The N-glycosylation defect of $cwh8\Delta$ yeast cells causes a distinct defect in sphingolipid biosynthesis

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Running head:

Sphingolipid biosynthesis in $cwh8\Delta$
Abstract

CWH8/YGR036c of Saccharomyces cerevisiae has been identified as a dolichylpyrophosphate phosphatase that removes a phosphate from the dolichylpyrophosphate (Dol-PP) generated by the oligosaccharyltransferase while it adds N-glycans to nascent glycoproteins in the ER. Lack of CWH8 was proposed to interrupt the so called dolichol cycle by trapping dolichol 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)₂C) is not affected. Microsomes of cwh8Δ contain normal ceramide synthase and IPC synthesis activities. Within a large panel of mutants affecting dolichol dependent pathways such as N- or O-glycosylation, or 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 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.

(203 words)

Keywords: Dolichol, Golgi, unfolded protein response, AUR1
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 CPY or invertase are N-glycosylated. The structure of the transferred oligosaccharides is however normal (van Berkel et al., 1999). Cwh8Δ accumulate dolicholpyrophosphate (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 4 transmembrane domains having a lumenally oriented active site (Fernandez et al., 2001). Indeed, Dol-PP is generated in the 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 dolichol (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-GlcNAc2-Man5, which all are then flipped to the luminal leaflet where they serve as donors for N- and O-glycosylation as well as GPI anchor biosynthesis. The flippase for Dol-PP-GlcNAc2-Man5 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 \textit{cwh8}$\Delta$ (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 \textit{cwh8} and \textit{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, \textit{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 \textit{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 [\textsuperscript{3}H]Inositol ([\textsuperscript{3}H]Ins) we incidentally observed that \textit{cwh8}$\Delta$ cells display a strong reduction of inositolphosphorylceramides (IPCs) similar to what was recently found in \textit{erg26-1} and \textit{aro1}$\Delta$ mutants (Swain et al., 2002a; Swain et al., 2002b). Here we investigate the reason for this abnormality and try to evaluate if 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.
Results

The cwh8Δ mutants show an abnormal profile of [3H]Inositol labeled lipids

The biosynthetic pathway for the elaboration of yeast sphingolipids is shown in Fig. 1. As can be seen in Fig. 2A and as quantified in Fig. 2B, the incorporation of [3H]inositol ([3H]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 (Fig. 2A, lanes 3-5). The identity of IPCs was confirmed by mild base deacylation (not shown). Interestingly, the level of M(IP)_2C is not generally affected while the IPC/D-MIPC band usually is low but sometimes appears normal. (Our TLC system resolves IPC/D and MIPC only poorly.)

As shown in Fig. 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 [3H]Ins-labeled cells are further incubated for 20 hours allowing [3H]Ins to be chased by endogenously produced cold inositol, a conspicuous absence of IPC/C, IPC/D and MIPC in cwh8Δ mutants persists but M(IP)_2C is normal or increased (Fig. 3B). [3H]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 are normal

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 (Fig. 1)(Buede et al., 1991). Yet, the addition of PHS or DHS to the medium did not stimulate the incorporation of [3H]Ins into
IPC/C (Fig. 4A) in *cwh8Δ* cells. This suggests that PHS production is not limiting for IPC/C biosynthesis in *cwh8Δ*. Moreover, as can be seen from Fig. 4B, and as verified by quantitation of the various spots, microsomes from *cwh8Δ*, when incubated with [3H]dihydrosphingosine ([3H]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 [3H]ceramides (Fig. 4C). Finally, IPC synthesis of *cwh8Δ* cells was also found to be normal when the incorporation of short chain [3H]ceramides into IPCs was measured in intact cells (Fig. 4D).

Fatty acid elongation, which generates C26:0-CoA, the preferred substrate of ceramide synthase (Fig. 1), appears to be functioning normally in *cwh8Δ* as the TLC mobility of the remaining IPCs and of M(IP)_2C is normal (Fig. 2A lanes 1, 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 lab 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 UDP-[³H]GlcNAc (Fig. 5A). A normal set of GPI lipids was made by \textit{cwh8Δ} microsomes. To see if the GPI lipids are transferred onto GPI proteins at a normal rate we analyzed the GPI protein Gas1p, whose ER form migrates at 105kD and whose mature form, produced after arrival in the Golgi, migrates at 125kD by 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 105kD form. Indeed, any significant reduction of GPI lipid biosynthesis leads to an increase of the immature 105 kD form of Gas1p (Benghezal et al., 1995). As can be seen from Fig. 5B, the mature form of Gas1p was present in reduced amounts in \textit{cwh8Δ} and was slightly underglycosylated. Tunicamycin (Tm) pretreatment of wt cells shows that the ER form lacking N-glycans runs at 83 kD (lane 3) while the absence of all N- and O-glycans results in a 60 kD form as seen in \textit{sec59} at 37°C. In \textit{cwh8Δ} the zone between 105 kD and 60 kD is empty, arguing that there is no accumulation of any immature ER form, and hence no lag in GPI anchor addition. The reduction of mature Gas1p in \textit{cwh8Δ} was consistently observed in several experiments, but it does not necessarily mean that there is a problem with GPI anchoring, since mutants affecting other Dol-P dependent reactions can lead to a similar reduction of Gas1p. Thus, as shown in Fig. 5B, Gas1p was found to be reduced in certain \textit{pmtΔ} double mutants deficient on O-glycosylation. The dolichokinase mutant \textit{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 (Fig. 5B). The reduction of Gas1p in \textit{cwh8Δ} mutants is not a consequence of reduction of IPC/C, since blocking IPC biosynthesis for 2 hours using aureobasidin A (AbA) (Fig. 1) does not reduce Gas1p levels (Fig. 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 $cwh8\Delta$ cells with $[^3]$H]Ins, isolated the lipids of the GPI anchors and analyzed them by TLC. As can be seen in Fig. 5C, the spectrum of anchor lipids made by the mutants was normal, the prevalent lipids being IPC/B and a 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 $cwh8\Delta$ mutants. Also, in the experiment shown in 5C, the incorporation of $[^3]$H]Ins into proteins was the same for mutant and wt cells, further demonstrating that $cwh8\Delta$ 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 shown in Table I, some cells containing the $pmt1\Delta$ deletion combined with the $pmt2\Delta$ or the $pmt5\Delta$ 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)$_2$C (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 \textit{pmt1\Delta}, \textit{pmt2\Delta}, \textit{pmt4\Delta} and \textit{sec59} mutants (Fig. 6)\cite{gentzsch1997}. Removal of O-glycans using HF reduces its molecular mass from 150 to 47 kDa \cite{russo1992}. Yet, \textit{cwh8\Delta} make normally glycosylated Hsp150 with only small amounts being underglycosylated (Fig. 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 \textit{cwh8\Delta} mutants and that an O-glycosylation defect therefore cannot explain the deficiency in IPC/C of \textit{cwh8\Delta} mutants.

\textit{Influence of the N-glycosylation defects on IPC biosynthesis}

Treatment of wt cells with the N-glycosylation inhibitor tunicamycin (Tm), which specifically inhibits the transfer of GlcNAc-P onto Dol-P, induces a drastic decrease of \textsuperscript{3}H\textsuperscript{Ins} incorporation into IPC (Fig. 7A, 7B). Cells incorporated \textsuperscript{3}H\textsuperscript{Ins} efficiently into M(IP)_2C and this was true even after prolonged preincubation in 10 \mu g/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 tunicamycin seems to faithfully mimic the sphingolipid abnormality of \textit{cwh8\Delta} mutants (Fig. 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 \textit{ALG3, ALG6, ALG8} and \textit{ALG9}, which cannot add all mannoses and glucoses to Dol-PP-GlcNAc\textsubscript{2}-Man\textsubscript{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 \textit{och1Δ}, a mutant in which elongation of N-glycans is completely abolished (Nakanishi-Shindo et al., 1993). The \textit{och1Δ} cells made normal amounts of IPC/ but showed a moderate reduction of IPC/D-MIPC and a compensatory increase of M(IP)\textsubscript{2}C (Table II). IPC levels were however depressed in certain mutants having defects in the oligosaccharyltransferase such as the \textit{ost5Δ alg5Δ} and in also cells depleted of Rft1p but not in others such as \textit{ost3Δ ost6Δ} (Fig. S1 and 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 (Fig. 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 \textit{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 Fig. 7D, the \textit{rer2-2} strain exhibited only a moderate decrease of IPC-C levels, but CPY also was less severely underglycosylated than in \textit{cwh8Δ}.

\textit{The effects of the unfolded protein response on the sphingolipid profile}

Underglycosylation and tunicamycin treatment induce an UPR (Travers et al., 2000). Using a plasmid carrying an UPR responsive element (UPRE) in the promoter in front of lacZ we found that the UPR was indeed constitutively activated in \textit{cwh8Δ} (data not shown). Also, secretion of Kar2p, an ER chaperone, has been recognized as a sign of UPR activation. As shown in Fig. 8A, \textit{cwh8Δ} cells secrete significant amounts of Kar2p, significantly more than what is secreted by wt cells exposed to Tm or aureobasidin A. The UPR strongly induces the transcription of \textit{INO1}, the rate limiting enzyme for inositol and PI biosynthesis in yeast (Fig. 1)(Cox et
We indeed observed that \textit{cwh}8\textDelta\ overproduce and secrete inositol, i.e. that they display the so called \textit{opi} phenotype (Figure S2). We wondered if the decreased incorporation of [\textsuperscript{3}H]Ins into IPC/C in \textit{cwh}8\textDelta mutants and tunicamycin treated cells could be explained by an increase in inositol and PI biosynthesis, so that \textit{cwh}8\textDelta\ cells, when labeled with [\textsuperscript{3}H]Ins, would make PI and IPC/C of lower specific activity than wt but in normal amounts. This hypothesis seemed unlikely because \textit{cwh}8\textDelta\ cells incorporate normal amounts of [\textsuperscript{3}H]Ins into M(IP)\textsubscript{2}C (Figs. 2A and 3). It was further rendered unlikely by experiments, in which cold inositol (up to 50 mg/L) was added to the labeling medium. Addition of cold inositol did not significantly change the fraction of [\textsuperscript{3}H]Ins incorporated into sphingolipids (Fig. S3). Furthermore, the relative lack of IPC/C could also be seen, when the sphingolipids of \textit{cwh}8\textDelta\ cells were revealed by charring (Fig. 8B). Finally, we tested other mutants displaying a strong \textit{opi} phenotype such as \textit{sec}14 (Henry and Patton-Vogt, 1998), or cells in which the UPR is constitutively induced by the presence of \textit{HAC1-i}, a spliced, i.e. active version of the \textit{HAC1} transcription factor. Neither one of these cells showed a decreased IPC/C biosynthesis (Table III).

Treatment with Tm or deletion of \textit{CWH}8 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 \textit{ire}1\Delta\ cells which are unable to induce an UPR (Sidrauski and Walter, 1997). As shown in Fig. 8C, IPC/C biosynthesis is still Tm sensitive in \textit{ire}1\Delta\ cells.

Thus, it seems that the altered sphingolipid profile of the \textit{cwh}8\textDelta\ mutant is not a consequence of its constitutive UPR or its increased biosynthesis of inositol, 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 (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 7 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 HA tag. As shown in Fig. 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 3 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 (not shown). The spore 8A (cwh8Δ AUR1-HA) showed the expected defect in IPC biosynthesis (Fig. 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 (Fig. 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 (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 (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 while 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 recently been shown to alter sphingolipid biosynthesis in two recent reports: One report demonstrates that *erg26-1* mutants specifically decrease IPC and MIPC while M(IP)_2C remains normal (Swain et al., 2002a); another report shows that cells lacking Arv1p, a multispansing 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 *cwh8*Δ mutants which show a sphingolipid profile similar to the one of *erg26-1* cells, since we find IPCs to be reduced whereas M(IP)_2C levels are normal. Our data on *cwh8*Δ 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 *cwh8*Δ 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, while O-glycosylation and GPI anchoring are not or only slightly
affected (Figs. 5, 6, Table I). As pointed out before, the N-
underglycosylation could be due either to a relative lack of Dol-P at the
cytosolic side of the ER membrane or to an inhibitory effect of the
accumulated Dol-PP (Fernandez et al., 2001). However, the profile of Dol-
linked oligosaccharides of \( cwh8\Delta \) (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 this finding
suggests that \( cwh8\Delta \) may rather suffer from a relative lack of cytosolic Dol-
P than Dol-PP accumulation. The lack of cytosolic Dol-P of \( cwh8\Delta \) cells is
predicted by the so called Dol cycle model. Our calculations (see
supplemental 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 \( cwh8\Delta \) 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 \( cwh8\Delta \) 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 \( cwh8\Delta \), 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 $cwh8\Delta$ cells as they have a similar problem as the dolichol 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 $cwh8\Delta$ 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)$_2$C (Hechtberger and Daum, 1995). The result may be interpreted as to mean that the relocalization of Aur1p, Csg1p, Csh1p, and the M(IP)$_2$C-synthase Ipt1p (Fig. 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)$_2$C. 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 (fig. 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 if 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, since 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 (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 (YPD) or minimal media (e.g. SD) containing salts (S), 2% glucose (D) or 2% galactose (G) as a carbon source, uracil (U), adenine (A) and amino acids (aa) or casein hydrolysate (C) as described (Sherman, 2002) but lacking inositol. Wild type strains were BY4742 MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0; SS328 MATa ade2-101 ura3-52 his3Δ200 lys2-801; SEY6210 MATa ura3-52 leu2-3,112 his3Δ200 lys2-801 trp1Δ901 suc2-Δ9; SEY6211 MATa ura3-52 leu2-3,112 his3Δ200 ade2-101 trp1Δ901 suc2-Δ9; R1158 MATa ura3::CMV-tTA his3-1 leu2Δ0 met15Δ0. Mutant strains were cwh8Δ-1 YGR036c::kanMX4 in BY4742; cwh8Δ-2 YGR036c::HIS3 in SS328; dpm1 MATa dpm1-6 ura3; SNH023-7D MATa rer2-2 mfa1::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; ost5Δalg5Δ ost5::HIS3 alg5:: HIS3 in SS328; RKY327 ost3Δost6Δ MATa ade2-101 ura3-52 his3Δ200 ost3::HIS3 ost6Δ; pmt1 pmt2 pmt1::URA3 pmt2::LEU2 in SEY6210; pmt1 pmt3 pmt1::URA3 pmt3::HIS3 in SEY6210; pmt1 pmt5 pmt1::HIS3 pmt5::URA3 in SEY6210; pmt2 pmt4 pmt2::URA3 pmt4::TRP1; TetRft1 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 (trp1? lys2? suc2? met15?); och1Δ YGL038c::kanMX4 in BY4742; NY758 sec7-1; NY878 MATa sec7 ura3 leu2; NY966 MATa sec7-1 ura3 leu2 his3.

Plasmids pCWH8, pMPG1, pSV463VIG4 and pRC43 contain CWH8, MPG1, VIG4 and HAC1-i (spliced, constitutively active HAC1), respectively.
All chemicals were from commercial suppliers. Tunicamycin was from Fluka. Antibody against Hsp150p was kindly provided by Marja Makarow (Helsinki), antibodies against Kar2p were a gift from Dr. Marc Rose and antibodies against CPY and the HA tag were from MOLECULAR PROBES.

**Metabolic labeling of cells with [³H]inositol**

Cells growing in YPDUA were labeled in SDaaUA at 30°C during 2h. To 2.5 OD₆₀₀ units of cells in 250µl of medium, preincubated for 10 minutes, 4 µCi [³H]Ins per OD₆₀₀ unit were added. After 40 minutes, the culture was diluted with 750µl 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 1ml water. Lipids were extracted twice using 500 µl of EtOH:H₂O:Et₂O:pyridine:25%NH₄OH,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-[³H]GlcNAc as described (Canivenc-Gansel et al., 1998). Lipids were reextracted with 500µl CHCl₃:MeOH:H₂O 10:10:3.
**Lipid analysis**

Lipids were separated by TLC on silica gel 60 plates developed in CHCl₃:MeOH:H₂O, 10:10:3, or CHCl₃:MeOH:KCl 0.25%, 55:45:5, or CHCl₃:MeOH:KCl 0.25%, 55:45:10. Plates were read with a Berthold radioscanner and then sprayed with EN³HANCE and laid on X-Omat film (KODAK) for autoradiography. Desalted lipids were deacylated in 500µl of 33% monomethylamine (MMA) at 53°C for 2h. 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 100µl Tris buffer, pH 7.5 supplemented with 1 mM of each, ATP, GTP, CTP, GDPMan, CoA, 10mg/ml creatine phosphate, 10µg/ml creatine kinase, 300µM C26-CoA (in Zwittergent) and 10 µCi of [³H]DHS, C₂-[³H]DHS, C₄-[³H]DHS or C₆-[³H]DHS at 37°C for 1h. Products were separated on TLC with CHCl₃:MeOH:2M NH₄OH, 40:10:1 (ceramide synthase) or with CHCl₃: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 PVDF membranes and detected with ECL technology (AMERSHAM).
Acknowledgements

We would like to thank the labs of Drs. S. Strahl-Bolsinger, L. Lehle, P. Robins, S. Munro, Y. Noda, P. Novick, H. Riezman, R. Schekman, K. Sato, P. Orlean, A. Nakano, G. Palamarczyk and P. Walter for yeast strains and plasmids and Dr. Claude Jakob (ETHZ, Zürich, Switzerland) for the communication of unpublished results. Thanks to Marco Celio for letting D.U. work for this project. The work was supported by grant 31-67188.01 from the Swiss National Science Foundation.

Abbreviations

DHS, dihydrosphingosine; Dol, dolichol; Dol-P, dolichylphosphate; Dol-PP, dolichylpyrophosphate; ER, endoplasmic reticulum; GPI, glycosylphosphatidyl inositol; Ins, inositol; IPC, inositolphosphorylceramide; MIPC, mannosyl-IPC; M(IP)_2C, inositolphosphoryl-MIPC; PI, phosphatidyl inositol; TLC, thin layer chromatography; Tm, Tunicamycin; UPR, unfolded protein response; wt, wild type.
References


luminally oriented active site in the endoplasmic reticulum of


*Glycobiology, 9*, 243-253.
Figure legends

Figure 1. Sphingolipid biosynthesis in yeast. DAG, diacylglycerol; MIPC, mannosyl-IPC; M(IP)₂C, inositolphosphoryl-MIPC; PI, phosphatidylinositol. Genes are in italics.

Figure 2. Sphingolipid biosynthesis is defective in the absence of CWH8. (A) Strains with cwh8Δ deletions in the BY4742 or SS328 background were labeled with [³H]Ins, lipids were extracted and separated by TLC. Incorporation of [³H]Ins incorporation 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%.

Figure 3. Kinetics of [³H]inositol incorporation in cwh8Δ. (A) Wt (BY4742) and cwh8Δ cells were labeled with [³H]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 [³H]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.

Figure 4. The cwh8Δ cells make DHS, PHS and ceramide normally. (A) Cells were preincubated (10 min) and labeled with [³H]Ins in presence or absence of 5µM DHS or PHS and lipids were separated on TLC and quantitated. (B) Ceramide synthase in vitro. Microsomes were incubated with [³H]DHS and C26-CoA during 5 to 60 min. The amount of C₂₆-DHS made as a fraction of the total radioactivity is given below each lane. (C)
C$_2$- or C$_4$-[3H]DHS was incubated alone (lanes 1, 4) or in the presence of microsomes (lanes 2, 3, 5, 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$_6$-[3H]DHS for 2h 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.

Figure 5. *The cwh8Δ cells add GPI anchors normally.* (A) Microsomes were labeled with UDP-[3H]GlcNAc in the presence of GDP-mannose. Lipids were extracted and migrated on a TLC. CP = complete precursor (CP). (B) Proteins were extracted from the indicated strains and processed for Western blotting. Blots were probed with anti-Gas1p. Aureobasidine A (Aba) was added at 1μg/ml for 2h and sec59 cells were grown for 4h at 37°C before extraction. The immature (i) and mature (m) forms of Gas1p are indicated. (C) Cells were labeled with [3H]Ins at 30°C for 2h, 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).

Figure 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).

Figure 7. *Tunicamycin causes a reduction in IPC/C levels.* (A) Cells were preincubated for 2h in SDaaUA with or without tunicamycin (Tm, 2.5μg/ml) and labeled with [3H]Ins. (B) Quantification of lipids in panel A. (C) Cells were labeled with [3H]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 temperature (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.

Figure 8. The abnormal sphingolipid profile of cwh8Δ is not caused by its unfolded protein response. (A) Strains were inoculated at 0.5 OD₆₀₀ cultured for 1 or 3 h at 30°C and media were collected, TCA precipitated and processed for Western blot detection of Kar2p, a 70 kD 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 [³H]Ins-labeled lipids leads to the disappearance (precipitation) of M(IP)₂C (not shown). (C) Aliquots of ire1Δ were labeled with 10 μCi of [³H]Ins using different preincubation times, labeling times and concentrations of Tm. Total incorporation into lipids is given for each condition (μCi).

Figure 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 45kD protein. Underglycosylation of CPY in spores A and B indicates the presence of the cwh8::KanMX allele. (B) Cells were labeled with [³H]Ins, and lipids were quantified by TLC.
Tables

*Table I. O-glycosylation mutants*

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### Table II. N-glycosylation mutants

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

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* three different sec7 strains
Supplemental material

Supplemental Materials and methods

Detection of opi phenotype

Cells to be tested were plated onto SDaaUA medium to obtain individual colonies. After 24h at the appropriate temperature (24°C for sec14 strain and 30°C for other strains), a tester strain (MATa ino1-13, ade1) was sprayed onto the plate. Colonies were observed 2 days later. Appereance of the red tester cells on top of the white colonies tested is indicative of inositol secretion.

Electron microscopy

Cells grown in YPDUA were collected between 1 and 2 OD600. They were washed in phosphate buffered saline, fixed in 2.5% glutaraldehyde in 0.1M phosphate, pH 7.2 (buffer 1), at 4°C for 30 min. Cells are washed in three times in buffer 1. Postfixation was done with 1% osmium tetroxide in buffer 1 for 30 min at room temperature. Fixed cells were washed 3 times in buffer 1 and resuspended in 2% of low melting Agarose to form blocks. Blocks were dehydrated by incubation for two times 5 min in 30 %, then 50 %, the 70 % ethanol. Contrasting was achieved by adding 1% uranyl acetate in 70% ethanol for 45 min. After rinsing twice with 50% ethanol blocks were further dehydrated by placing them into 70 %, 80 %, 95 % and finally 100% ethanol for two times 15 min. Dehydration was terminated by placing blocks in propylen oxide for two times 10 min. Block were embedded in Epon-Propylen 1:3 for 1h, 1:1 for 2h, 3:1 for 2h, Epon only for 2h and overnight. Epon was allowed to olymerize for 2 days at 60°C. Ultrathin sections
were prepared and disposed on grids. Grids were then contrasted in 0.3% KMnO₄ in 0.05M phosphate buffer pH 8.0 for 5 min, 10% uranyl acetate for 30 min and finally in Reynolds solution for 10 min. Grids were observed at 80kV.

**Supplemental Figure legends**

Figure S1. *Sphingolipids of oligosaccharyltransferase mutants*. Indicated strains were labeled with [³H]Ins, lipids were extracted and analyzed by TLC and autoradiography.

Figure S2. *Opi phenotype in cwh8Δ cells*. Strains to be tested were grown on a plate for 1 day and sprayed with a red, Ins-requiring tester strain and incubated for 2 more days. One representative colony is shown for each strain. *Sec14* at non-permissive temperature (37°) served as a positive control for the *opi* phenotype.

Figure S3. *Effect of inositol in the media*. Wild type cells were labeled with [³H]Ins in the presence of various concentrations of cold inositol. Lipids were extracted and quantitated. The incorporation [³H]Ins into cellular lipids was the same in all conditions and amounted to about 80% of added radioactivity.

Figure S4. *Morphology of cwh8Δ cells*. Electron microscopy pictures of wt, *cwh8Δ* and *cwh8Δ* complemented by pCWH8 or by the empty control vector are shown.
The plausibility of the Dol cycle model is supported by the following rough calculations: We can estimate that in cells growing on glucose, a membrane preparation containing 1 g of membrane protein also contains 0.6 g carbohydrate, that 20% of the latter is added in the ER and that thereof, 2/3 is \(N\)-linked. According to the thesis work of K. Grabinska, 2002 (Inst. of Biochemistry and Biophysics, Warsaw, Poland) the amount of Dol in microsomal membrane fractions is \(2.2 \times 10^{-7}\) mol Dol per g membrane protein and another report allows to calculate that one g of membrane protein contains \(4.0 \times 10^{-7}\) mol Dol-P (Heller et al., 1992). Calculating under these assumptions we get numbers saying that the ER membrane contains 450 fold more Dol-born sugars in its N-glycans than free Dol and Dol-P. If in \(cwh8\Delta\) cells one Dol-PP would irreversibly be trapped for each \(\text{GlcNAc}_2\text{-Man}_\nu\text{-Glc}_3\) added, the biosynthetic process adding \(\text{GlcNAc}_2\text{-Man}_\nu\text{-Glc}_3\) to nascent glycoproteins in normal quantity would consume 57 fold more Dol-P per gram of membrane made than the amount of Dol plus Dol-P one gram of membrane actually contains.

<table>
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<th>Supplemental Tables</th>
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Pittet et al., Fig 1
Figure 2

A

B

% of labeled lipids
Figure 3

A

BY4742

 labeling time (min)

 cpm X 10^6

 80

 60

 40

 20

 0

 5 15 30 60

 cwhΔ

 labeling time (min)

 cpm X 10^6

 80

 60

 40

 20

 0

 5 15 30 60 80

 B

 % of labeled lipids

 BY4742

 cwhΔ

 IPC/C
 IPC/C+MIPC
 MIPC/C

 http://doc.rero.ch
Figure 4

A

B

C

D

label C2-DHS
C2-DHS' C2-IPC

label C2-DHS
C2-DHS' C2-IPC
Figure 6
Figure 9

A

Aur1p-HA

CPY

TLY138

cw108A

spore 8A

spore 8B

spore 8C

spore 8D

B

% of labeled lipids

BY4742

BY4742-1

cw108A

spore 8A

PI

IPC/C

IPC/D+MIPC

M(IP)_2C
Figure S1

PI-
IPC/C-
IPC/D+MIPC-
LysoPI-

M(IP)$_2$C-
SS328
cwh8Δ
ost5Δ alg5Δ
ost3Δ ost6Δ

Figure S2

BY4742, 30°C  cwh8A, 30°C

sec14, 24°C  sec14, 37°C
Figure S3

![Graph showing the effect of cold inositol (mg/l) on the percentage of labeled lipid in YPDUA and SDaaUA media. The graph includes four conditions: PI+lysoPI, IPC/C, IPC/D+MIPC, and M(IP)2C.](http://doc.rero.ch)
Figure S4

BY4742

cwh8Δ

cwh8Δ+pCWH8

cwh8Δ+pYEp352

1μm