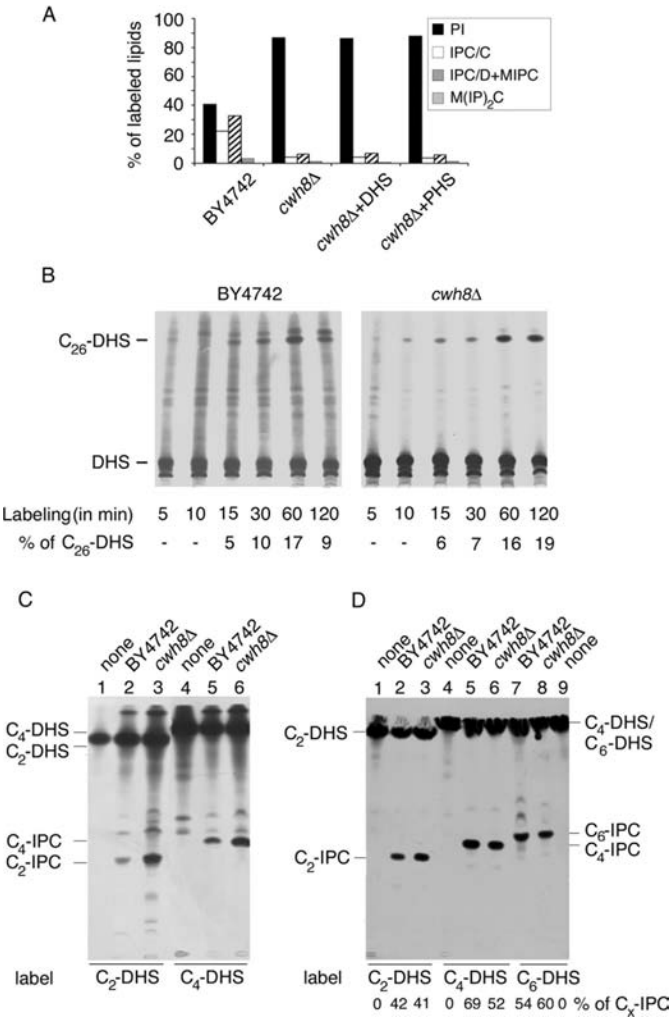

Fig. 4. The *cwh8Δ* cells make DHS, PHS, and ceramide normally. (**A**) Cells were preincubated (10 min) and labeled with [^{3}H]Ins in presence or absence of 5 μM DHS or PHS, and lipids were separated on TLC and quantitated. (**B**) Ceramide synthase *in vitro*. Microsomes were incubated with [^{3}H]DHS and C_{26}-CoA for 5–60 min. The amount of C_{26}-DHS made as a fraction of the total radioactivity is given below each lane. (**C**) C_2- or C_4-[^{3}H]DHS was incubated alone (lanes 1 and 4) or in the presence of microsomes (lanes 2, 3, 5, and 6) for 60 min at 30°C to measure IPC-synthase activity. Lipids were extracted and separated by TLC. (**D**) Intact cells were incubated with C_2-, C_4-, or C_6-[^{3}H]DHS for 2 h at 30°C. Lipids were extracted and separated by TLC. The amount of the IPC made is given as a percentage of total amount of label present in each lane.

normal when the incorporation of short chain [^{3}H]ceramides into IPCs was measured in intact cells (Figure 4D).

Fatty acid elongation, which generates C26:0-CoA, the preferred substrate of ceramide synthase (Figure 1), appears to be functioning normally in *cwh8Δ* as the TLC mobility of the remaining IPCs and of $M(IP)_2C$ is normal (Figure 2A, lanes 1 and 2).

Thus, we could not detect any deficiency in the enzymes that are required to make IPC in *cwh8Δ*.

The cwh8Δ *mutants anchor GPI proteins normally*

The cessation of the Dol cycle in *cwh8Δ* ought to cause a lack of Dol-P and thereby compromise Dol-P-Man biosynthesis at the cytosolic leaflet of the ER. Dol-P-Man is required for the biosynthesis of *N*- and *O*-glycans and of GPI anchors. A reduction in GPI anchoring by itself would not be sufficient to cause a depression of IPC synthesis, because numerous *gpi* mutants analyzed in our laboratory showed normal biosynthesis of IPCs (Canivenc-Gansel *et al*., 1998; Meyer *et al*., 2000; Fraering *et al*., 2001), but a GPI deficiency could nevertheless be required for the depression of IPC levels in *cwh8Δ* cells. To assay the biosynthesis of GPI lipids in *cwh8Δ* mutants, microsomes were labeled with uridine diphosphate (UDP)-[^{3}H]GlcNAc (Figure 5A). A normal set of GPI lipids was made by *cwh8Δ* microsomes. To see whether the GPI lipids are transferred onto GPI proteins at a normal rate we analyzed the GPI protein Gas1p, whose ER form migrates at 105 kDa and whose mature form, produced after arrival in the Golgi, migrates at 125 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). As long as a GPI anchor is not added to Gas1p, the protein is not packaged into COPII vesicles and not transported to the Golgi so that it accumulates as an immature 105 kDa form. Indeed, any significant reduction of GPI lipid biosynthesis leads to an increase of the immature 105 kDa form of Gas1p (Benghezal *et al*., 1995). As can be seen from Figure 5B, the mature form of Gas1p was present in reduced amounts in *cwh8Δ* and was slightly underglycosylated. Tunicamycin (Tm) pretreatment of wt cells shows that the ER form lacking *N*-glycans runs at 83 kDa (lane 3), whereas the absence of all *N*- and *O*-glycans results in a 60 kDa form as seen in *sec59* at 37°C. In *cwh8Δ* the zone between 105 and 60 kDa is empty, arguing that there is no accumulation of any immature ER form, and hence no lag in GPI anchor addition.

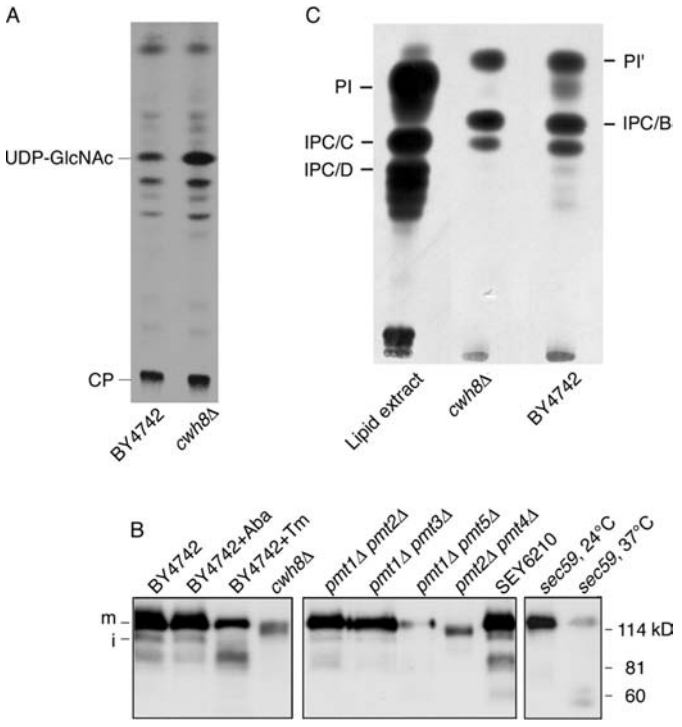

Fig. 5. The *cwh8Δ* cells add GPI anchors normally. (**A**) Microsomes were labeled with UDP-[^{3}H]GlcNAc in the presence of GDP-mannose. Lipids were extracted and migrated on a TLC. CP, complete precursor. (**B**) Proteins were extracted from the indicated strains and processed for western blotting. Blots were probed with anti-Gas1p. AbA was added at 1 μg/mL for 2 h, and *sec59* cells were grown for 4 h at 37°C before extraction. The immature (i) and mature (m) forms of Gas1p are indicated. (**C**) Cells were labeled with [^{3}H]Ins at 30°C for 2 h, GPI anchored proteins were isolated, anchor peptides were prepared, and their lipid moieties released by HNO_2 treatment as described (Guillas *et al.*, 2000). The liberated anchor lipids were analyzed by TLC. The lipid extract of the labeled BY4742 cells served as standard (lane 1).

The reduction of mature Gas1p in *cwh8Δ* was consistently observed in several experiments, but it does not necessarily mean that there is a problem with GPI anchoring, because mutants affecting other Dol-P dependent reactions can lead to a similar reduction of Gas1p. Thus, as shown in Figure 5B, Gas1p was found to be reduced in certain *pmtΔ* double mutants deficient in O-glycosylation. The dolicholkinase mutant *sec59*, which lacks Dol-P at 37°C and has a combined N-glycosylation, O-glycosylation, and GPI anchoring defect (Heller *et al.*, 1992), shows equally a severe reduction of Gas1p (Figure 5B). The reduction of Gas1p in *cwh8Δ* mutants is not a consequence of reduction of IPC/C because blocking IPC biosynthesis for 2 h using aureobasidin A (AbA) (Figure 1) does not reduce Gas1p levels (Figure 5B).

Remodeling of GPI anchor lipids is thought to be operated by an enzyme that is similar to the IPC-synthase Aur1p (Reggiori and Conzelmann, 1998). As the loss of Cwh8p may affect the IPC-synthase, we wondered if this defect also affects GPI anchor remodeling. For this, we labeled *cwh8Δ* cells with [^{3}H]Ins, isolated the lipids of the GPI anchors and analyzed them by TLC. As can be seen in Figure 5C, the spectrum of anchor lipids made by the mutants was normal, the prevalent lipids being IPC/B and a phosphatidyl inositol (PI) having C26:0 in sn2 causing a higher R_f than seen in the ordinary PI of cellular membranes (Sipos *et al.*, 1997). Thus, GPI anchor remodeling seems to proceed normally in *cwh8Δ* mutants. Also, in the experiment shown in Figure 5C, the incorporation of [^{3}H]Ins into proteins was the same for mutant and wt cells, further demonstrating that *cwh8Δ* mutants do not have any problem with GPI anchoring.

Influence of O-glycosylation defects on IPC biosynthesis

Protein O-glycosylation in the ER also uses Dol-P-Man. We therefore tested whether mutations specifically affecting O-glycosylation could alter the sphingolipid profile. The *pmt* genes encode for ER-localized protein mannosyltransferases of partially overlapping acceptor specificity (Strahl-Bolsinger *et al.*, 1999). As summarized in Table I, some cells containing the *pmt1Δ* deletion combined with the *pmt2Δ* or the *pmt5Δ* deletion show a moderate decrease of IPC biosynthesis. Moreover, *dpm1-6* mutants have a temperature sensitive defect in Dol-P-Man synthase. At 37°C these cells do not transfer any *O*-glycans, are unable to synthesize GPI lipids but transfer incomplete $GlcNAc_2$-Man_5-Glc_3 oligosaccharides to proteins (Orlean, 1990). The *dpm1-6* mutant shows only a moderate decrease of the IPC biosynthesis although it has a significant difficulty in making IPC/D-MIPC and $M(IP)_2C$ (Table I), this concomitant decrease being unique for this strain.

Hsp150, a secretory protein which carries a high amount of *O*-glycans, but no *N*-glycans, is underglycosylated or fails to be secreted in *pmt1Δ*, *pmt2Δ*, *pmt4Δ*, and *sec59* mutants (Figure 6) (Gentzsch and Tanner, 1997). Removal of *O*-glycans using hydrofluoric acid reduces its molecular mass from 150 to 47 kDa (Russo *et al.*, 1992). Yet, *cwh8Δ* makes normally glycosylated Hsp150 with only small amounts being underglycosylated (Figure 6). Thus, it would appear that O-glycosylation defects in principle can slightly depress IPC/C levels but that, in contrast to N-glycosylation, the O-glycosylation is not affected in *cwh8Δ* mutants and that an O-glycosylation defect therefore cannot explain the deficiency in IPC/C of *cwh8Δ* mutants.

Influence of the N-glycosylation defects on IPC biosynthesis

Treatment of wt cells with the N-glycosylation inhibitor Tm, which specifically inhibits the transfer of GlcNAc-P onto Dol-P, induces a drastic decrease of [^{3}H]Ins incorporation into

Table I. O-Glycosylation mutants

Strain	PI + LysoPI	IPC/C	IPC/D + MIPC	$M(IP)_2C$
SEY6210	48.0	15.7	24.1	10.6
SEY6211	24.7	29.1	27.1	17.5
pmt1Δ pmt2Δ	36.8	10.5	44.5	6.2
pmt1Δ pmt3Δ	29	23.1	26.9	18.5
pmt1Δ pmt5Δ	57	11.2	19.1	11.4
pmt2Δ pmt4Δ	28.8	35.1	20.7	14.1
dpm1-6, 24°C	39.2	29.2	20.6	9.9
dpm1-6, 37°C	60.8	21.9	8.2	5

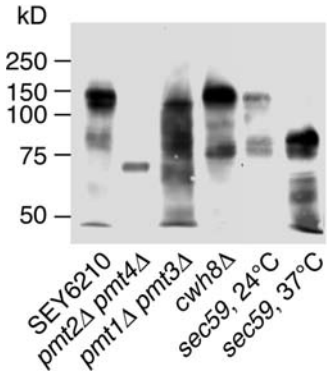

Fig. 6. *Cwh8Δ* cells O-glycosylate proteins normally. Cells were grown at 30°C or the indicated temperatures, and cell lysates were analyzed by western blotting using antibodies against Hsp150 (Pir2p).

IPC (Figure 7A and B). Cells incorporated [^{3}H]Ins efficiently into M(IP)$_2$C, and this was true even after prolonged preincubation in 10 μg/mL of Tm or when the labeling was extended to 20 h allowing the label to be chased by the endogenous Ins production. Thus, inhibition of N-glycosylation using Tm seems to faithfully mimic the sphingolipid abnormality of *cwh8Δ* mutants (Figure 7C).

To evaluate if a particular structural feature of *N*-glycans had an impact on IPC/C levels, we tested a series of N-glycosylation mutants. Strains deleted for *ALG3*, *ALG6*, *ALG8*, and *ALG9*, which cannot add all mannoses and glucoses to Dol-PP-GlcNAc$_2$-Man$_5$ in the lumen of the ER, did not present any defect of sphingolipid biosynthesis (Table II). The truncated oligosaccharides added in these mutants can still get elongated in the Golgi, whereby the molecular mass of glycoproteins can be massively increased. Therefore we also tested *och1Δ*, a mutant in which elongation of *N*-glycans is completely abolished (Nakanishi-Shindo *et al*., 1993). The *och1Δ* cells made normal amounts of IPC but showed a moderate reduction of IPC/D-MIPC and a compensatory increase of M(IP)$_2$C (Table II). IPC levels were, however, depressed in certain mutants having defects in the OST such as the *ost5Δ alg5Δ* and in also cells depleted of Rft1p but not in others such as *ost3Δ ost6Δ* (Figure S1; Table II). Analysis of the N-glycosylation status of CPY showed that the mutants having decreased IPC/C levels also had severe underglycosylation of CPY (Figure 7D). Thus, it appears that a defect of IPC synthesis is not correlated with the absence of some particular structural feature of the *N*-glycan core, but rather maybe the consequence of severe N-underglycosylation. The correlation also holds in *rer2-2*, a mutant that has been reported to have a severe hypoglycosylation phenotype affecting not only N- but also O-glycosylation and GPI anchoring (Sato *et al*., 1999). As shown in Table II and Figure 7D, the *rer2-2* strain exhibited only a moderate decrease of IPC-C levels, but CPY also was less severely underglycosylated than in *cwh8Δ*.

The effects of the unfolded protein response on the sphingolipid profile

Underglycosylation and Tm treatment induce an unfolded protein response (UPR) (Travers *et al*., 2000). Using a plasmid

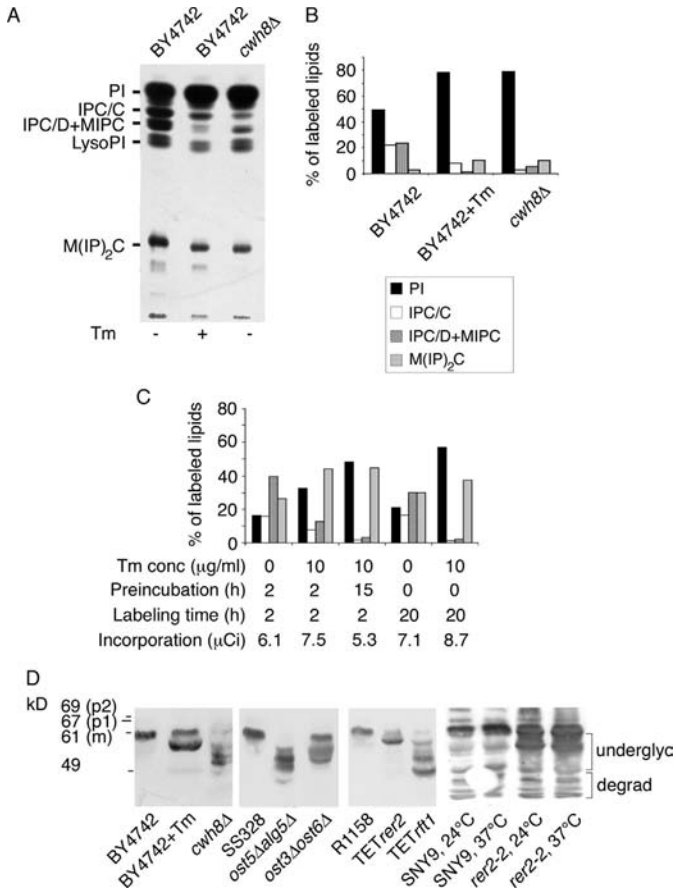

Fig. 7. Tm causes a reduction in IPC/C levels. (**A**) Cells were preincubated for 2 h in SDaaUA with or without Tm (2.5 μg/mL) and labeled with [^{3}H]Ins. (**B**) Quantification of lipids in panel **A**. (**C**) Cells were labeled with [^{3}H]Ins during 2 or 20 h after preincubations of 0, 2, or 15 h in presence of Tm; labeled lipids were analyzed by TLC. Total incorporation into lipids is given for each condition (μCi). (**D**) CPY western blot analysis. Proteins were extracted from cells having been exposed to Tm (10 μg/mL) for 2 h, doxycyclin (150 μg/mL) for 15 h, or elevated temeprature (37°C) for 2 h. The completely deglycosylated mature form of CPY has a peptide mass of 47.4 kDa; smaller fragments are presumably degradation products.

carrying an UPR responsive element (UPRE) in the promoter in front of lacZ we found that the UPR was indeed constitutively activated in *cwh8Δ* (data not shown). Also, secretion of Kar2p, an ER chaperone, has been recognized as a sign of UPR activation. As shown in Figure 8A, *cwh8Δ* cells secrete significant amounts of Kar2p, significantly more than what is secreted by wt cells exposed to Tm or AbA. The UPR strongly induces the transcription of *INO1*, the rate limiting enzyme for Ins and PI biosynthesis in yeast (Figure 1) (Cox *et al*., 1997). We indeed observed that *cwh8Δ* overproduce and secrete Ins, that is, that they display the so called *opi* phenotype (Figure S2). We wondered if the decreased incorporation of [^{3}H]Ins into IPC/C in *cwh8Δ* mutants and Tm-treated cells could be explained by an increase in Ins and PI biosynthesis, so that *cwh8Δ* cells, when labeled with [^{3}H]Ins, would make PI and IPC/C of lower specific activity than wt but in normal amounts. This hypothesis seemed unlikely because *cwh8Δ* cells incorporate normal amounts of [^{3}H]Ins into M(IP)$_2$C (Figures 2A and 3).

Table II. N-Glycosylation mutants

Strain	PI + LysoPI	IPC/C	IPC/D + MIPC	$M(IP)_2C$
BY4742	13.1	34.5	31.7	19.3
cwh8Δ	60.6	4.1	5.3	28.1
alg3Δ	44.1	19.8	21.1	14.0
alg6Δ	45.8	19.6	23.1	10.7
alg8Δ	47.9	15.4	22.7	13.2
alg9Δ	44.4	19.6	25.2	10.0
och1Δ	23.7	32.5	15.2	27.9
R1158	24.5	28.9	31.7	11.3
Tetrft1	49.7	9.2	6.6	33.1
SS328	23.6	26.0	34.4	13.5
ost5Δ alg5Δ	80.2	2.4	5.2	11.2
ost3Δ ost6Δ	23.7	20.1	31.5	23.4
SNY9	26.7	25.2	30.7	16.0
rer2-2, 24°C	42.3	17.0	30.7	7.9
rer2-2, 37°C	33.2	18.6	40.7	5.8

Table III. Influence of UPR and secretion blocks on IPC/C level

Strain	PI + LysoPI	IPC/C	IPC/D + MIPC	$M(IP)_2C$
BY4742	56.6	11.0	8.4	22.7
cwh8Δ	79.6	1.8	1.7	15.2
SS328	43.8	22.6	26.6	5.9
SS328 + p*HAC-i*	30.8	23.2	36.9	8.0
sec14, 24°C	50.5	12.0	8.9	27.3
sec14, 37°C	38.8	21.5	26.0	10.8
sec7,[a] 24°C	25.5	19.5	29.8	24.4
sec7,[a] 37°C	30.9	38.2	13.3	16.6
sec7,[a] 24°C	32.8	30.9	19.9	15.7
sec7,[a] 37°C	40.6	39.3	8.7	9.4
sec7,[a] 24°C	45.2	28.0	18.0	8.0
sec7,[a] 37°C	34.4	39.4	9.7	15.3

[a]Three different *sec7* strains.

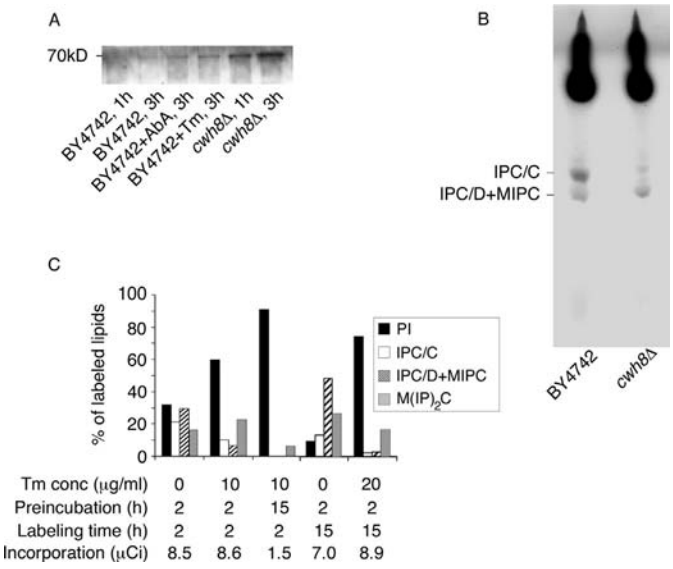

Fig. 8. The abnormal sphingolipid profile of *cwh8Δ* is not caused by its UPR. (**A**) Strains were inoculated at 0.5 OD600, cultured for 1 or 3 h at 30°C, and media were collected, trichloroacetic acid (TCA) precipitated, and processed for western blot detection of Kar2p, a 70-kDa protein. (**B**) Lipids were extracted from unlabeled cells, deacylated, and 25 OD600/strain were separated by TLC. Lipids were detected by charring. Control experiments showed that the admixture of the same amount of cold lipids to [^{3}H]Ins-labeled lipids leads to the disappearance (precipitation) of $M(IP)_2C$ (data not shown). (**C**) Aliquots of *ire1Δ* were labeled with 10 μCi of [^{3}H]Ins using different preincubation times, labeling times, and concentrations of Tm. Total incorporation into lipids is given for each condition (μCi).

It was further rendered unlikely by experiments, in which cold Ins (up to 50 mg/L) was added to the labeling medium. Addition of cold Ins did not significantly change the fraction of [^{3}H]Ins incorporated into sphingolipids (Figure S3). Furthermore, the relative lack of IPC/C could also be seen, when the sphingolipids of *cwh8Δ* cells were revealed by charring (Figure 8B). Finally, we tested other mutants displaying a strong *opi* phenotype such as *sec14* (Henry and Patton-Vogt, 1998), or cells in which the UPR is constitutively induced by the presence of *HAC1-i*, a spliced, that is, active version of the *HAC1* transcription factor. Neither one of these cells showed a decreased IPC/C biosynthesis (Table III).

Treatment with Tm or deletion of *CWH8* leads to an accumulation of underglycosylated proteins, which invariably induce an UPR. To test if the UPR, although not sufficient, is required to provoke reduced IPC biosynthesis under Tm treatment, we tested if Tm can induce the reduction of IPC in *ire1Δ* cells which are unable to induce an UPR (Sidrauski and Walter, 1997). As shown in Figure 8C, IPC/C biosynthesis is still Tm sensitive in *ire1Δ* cells.

Thus, it seems that the altered sphingolipid profile of the *cwh8Δ* mutant is not a consequence of its constitutive UPR or its increased biosynthesis of Ins but is caused solely by the N-underglycosylation and that the associated UPR response is not required for the appearance of the abnormal sphingolipid profile.

Isc1p, an IPC hydrolase may, if mislocalized, selectively destroy IPC (Sawai *et al.*, 2000). However, Tm causes the characteristic drop in IPC/C and IPC/D-MIPC labeling also in *isc1Δ* cells (data not shown). This argues that Isc1p is not involved in the depression of IPC levels when N-glycosylation is repressed.

Quantification of Aur1p in cwh8Δ *mutants*

Aur1p is an integral membrane protein with seven membrane spanning domains and three potential N-glycosylation sites, two of which are predicted to be lumenally exposed by the program displayed at http://www.cbs.dtu.dk/services/TMHMM/. The *cwh8Δ* mutants were crossed with TLY139, a strain, in which the genomic *AUR1* gene had been tagged with an hemagglutinin antigen (HA) tag. As shown in Figure 9A, spore 8A (*cwh8Δ*) contains as much Aur1p-HA as spore 8C (*CWH8*), and the Aur1p does not seem to be underglycosylated in the *cwh8Δ* background, suggesting that none of its

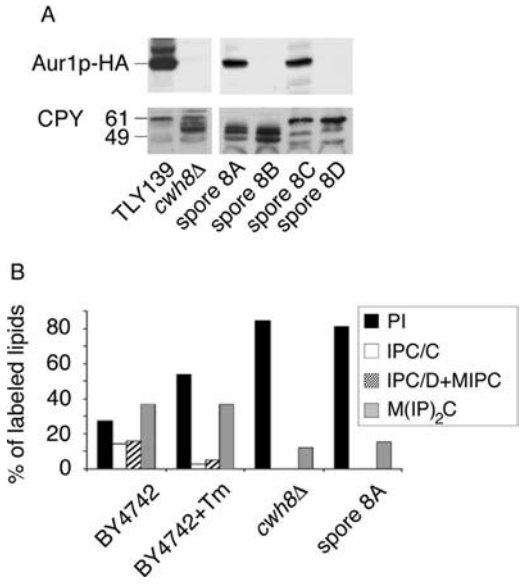

Fig. 9. Aur1p expression is normal in *cwh8Δ*. (**A**) TLY139, a wt strain containing a HA-tagged version of Aur1p was crossed with *cwh8Δ*, and a tetrad obtained from the diploid was analyzed by western blotting with antibodies against the HA tag and CPY. Aur1p-HA is a 45-kDa protein. Underglycosylation of CPY in spores **A** and **B** indicates the presence of the *cwh8::KanMX* allele. (**B**) Cells were labeled with [^{3}H]Ins, and lipids were quantified by TLC.

three potential N-glycosylation sites is used. Also, the apparent molecular weight of Aur1p-HA was unchanged, when TLY139 (*AUR1-HA*) cells were grown in Tm (data not shown). The spore 8A (*cwh8Δ AUR1-HA*) showed the expected defect in IPC biosynthesis (Figure 9B). We thus conclude that the relative lack of IPC biosynthesis in *cwh8Δ* mutants cannot be explained by a loss or by underglycosylation of Aur1p.

The *cwh8Δ* cells accumulate internal membranes. Electron microscopy pictures of *rer2* cells, being deficient in cytosolic Dol-P as *cwh8Δ* cells, show an aberrant proliferation of ER and Golgi membranes (Sato *et al*., 1999). Electron microscopy of *cwh8Δ* cells typically shows fragmentation of vacuoles and a discrete proliferation of internal membranes that has ER-like morphology (Figure S4). Vacuolar fragmentation is equally seen using FM4-64, a compound that stains vacuolar membranes (data not shown). This is somewhat in contrast to the morphological classification of *cwh8Δ* as class C *vps* mutant (Bonangelino *et al*., 2002), as the presence of fragmented vacuoles would place them rather into the *vps* mutant class B (Raymond *et al*., 1992). The accumulation of internal membranes is reminiscent of what was described for *rer2-2* mutants. In our hands the accumulation of membranes in *rer2-2* was not more pronounced than what was observed in *cwh8Δ* (data not shown).

General properties of cwh8Δ *mutants*

For many sphingolipid biosynthesis mutants the concentrations of Ca^{2+} and Zn^{2+} in the medium are critical, but growth of *cwh8Δ* mutants was not influenced by the presence of these cations in the medium (data not shown). Also, they are respiration competent.

Discussion

In *S. cerevisiae*, the main sphingolipids are IPC, MIPC, and $M(IP)_2C$ and represent major components of the plasma membrane (Dickson and Lester, 1999). Aur1p, the IPC synthase is essential, whereas the later acting genes required for making MIPC and $M(IP)_2C$ have been shown to be dispensable (Lisman *et al*., 2003). Mutations in ergosterol biosynthesis have been shown to alter sphingolipid biosynthesis in two recent reports: One report demonstrates that *erg26-1* mutants specifically decrease IPC and MIPC, whereas $M(IP)_2C$ remains normal (Swain *et al*., 2002a); another report shows that cells lacking Arv1p, a multispanning integral membrane protein required for sterol uptake and intracellular sterol distribution, greatly reduce the biosynthesis of all IPCs including $M(IP)_2C$ (Swain *et al*., 2002b). It is presently unknown, by what mechanism the ergosterol and sphingolipid biosynthesis pathways influence each other.

Here we examined *cwh8Δ* mutants which show a sphingolipid profile similar to the one of *erg26-1* cells because we find IPCs to be reduced, whereas $M(IP)_2C$ levels are normal. Our data on *cwh8Δ* indicate that the flux through the pathway leading to the synthesis of $M(IP)_2C$ is normal but that intermediates IPC/C and often IPC/D-MIPC are strongly reduced. The *cwh8Δ* cells contain normal amounts of ergosterol and of the biosynthetic intermediates of the ergosterol pathway (Claude Jakob *et al.*, personal communication) so that the abnormality of its sphingolipid profile cannot be attributed to a deficiency in ergosterol biosynthesis. Rather the abnormal sphingolipid profile seems to be caused by a defect in N-glycosylation.

Indeed the lack of *CWH8* seems to affect mainly the N-glycosylation pathway, whereas O-gylcosylation and GPI anchoring are not or only slightly affected (Figures 5 and 6; Table I). As pointed out before, the N-underglycosylation could be due to either a relative lack of Dol-P at the cytosolic side of the ER membrane or an inhibitory effect of the accumulated Dol-PP (Fernandez *et al*., 2001). However, the profile of Dol-linked oligosaccharides of *cwh8Δ* (van Berkel *et al*., 1999) suggests that there is no inhibiton of the lumenal glycosylation reactions transforming Dol-PP-$GlcNAc_2$-Man_5 into Dol-PP-$GlcNAc_2$-Man_9-Glc_3 and of the OST. This finding suggests that *cwh8Δ* suffers from a relative lack of cytosolic Dol-P than Dol-PP accumulation. The lack of cytosolic Dol-P of *cwh8Δ* cells is predicted by the so called Dol cycle model. Our calculations (Table SI) indicate that indeed the content of Dol in ER membranes is by far insufficient to support all the manifold glycosylation reactions that occur unless Dol can be recycled. Most importantly, the finding that Tm treatment and certain *ost* or *rft1* mutants faithfully reproduce the abnormal sphingolipid profile of *cwh8Δ* cells clearly indicates that the lack of Dol-P or the accumulation of Dol-PP do not affect sphingolipid biosynthesis directly, but they do so through the ensuing N-underglycosylation.

If a relative lack of cytosolic Dol-P of *cwh8Δ* cells can affect only N- but no other glycosylation reactions, we also have to assume that the various pathways that depend on cytosolic Dol-P have different affinities for, or different accessibility to Dol-P. Even though only Dol-PP-GlcNAc synthesis is affected in *cwh8Δ*, the total amount of Dol-P required is significantly reduced in this mutant as each Dol-PP-GlcNAc that is not made spares the system the need of making further 4 Dol-P-Man and 3 Dol-P-Glc.

By what mechanism does N-underglycosylation depress IPC/C levels? For the moment we speculate that severe underglycosylation leads either to some perturbation of the distribution of the sphingolipid biosynthetic enzymes, or to a size reduction of some biosynthetic compartment ("compartment size hypothesis"), or to the fusion of some normally distinct compartments ("compartment fusion hypothesis").

Protein mislocalization may occur in *cwh8Δ* cells as they have a similar problem as the Dol biosynthesis mutant *rer2*. The *rer2* cells contain only 25% of normal levels of Dol and show defects in N- and O-glycosylation as well as in GPI biosynthesis (Sato *et al.*, 1999). They mislocalize several ER components such as Sec12p, Sec71p, and Sec63p to later secretory compartments but the distribution of Golgi proteins in *rer2* cells has not been investigated. Hence, the defect in sphingolipid biosynthesis of *cwh8Δ* cells may be due to mislocalization of some essential component of IPC biosynthesis to a late Golgi compartment.

The potential validity of the "compartment fusion hypothesis" is underscored by the observation that brefeldin A drastically reduces the IPC and MIPC biosynthesis without affecting biosynthesis of $M(IP)_2C$ (Hechtberger and Daum, 1995). The result may be interpreted as to mean that the relocalization of Aur1p, Csg1p, Csh1p, and the $M(IP)_2C$-synthase Ipt1p (Figure 1) from the Golgi into the ER under brefeldin A, while reducing the overall activity of these enzymes, will allow whatever product is made to get immediate access to all enzymes in the pathway, thus allowing for the disappearance of intermediates in face of a slightly enhanced synthesis of $M(IP)_2C$. The analysis of the relative abundance of IPC/C and MIPC in the secretion mutants *sec7* and *sec14* equally argues that the sphingolipid biosynthesis in the yeast Golgi is compartmentalized. Although not pointed out, the data in two earlier reports (Puoti *et al.*, 1991; Hechtberger *et al.*, 1994) clearly indicate that IPC and MIPC synthesis may reside in different Golgi compartments: *sec7* mutants (blocking early in the Golgi) accumulate IPC but make relatively little MIPC, whereas *sec14* mutants (blocking exit from late Golgi) accumulate high amounts of both. These experiments were reproduced and quantitated in this study (Table III). The data clearly suggest that Aur1p can act in an earlier Golgi compartment than the mannosyltransferases Csg1p and Csh1p. This may seem to be in contradiction with a recent report showing that the immunoisolation of Aur1p-HA-containing microsomes brings down the same fraction of total Csg1p as of total Aur1p, suggesting that these two proteins reside in the same compartment (Lisman *et al.*, 2003). Yet, this result does not exclude the existence of a compartment containing only Aur1p but no Csg1p, and the immunofluorescence studies reported in the same paper show only partial overlap between Csg1p and Aur1p (Lisman *et al.*, 2003). Under the assumption that Aur1p, Csg1p, and maybe Ipt1p (Figure 1) reside in different compartments, the level of IPC and MIPC present in a given cell may be a reflection of the relative size of the early and mid Golgi compartment. It seems conceivable that the drastic reduction of Golgi-based *N*-glycan elongation in *cwh8Δ* mutants may lead to a size reduction of the $Aur1p^{+}/Ipt1p^{-}$ Golgi compartment and thus reduce the time during which IPC/C stays in that compartment. To test this "compartment size hypothesis" we tried to see whether other changes in Golgi morphology would change the relative amounts of IPC. For this we used cells overexpressing Vig4p, the GDP-mannose transporter of the Golgi because overexpression of this gene has been shown to lead to the appearance of very massive Golgi-like structures in yeast (Hashimoto *et al.*, 2002). However, overexpression of *VIG4* did not increase the relative amount of IPC or MIPC as compared to the total amount of sphingolipids (data not shown). Studies to test the "compartment fusion hypothesis" are ongoing.

In summary, this and other recent studies reveal that, beyond the genes that directly are involved in the biosynthesis of yeast sphingolipids such as *ELO2*, *AUR1*, *CSG1*, *CSH1,* and *IPT1*, sphingolipid biosynthesis is indirectly affected by alterations in sterol biosynthesis, N-glycosylation, and vesicle traffic, although further efforts are required to unravel the mechanisms by which this occurs.

Materials and methods

Materials

Cells were grown at 30°C in rich medium yeast extract peptone dextrose (YPD) or minimal media (e.g., salt dextrose) containing salts (S), 2% glucose (D) as a carbon source, uracil (U), adenine (A), and amino acids or casein hydrolysate (C) as described (Sherman, 2002). Wt strains were BY4742 *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*; SS328 *MATα ade2-101 ura3-52 his3-Δ200 lys2-801*; SEY6210 *MATα ura3-52 leu2-3,112 his3-Δ200 lys2-801 trp1-Δ901 suc2-Δ9*; SEY6211 *MATα ura3-52 leu2-3,112 his3-Δ200 ade2-101 trp1-Δ901 suc2-Δ9*; and R1158 *MATα ura3::CMV-tTA his3-1 leu2Δ0 met15Δ0*. Mutant strains were *cwh8Δ-1 YGR036c::kanMX4* in BY4742; *cwh8Δ-2 YGR036c::HIS3* in SS328; *dpm1 MATα dpm1-6 ura3*; SNH023-7D *MATα rer2-2 mfα1::ADE2 mfa2::TRP1 bar1::HIS3 ura3 trp1 leu2 his3 ade2 lys2*; HMSF169 *MATa sec14-3; alg3Δ YBL082c::kanMX4* in BY4742; *alg6Δ YOR002w::kanMX4* in BY4742; *alg8Δ YOR067c::kanMX4* in BY4742; *alg9Δ YNL219c::kanMX4* in BY4742; *ost5Δ alg5Δ ost5::HIS3* alg5::HIS3 in SS328; RKY327 *ost3Δost6Δ MATα ade2-101 ura3-52 his3Δ200 ost3::HIS3 ost6Δ*; *pmt1Δ pmt2Δ pmt1::URA3 pmt2::LEU2* in SEY6210; *pmt1Δ pmt3Δ pmt1::URA3 pmt3::HIS3* in SEY6210; *pmt1Δ pmt5Δ pmt1::HIS3 pmt5::URA3* in SEY6210; *pmt2Δ pmt4Δ pmt2::URA3 pmt4::TRP1*; Tet*rft1 pRFT1::kanR-tet07-TATA URA3::CMV-tTA; ire1Δ YHR079c::kanMX4* in BY4742; TLY139 *aur1::AUR1-3XHA-HIS5(S.p)* in SEY6210; FBY337 *YGR036c::kanMX4 aur1::AUR1-3XHA-HIS5(S.p) his3 leu2 ura3*; *och1Δ YGL038c::kanMX4* in BY4742; NY758 *sec7-1*; NY878 *MATa sec7 ura3 leu2*; and NY966 *MATa sec7-1 ura3 leu2 his3*. Plasmids p*CWH8*, p*MPG1*, pSV463VIG4, and pRC43 contain *CWH8*, *MPG1*,

VIG4, and *HAC1-i* (spliced, constitutively active *HAC1*), respectively.

All chemicals were from commercial suppliers. Tm was from Fluka (Buchs, Switzerland). Antibody against Hsp150p was kindly provided by Marja Makarow (Helsinki, Finland), antibodies against Kar2p were a gift from Dr. Marc Rose, and antibodies against CPY and the HA tag were from Molecular Probes (Eugene, OR).

Metabolic labeling of cells with [^{3}H]Ins

Cells growing in YPDUA were labeled in SDamino acids UA at 30°C during 2 h. To 2.5 OD600 units of cells in 250 µL of medium, preincubated for 10 min, 4 µCi [^{3}H]Ins per OD600 unit were added. After 40 min, the culture was diluted with 750 µL of fresh medium and further incubated. The labeling was stopped by the addition of trichloroacetic acid (TCA) to a final concentration of 5%. After 5 min on ice, the cells were centrifuged and washed twice with 1 mL of water. Lipids were extracted twice using 500 µL of EtOH : H_2O : Et_2O : pyridine : 25% NH_4OH, 15:15:5:1:0.018 for 20 min at 60°C. Lipids were desalted by butanol/water partitioning and stored at –20°C. In lipid extracts stored in chloroform/methanol/water, $M(IP)_2C$ becomes insoluble over time, especially at –20°C (Jens Knudsen, personal communication). This explains the relative low abundance of this species in certain experiments.

Microsomal assays

Microsomes were prepared and labeled with UDP-[^{3}H]GlcNAc as described (Canivenc-Gansel *et al*., 1998). Lipids were reextracted with 500 µL $CHCl_3$: MeOH : H_2O, 10:10:3.

Lipid analysis

Lipids were separated by TLC on silica gel 60 plates developed in $CHCl_3$: MeOH : H_2O, 10:10:3, or $CHCl_3$: MeOH : KCl 0.25%, 55:45:5, or $CHCl_3$: MeOH : KCl 0.25%, 55:45:10. Plates were read with a Berthold radioscanner and then sprayed with EN^{3}HANCE and laid on X-Omat film (Kodak, Renens, Switzerland) for autoradiography. Desalted lipids were deacylated in 500 µL of 33% monomethylamine (MMA) at 53°C for 2 h. Lipids were detected by charring as described (Haak *et al*., 1997).

Assay of ceramide synthase and IPC synthase

Ceramide synthase and IPC synthase activities were assayed in 100 µL Tris-buffer, pH 7.5 supplemented with 1 mM of each, ATP, GTP, CTP, GDPMan, CoA, 10 mg/mL creatine phosphate, 10 µg/mL creatine kinase, 300 µM C26-CoA (in Zwittergent), and 10 µCi of [^{3}H]DHS, C_2-[^{3}H]DHS, C_4-[^{3}H]DHS, or C_6-[^{3}H]DHS at 37°C for 1 h. Products were separated on TLC with $CHCl_3$: MeOH : 2 M NH_4OH, 40:10:1 (ceramide synthase) or with $CHCl_3$: MeOH : KCl (0.25%), 55:45:10 (IPC synthase).

Western blotting

Washed cells were extracted as described (Kushnirov, 2000). Proteins were separated under reducing conditions by SDS–PAGE, transferred to polyvinylidene fluoride (PVDF) membranes, and detected with enhanced chemiluminescence technology (Amersham Biosciences, Buckinghamshire, UK).

Supplementary data

Supplementary data are available at *Glycobiology* online (http://glycob.oxfordjournals.org/).

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Abbreviations

AbA, aureobasidin A; CPY, carboxypeptidase; DHS, dihydrosphingosine; Dol, dolichol; Dol-P, dolichylphosphate; Dol-PP, dolichylpyrophosphate; ER, endoplasmic reticulum; GPI, glycosyl-phosphatidyl inositol; HA, hemagglutinin antigen; Ins, inositol; IPC, inositolphosphorylceramide; $M(IP)_2C$, inositolphosphoryl-MIPC; MIPC, mannosyl-IPC; OST, oligosaccharyltransferase; PHS, phytosphingosine; PI, phosphatidyl inositol; TLC, thin-layer chromatography; Tm, tunicamycin; UDP, uridine diphosphate; UPR, unfolded protein response; wt, wild type; YPD, yeast extract peptone dextrose.

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