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CWH43 is required for the introduction of ceramides into GPI anchors in *Saccharomyces cerevisiae*

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Summary

After glycosylphosphatidylinositols (GPIs) are added to GPI proteins of *Saccharomyces cerevisiae*, the fatty acid in *sn*-2 of the diacylglycerol moiety can be replaced by a C26:0 fatty acid by a deacylation–reacylation cycle catalysed by Per1p and Gup1p. Furthermore the diacylglycerol moiety of the yeast GPI anchor can also be replaced by ceramides. *CWH43* of yeast is homologous to PGAP2, a gene that recently was implicated in a similar deacylation reacylation cycle of GPI proteins in mammalian cells, where PGAP2 is required for the reacylation of monoradylglycerol-type GPI anchors. Here we show that mutants lacking *CWH43* are unable to synthesize ceramide-containing GPI anchors, while the replacement of C18 by C26 fatty acids on the primary diacylglycerol anchor by Per1p and Gup1p is still intact. *CWH43* contains the COG3568 metal hydrolase motif, which is found in many eukaryotic and prokaryotic enzymes. The conserved His 802 residue of this motif was identified as being essential for ceramide remodelling. Ceramide remodelling is not required for the normal integration of GPI proteins into the cell wall. All remodelling reactions are dependent on prior removal of the inositol-linked fatty acid by Bst1p.

Introduction

The lipid moieties of mature glycosylphosphatidylinositol (GPI) anchors of *Saccharomyces cerevisiae* usually do not contain the diacylglycerol present on the GPI lipid at the stage when it is transferred by the transamidase to GPI proteins, and which probably contains the typical C16:0 and C18:1 fatty acids found in yeast phosphatidylinositol (PI) (Conzelmann *et al*., 1992; Sipos *et al*., 1997; Schneiter *et al*., 1999). The majority of mature GPI proteins of yeast contain a ceramide moiety, whereas a minor fraction contains a modified diacylglycerol containing C26 in *sn*-2 (Fankhauser *et al*., 1993; Sipos *et al*., 1997). GPI anchor ceramides contain phytosphingosine and C26:0 or C26:0-OH, as do the bulk of ceramide moieties present in yeast sphingolipids, which are the inositolphosphorylceramides (IPCs) and their derivatives (Fankhauser *et al*., 1993). Thus, all mature GPI proteins of yeast contain large lipid moieties with C26:0 or hydroxylated C26:0 fatty acids, either in the form of a ceramide or in the form of a special diacylglycerol and these lipids are introduced by remodelling enzymes that replace the primary lipid moiety of the anchor. Ceramides are also found in the GPI anchors in other fungi, certain plants and protozoan organisms and there is evidence that they may be introduced into the anchors through similar remodelling reactions (for review see Bosson and Conzelmann, 2007).

The yeast remodelling activity introducing ceramide (ceramide remodelase) can be monitored by metabolic labelling experiments using tritiated inositol ([^{3}H]inositol) or dihydrosphingosine ([^{3}H]DHS) (Reggiori *et al*., 1997; Sipos *et al*., 1997). When given to cells, these tracers are rapidly incorporated into all sphingolipids as well as ceramide-containing GPI proteins but no other proteins. All [^{3}H]inositol- or [^{3}H]DHS-derived label can be removed from the metabolically labelled proteins in the form of PIs or IPCs using nitrous acid, a reagent that releases the inositolphosphoryl-lipid moieties from GPI anchors by cleaving the link between glucosamine and inositol (Ferguson *et al*., 1988; Reggiori *et al*., 1997).

Recently, two enzymes required to introduce C26 fatty acids into the primary GPI anchor have been identified as shown in Fig. 1A. *PER1* encodes a phospholipase A2 that removes the C18:1 fatty acid of the primary anchor (Fujita *et al*., 2006). *GUP1* is an acyltransferase required for the addition of a C26 fatty acid to the liberated *sn*-2 position, thus generating a pG1-type anchor (Bosson *et al*., 2006) (Fig. 1A). Per1p and Gup1p are also required for the introduction of ceramide anchors into yeast anchors, as *per1*Δ and *gup1*Δ mutants contain only limited amounts of mild base-resistant anchors migrating in the region of IPC/B and IPC/C. Also, significant amounts of more polar, base-resistant, inositol-containing anchors are observed in *gup1*Δ cells (Bosson *et al*., 2006; Fujita *et al*., 2006; M. Jaquenoud *et al*., unpublished).

Recent data show that a similar deacylation reacylation cycle as used for generating pG1-type anchors in yeast

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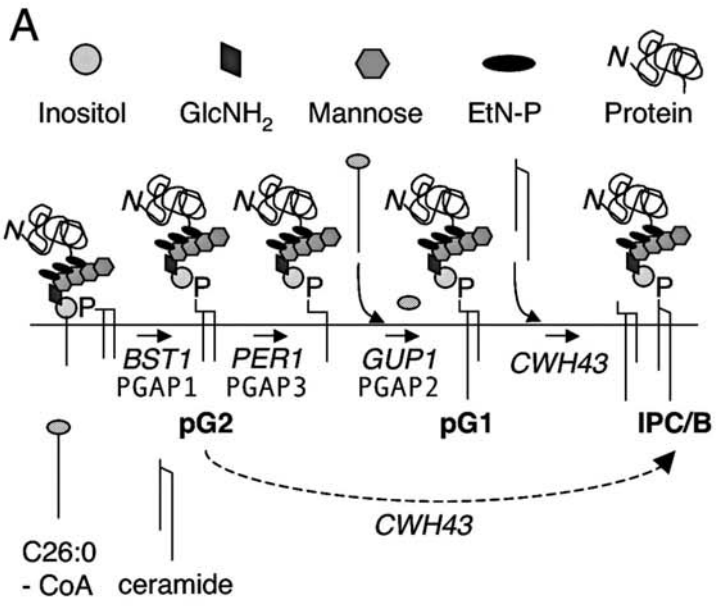

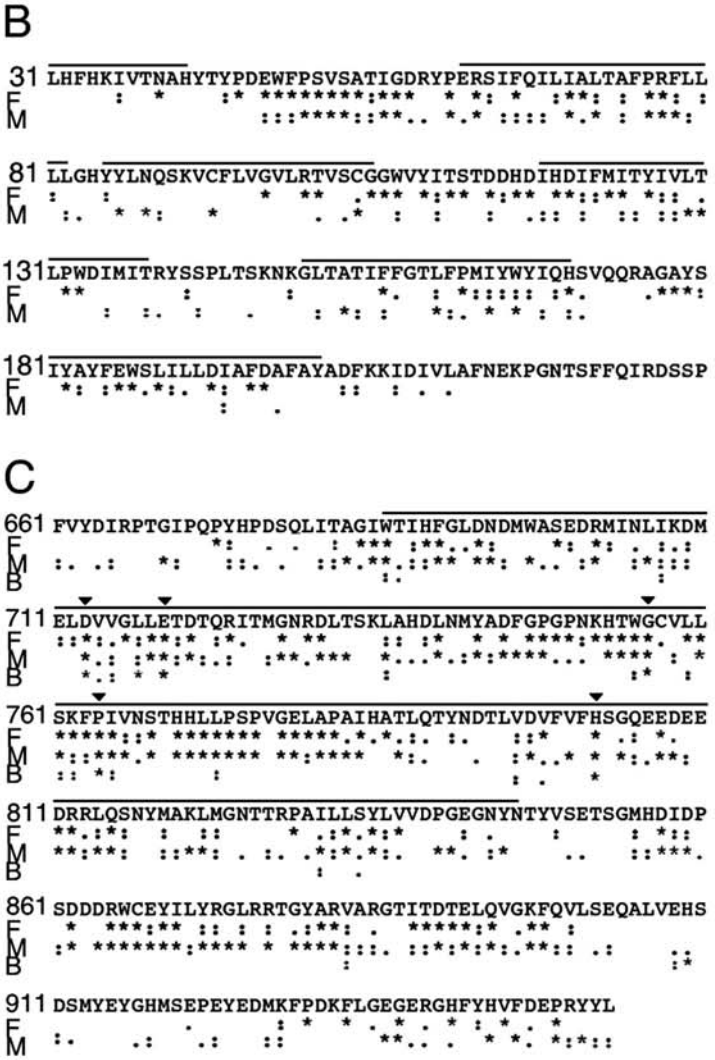

Fig. 1. GPI anchor lipid remodelling in *Saccharomyces cerevisiae*.
A. A likely model for the various remodelling reactions, which GPI proteins will be subjected to in the ER. Yeast genes implicated in the various steps are indicated in italics, while corresponding mammalian genes in non-italic. Metabolic labelling with [^{3}H]inositol shows that the reaction catalysed by Bst1p occurs within seconds after the GPI anchor is added to the protein, so that the left most structure is rarely detectable; Bst1p generates pG2-type anchors, which are gradually transformed into pG1- and IPC/B-type anchors over about 20–30 min (Sipos *et al*., 1997). Upon arrival in the Golgi apparatus, a small fraction of GPI anchors with a hydroxylated C26:0 fatty acid is generated (= IPC/ C-type anchors) (not shown).
B. The amino acid sequence corresponding to amino acids 31–230 of Cwh43p is depicted with its putative transmembrane domains (black lines, obtained from prodiv-TMHMM; http://www.pdc.kth.se/~hakanv/prodiv-tmhmm). Below we indicated the identities (*) and similarities (:, .) shared between *CWH43* and the most homologous members of the following gene families: F = 18 fungal genes showing high homology over the entire length of *CWH43*, and M = 23 metazoan sequences, all about 250 amino acids in length. Separate alignments of *CWH43* with each of these two phylogenetic groups were generated using CLUSTALV W.
C. The C-terminal hydrophilic domain of Cwh43p corresponding to amino acids 661–953 and comprising the COG3568 motif (grey line) is shown. The closest homologues, in descending order, are groups of 18 fungal (F), 9 metazoan (M) and 11 bacterial (B) sequences. They were aligned as for (B). Residues conserved in Cwh43p and all three phylogenetic groups are indicated by arrow heads.

takes place in mammalian cells (Tashima *et al*., 2006; Maeda *et al*., 2007). This cycle serves to replace a polyunsaturated fatty acid on *sn*-2 of the primary diradylglycerol moieties of GPI proteins by a saturated C18:0. Deacylation is operated by human PERLD1 (also named PGAP3), a homologue of *PER1* that also can functionally replace *PER1* in yeast cells (Fujita *et al*., 2006; Maeda *et al*., 2007) (Fig. 1A). PGAP2 then is required for the reacylation step (Fig. 1A). PGAP2, previously also known as FRAG1, is an integral membrane protein of the ER/Golgi, has no homology to *GUP1* and is widely conserved in eukaryotes. In cells deleted for PGAP2, the lyso-forms of GPI proteins generated by PGAP3 are transported to the plasma membrane, where their lipid moiety is cleaved off by a phospholipase D. By consequence, deficiency of PGAP2 causes GPI proteins to be secreted.

Saccharomyces cerevisiae contains a PGAP2 homologue, *CWH43*, a 953-amino-acid-long open reading frame with 19 predicted transmembrane domains. The N-terminus of *CWH43* shows about 26% identity with PGAP2 (Fig. 1B) (Martin-Yken *et al*., 2001). The 675 C-terminal amino acids of Cwh43p bear no homology to PGAP2, contain numerous transmembrane domains and a large C-terminal hydrophilic domain starting at amino acid 666. This latter domain harbours the COG3568, ElsH, metal-dependent hydrolase motif (Fig. 1C). The COG3568 motif is present in a vast number of prokaryotic as well as some eukaryotic proteins. Outside fungi, the closest homologues of this C-terminal part of *CWH43* are found in a family of hypothetical proteins of mammals and birds (Fig. 1C). The COG3568 motif is related to several other motifs among which pfam03372, a motif found in Mg^{2+}-dependent bacterial endonuclease/exonuclease/phosphatase enzymes, and to COG0708 XthA, a motif found in Exonuclease III.

Here we investigate the role of *CWH43* in GPI remodelling of yeast.

Results

cwh43Δ *cells are deficient in ceramide remodelling* in vivo

*cwh43*Δ and corresponding wild-type (wt) cells were metabolically labelled with [^{3}H]inositol or [^{3}H]DHS. After extraction, proteins were analysed by SDS-PAGE/fluorography, lipids by thin-layer chromatography (TLC). As can be seen in Fig. 2A, while proteins of wt cells could be labelled with both [^{3}H]inositol and [^{3}H]DHS, GPI proteins of *cwh43*Δ could be labelled only with [^{3}H]inositol but not with [^{3}H]DHS. GPI anchor lipids were released from [^{3}H]inositol-labelled GPI proteins by nitrous acid and the radioactive anchor lipids were analysed by TLC. Figure 2B shows the normal GPI anchor lipids pG1 and IPC/B of wt cells (Fig. 2B, lane 5; Fig. 1A). As pG1 is a PI containing a C26 fatty acid (Fig. 1A), it exhibits higher mobility than the bulk of the cell's free PI (Fig. 2B, lane 1). The two *cwh43*Δ strains analysed can generate pG1 (Fig. 2B, lanes 3 and 4), and this species, as expected, is completely mild base sensitive (lane 8). On the other hand, *cwh43*Δ cells make almost no IPC/B-type anchors (Fig. 2B, lanes 3 and 4), although a trace of IPC/B is still visible after mild base hydrolysis (lane 8). These lipids were not seen in each experiment, as traces of IPC/B were visible in only three out of five experiments, traces of IPC/C in five out of five. When IPC/Bs were observed they were present in similar amounts as IPC/C, a finding that argues against a contamination with free lipids, as the profile of free lipids contains mainly IPC/C, but very little IPC/B (Fig. 2B, lane 1). The data suggest that in *cwh43*Δ cells, Per1p and Gup1p correctly exchange a fatty acid on the primary PI moiety of GPI-anchored proteins to generate the pG1-type anchors (Bosson *et al*., 2006; Fujita *et al*., 2006), but that the ceramide-based anchor lipid IPC/B is no more made, or only very inefficiently. To see if *cwh43*Δ cells have a general defect in sphingolipid biosynthesis, the lipid extracts of [^{3}H]inositol-labelled cells were analysed by TLC. Figure 2C shows that the profile of [^{3}H]inositol-labelled sphingolipids in *cwh43*Δ cells is the same as in wt cells. (All sphingolipids of yeast contain inositol and are resistant to mild base treatment.) The profile of [^{3}H]DHS-labelled lipids of *cwh43*Δ is also normal (Fig. S1).

Transfection of *CWH43* under the *GAL1* promoter into *cwh43*Δ cells restored the capacity of these cells to make GPI anchors with IPC/B lipid moieties, when they were cultured on galactose, while lower expression of *CWH43* on raffinose allowed for only a small amount of IPC/B-type anchors (Fig. 3, lanes 2–6).

Importance of the COG3568 hydrolase motif for the remodelase activity of Cwh43p

During GPI lipid remodelling, no soluble protein intermediates have been detected, suggesting that some phosphodiesterase may exchange the diacylglycerol for a ceramide moiety through a transesterification reaction. The presence of a COG3568 in *CWH43* and the related pfam03372 motif (http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF03372) in many of its bacterial homologues raised the possibility that Cwh43p was carrying out such a transesterification reaction. Point mutations were made in highly conserved His and Asp residues of *CWH43* (arrow heads in Fig. 1C), as these residues are known to be active site residues in the huge pfam03372 metal-dependent phosphohydrolase family. As can be seen in Fig. 4A, the mutant alleles D713A and H802A could be overexpressed to a similar extent as wt *CWH43*. As shown in Fig. 4B, the D713A mutation reduced the amount of IPC-type anchors

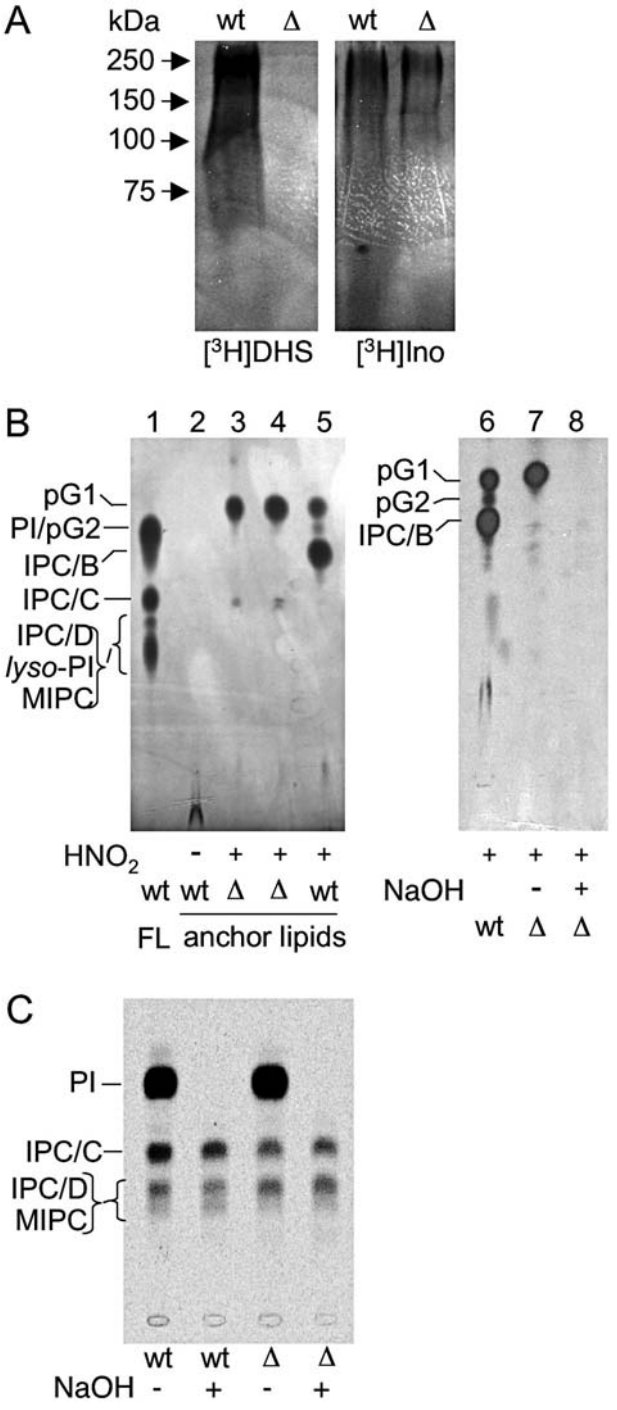

Fig. 2. *cwh43*Δ mutants do not incorporate ceramides into GPI anchors.
A. Exponentially growing BY4742 wt and *cwh43*Δ cells (Δ) were pre-incubated for 30 min in the presence of myriocin 40 μg ml^{-1} and metabolically labelled with either [^{3}H]DHS or [^{3}H]inositol for 120 min at 30°C in the presence of myriocin. (Myriocin enhances the efficiency of labelling with [^{3}H]DHS.) Proteins were extracted, purified by concanavalin A-Sepharose affinity chromatography (Guillas *et al*., 2000) and subjected to SDS-PAGE/fluorography.
B. Cells were labelled with [^{3}H]inositol as for (A) but without myriocin, proteins were delipidated, anchor peptides were prepared, treated with nitrous acid (+) or control incubated (–), and the released lipids were analysed by TLC/fluorography. Two different *cwh43*Δ strains (*MAT***a** and *MAT*α) are shown in lanes 3 and 4. The [^{3}H]inositol-labelled free lipids (FL) of wt cells were run in lane 1. In an analogous experiment the anchor lipids of *cwh43*Δ were deacylated by mild alkaline hydrolysis using NaOH (+) or control incubated (–) (lanes 7 and 8).
C. Free lipids of wt and *cwh43*Δ cells labelled in the experiment of (B), either deacylated or not by NaOH, were resolved by TLC and visualized by radioimaging.

(Fig. 4B, lanes 1 and 2 versus 5 and 6). A similar reduction of IPC/B was also seen in the anchor lipid fraction eluting from octyl-Sepharose at 25% propanol (Fig. 4C, lane 1 versus 3). Quantification using two-dimensional radioscanning showed that the relative amount of IPCs had dropped from 29% to 14% of total anchor lipids in this mutant. The H802A mutation abrogated the biosynthesis of IPC-type anchors almost completely, although small amounts of IPC/B and IPC/C were still visible on long exposures (Fig. 4B, lanes 3 and 4; Fig. 4C, lane 2). It is likely that these small amounts of IPC/B and IPC/C are anchor derived and not due to a contamination of anchor lipids with the ordinary IPCs, as these lipids were not observed in these same samples when nitrous acid treatment was omitted (Fig. 4B, lanes 8–10), and because

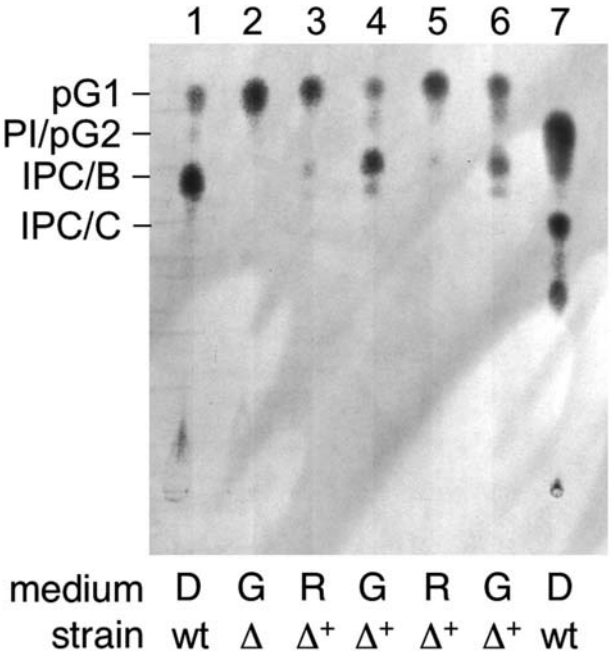

Fig. 3. Transfection of wt *CWH43* restores the ability to make IPC-type anchors in *cwh43*Δ cells. [^{3}H]inositol-labelled anchor lipids were prepared as for Fig. 2B from BY4742 (wt), *cwh43*Δ cells (Δ) or *cwh43*Δ cells complemented with pCWH43, a centromeric plasmid carrying *CWH43* under the control of the inducible *GAL1* promoter (Δ$^+$). Cells were grown for 16 h in glucose (D), for 16 h in raffinose (R) or for 14 h in raffinose, then 2 h on galactose (G) at 30°C before being labelled in the same media for 2 h. Lane 7 contains free lipids.

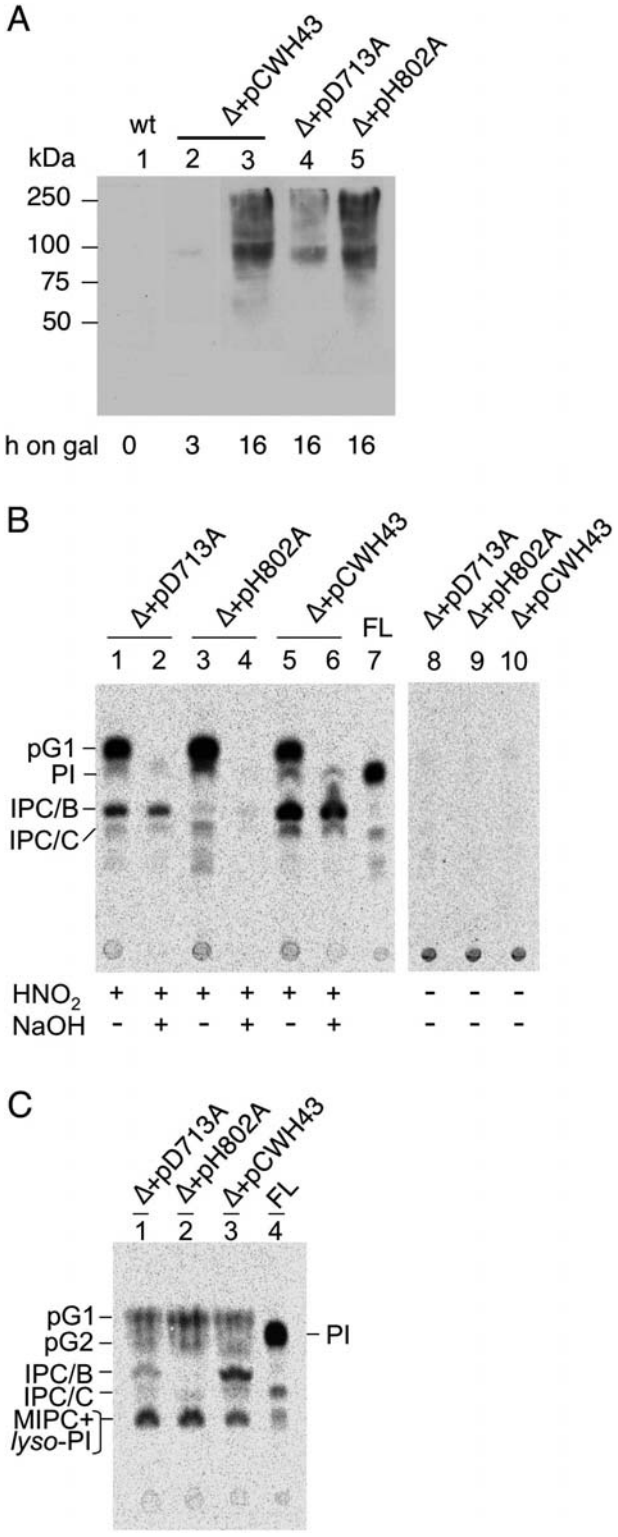

Fig. 4. His 802 is important for the incorporation of ceramides into GPI anchors.
A. BY4742 wt cells and *cwh43*Δ cells harbouring plasmids containing either HA-tagged *CWH43* (Δ+pCWH43) or *cwh43* mutant alleles D713A (Δ+pD713A) or H802A (Δ+pH802A) were grown in glucose and then further cultured in galactose (SGaa, no uracil) for 0, 3 or 16 h. Proteins were extracted and analysed by Western blotting using antibodies against the HA tag.
B. In parallel, the cells having been grown in galactose for 16 h were labelled with [^{3}H]inositol and anchor lipids were prepared and analysed as described for Fig. 2B. Free lipids (FL) of wt cells were run in lane 7.
C. To make sure that no anchors went unrecognized, the anchor lipids eluting from octyl-Sepharose at 25% propanol were equally analysed (see *Experimental procedures*; these fractions are contaminated by non-radioactive hydrophobic peptides and do not migrate very neatly on TLC).

IPC/B is only very minor among the free IPCs of the cell (lane 7). Quantification of the data in Fig. 4B using two-dimensional radioscanning revealed that the mild base-resistant IPC/B in Δ+pH802A amounted to 0.7% of total anchor lipids instead of the normal 24% found in Δ+pCWH43 cells overexpressing the wt form of *CWH43*. In a second identical experiment, IPC/B in *cwh43*Δ, Δ+pH802A and Δ+pCWH43 amounted to 0.7%, 1.6% and 38.9% of total anchor lipids (not shown). In view of the fact that traces of IPC/B-type anchor lipids are present in several experiments also in *cwh43*Δ cells, it is difficult to decide if His 802 is directly involved in catalysis of the addition of ceramides to GPI proteins or not. It also is conceivable that low amounts of IPC/B-type anchors could be generated in the mutated Cwh43^{H802A}p through the ordering effect of substrate binding, but without the contribution of the active site residue. Altogether the data suggest that the C-terminal, hydrophilic part of Cwh43p and the residues conserved in COG3568 and pfam03372 hydrolase and phosphodiesterase motifs are important for the role of Cwh43p in the introduction of ceramides into the yeast GPI anchor. This C-terminal part is oriented towards the ER lumen and thus is properly located to interact with GPI proteins, which are its presumed substrates (Kim *et al.*, 2006).

Ceramide remodelling is not required for the integration of GPI proteins into the cell wall

cwh43 is one among 52 complementation classes of recessive calcofluor white (CFW)-hypersensitive mutants, which were generated in order to get mutants deficient in cell wall assembly (Ram *et al.*, 1994). It is not surprising that some of them have been found to have mutations in genes encoding GPI proteins or enzymes required for GPI biosynthesis, as the bulk of yeast GPI proteins ends up in the yeast cell wall, where, after the loss of the GPI lipid moiety, the proteins can become covalently linked to β1,6-glucans (Klis *et al.*, 2006). Later studies showed that the hypersensitivity to CFW as well as to Congo red and killer toxins K1 and K2 and the secretion of the cell wall protein Cwp1p is pronounced only in the original *cwh43-2* mutant harbouring a G57R mutation but not in *cwh43*Δ

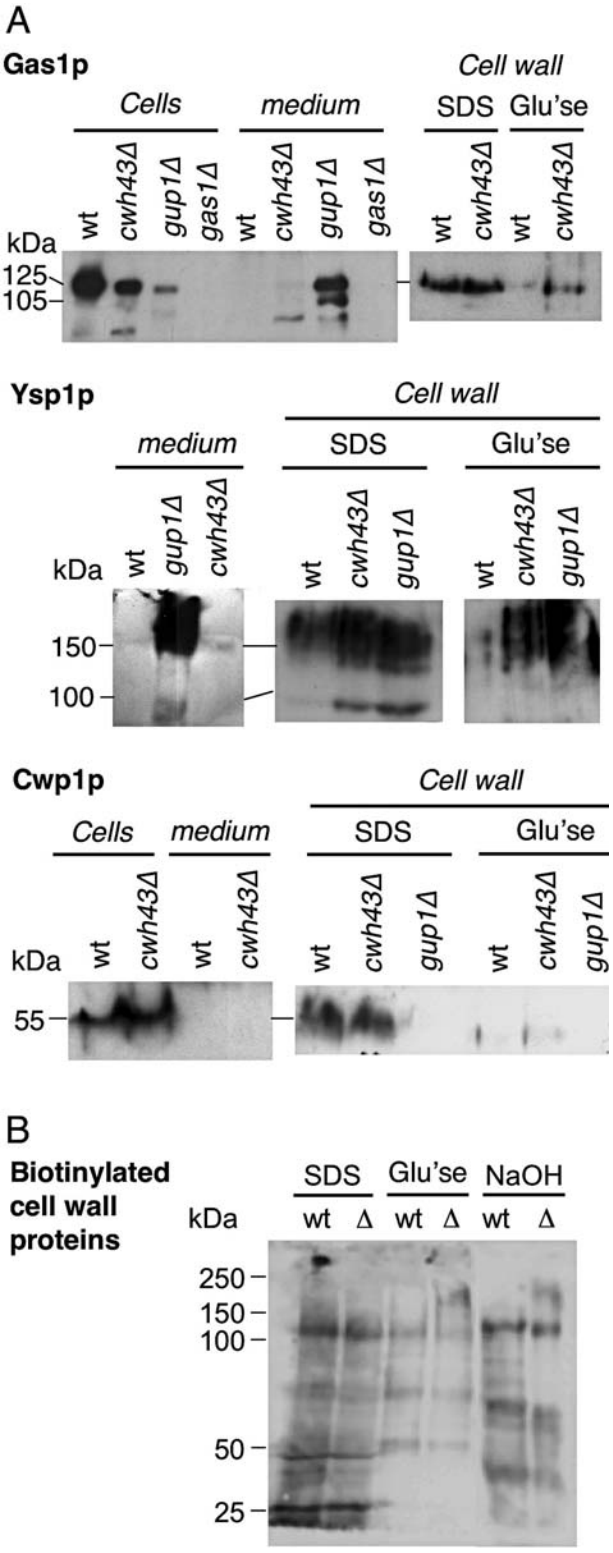

Fig. 5. GPI proteins of *cwh43*Δ cells are incorporated correctly into the cell wall and are not secreted.
A. Cells were grown in YPD to an absorbance (A_{600}) of 1.6. Media and cells were collected, proteins were extracted from whole cells and also from purified cell walls by boiling in SDS. Residual proteins were then liberated by treatment with β1,3-specific glucanase (Glu'se). Proteins were separated by SDS-PAGE and detected by Western blotting using antibodies against Gas1p, Yps1p and Cwp1p. Material from equivalent numbers of cells was loaded in each lane.
B. Wild-type and *cwh43*Δ (Δ) cells were grown in YPD medium to an A_{600} of about 4, and were incubated with the impermeable NHS-LC-Biotin to derivatize cell wall proteins. Cell walls were prepared and their proteins extracted first with SDS, and then with either NaOH or β1,3-specific glucanase (Glu'se). Extracted proteins were analysed by SDS-PAGE, blotting and probing with streptavidine-peroxidase.

cells (Martin-Yken *et al*., 2001). The study also revealed numerous polymorphisms in the DNA sequence of *CWH43* between different strains. We found that our *cwh43*Δ mutants were not more sensitive to CFW than wt cells (Fig. S2; data not shown). While mammalian PGAP2 mutants and yeast *gup1*Δ mutants were shown to secrete GPI proteins into the culture medium (Bosson *et al*., 2006; Tashima *et al*., 2006), we found that *cwh43*Δ mutants analysed here secrete neither Gas1p nor other GPI proteins such as Yps1p or Cwp1p into the medium (Fig. 5A). Moreover, these proteins appear in normal amounts in the SDS- and the glucanase-extractable cell wall fractions of *cwh43*Δ (Fig. 5A). Cell wall proteins were also derivatized by treating cells with an impermeable biotinylation reagent. This showed that the various classes of cell wall proteins, which can be extracted by SDS, glucanase or mild NaOH treatment, are present in normal amounts in the cell walls of *cwh43*Δ cells (Fig. 5B). We conclude that all types of cell wall proteins including GPI proteins get integrated efficiently into the cell wall in *cwh43*Δ cells and that ceramide remodelling is not required for GPI proteins to be incorporated into the cell wall.

Removal of fatty acid from inositol by Bst1p is a prerequisite for GPI lipid remodelling in yeast

An acyl group is added at an early stage of GPI biosynthesis by Gwt1p and subsequently removed again by Bst1p shortly after addition of the GPI lipid to a newly synthesized protein in the ER (Murakami *et al*., 2003; Umemura *et al*., 2003; Tanaka *et al*., 2004) (Fig. 1A). The persistence of this acyl chain in mammalian cells carrying mutations in PGAP1, the mammalian homologue of Bst1p, has been shown to be a hindrance to the efficient export of GPI proteins out of the ER, although this acyl chain is not removed in all cell types. The deletion of yeast *BST1* has been shown to delay the degradation of a misfolded form of Gas1p (Fujita *et al*., 2006), but a direct analysis of the GPI anchor lipids of *bst1*Δ cells has not been reported. As shown in Fig. 6, lanes 1, 2, when *bst1*Δ mutants were metabolically labelled with [^{3}H]inositol, none of the normal lipid moieties pG1, pG2, IPC/B and IPC/C could be liberated from their anchor peptides. Instead,

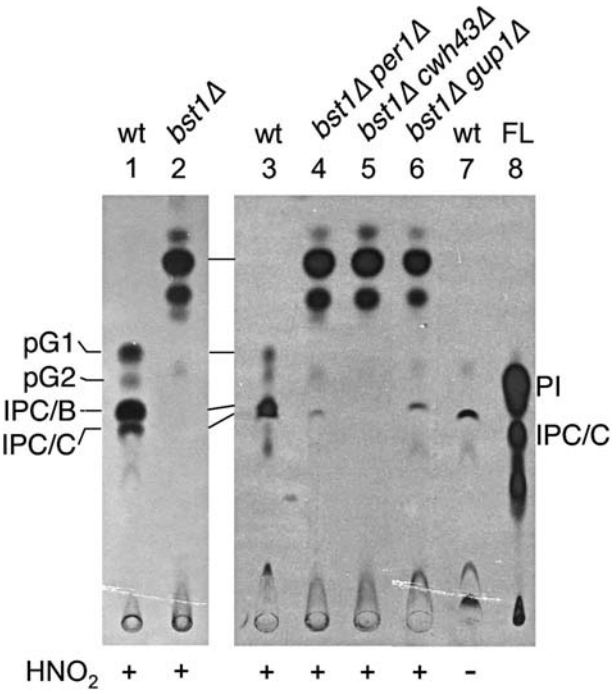

Fig. 6. Remodelling reactions are dependent on prior inositol deacylation by Bst1p. The *bst1*Δ cells as well as double mutants combining *bst1*Δ with *per1*Δ, *cwh43*Δ or *gup1*Δ mutations were labelled with [^{3}H]inositol and anchor lipids were prepared and analysed as described for Fig. 2B. Free lipids (FL) of wt cells were run in lane 8. Minor amounts of lipids migrating between pG1 and pG2 and with IPC/C cannot be regarded as anchor lipids, as they were also observed in the control peptides not treated with nitrous acid (lane 7).

four more hydrophobic lipids were present, as can be expected for anchor lipids, which contain an additional fatty acid on the inositol moiety. It seemed possible that the different lipids were inositol-acylated forms of pG1, pG2, IPC/B and IPC/C. To investigate this, we examined double mutants containing *bst1*Δ in combination with *per1*Δ, *gup1*Δ and *cwh43*Δ, as *PER1*, *GUP1* and *CWH43* are required to transform pG2 into pG1-, IPC/B- and IPC/C-type lipid moieties. As shown in Fig. 6, the GPI proteins of these double mutants and of the *bst1*Δ mutant essentially contain the same set of very hydrophobic lipid moieties. The data suggest that the various anchor lipids of *bst1*Δ are generated independently of known remodelling enzymes and that neither Per1p/Gup1p- nor Cwh43p-mediated remodelling occurs prior to the removal of the inositol-linked fatty acid. Similar observations were made in mammalian cells. While most GPI proteins with non-acylated inositol moieties contain saturated or mono-unsaturated fatty acids, unsaturated fatty acids are found on inositol-acylated GPI anchors (Ferguson *et al*., 2007). Moreover, the mature CD52 was shown to contain exclusively distearoyl-PI, while the inositol-acylated precursor of CD52 of the same tissue contained predominantly a stearoyl-arachidonoyl-PI (Treumann *et al*., 1995). The difference of anchor lipids between immature and mature mammalian GPI anchors was recently shown to be due to PGAP2/PGAP3-mediated remodelling steps, which are dependent on prior removal of the acyl-inositol by PGAP1 (Maeda *et al*., 2007). We have previously observed that isomers of different TLC mobility arise through spontaneous transacylation, by which the fatty acid in inositol-acylated PI can move from the C2 to the C3 and to further positions on the inositol ring (Sipos *et al*., 1997). Thus, we believe that the different anchor lipids of *bst1*Δ are isomers becoming apparent after removal of the GPI carbohydrate core from the inositol ring by nitrous acid.

Discussion

Recent data from several labs led to the identification of two novel enzymes, Per1p and Gup1p, involved in the generation of pG1-type anchors in yeast and at the same time revealed the existence of a similar GPI deacylation–reacylation cycle in mammalian cells (Bosson *et al*., 2006; Fujita *et al*., 2006; Tashima *et al*., 2006; Houjou *et al*., 2007; Maeda *et al*., 2007). Indeed, according to current understanding, the mammalian GPI anchors are modified sequentially by PGAP1, PGAP3 and PGAP2, while the yeast anchors are remodelled sequentially by Bst1p, Per1p and Gup1p (Fig. 1A). The first two steps in both of these organisms are dependent on orthologous genes. In yeast, the third step, the acylation of lyso-GPI proteins, is carried out by Gup1p. Gup1p is likely to be a catalytic subunit, because it harbours a membrane bound O-acyl transferase (MBOAT) motif, which is found in numerous confirmed acyltransferases. As mammals contain several genes showing homology to *GUP1*, it has been considered a possibility that one of them is also involved in reacylation of lyso-GPI proteins (Maeda *et al*., 2007). However, in mammals this reacylation step depends also on PGAP2, which has no recognizable motif that would give a clue to its mode of action.

Based on its homology with PGAP2 we expected that *CWH43* would be important for the generation of pG1-type anchors in yeast. It therefore was surprising to find that *CWH43* is involved in the replacement of the lipid moiety of pG1 by a ceramide. This step is likely to occur through a transesterification reaction, which could be operated by a phosphodiesterase exchanging either diacylglycerol for ceramide or phosphatidic acid for ceramide phosphate. Indeed, our data make it clear that the exchange reaction introducing ceramides is dependent on the C-terminal part *CWH43*, and in particular, on His 802, which our data pinpoint as the, or a possible active site residue. On the other hand, the generation of pG1-type anchors by Gup1p does not seem to require the aid of the N-terminal part of *CWH43*, which is homologous to PGAP2.

The bulk of the yeast GPI remodelling reactions takes place in the ER but IPC/C-type GPI anchors and the ongoing exchange of ceramide for ceramide occurs in a post-ER compartment, i.e. Golgi and/or plasma membrane (Reggiori *et al*., 1997). According to the database at http://yeastgfp.ucsf.edu/, Cwh43p as well as Per1p and Gup1p are localized in the ER, but an earlier report localized a functional Cwh43p-GFP to the cell periphery, possibly the plasma membrane (Martin-Yken *et al*., 2001; Huh *et al*., 2003). Thus, Cwh43p has been found in the compartments, where ceramide remodelling of GPI anchors has been observed.

A BLAST search with *CWH43* brings up homologues in a wide range of species. There is a group of 21 fungal homologues in 19 fungal species which show highest homology (32–63% identity) extending over the entire length of Cwh43p. The second best homology is found in a group of seven (six mammalian and one hen) hypothetical proteins, showing 25–30% identity with the C-terminal 675 amino acids of Cwh43p. Thus, it appears that mammals contain the entire genetic information of *CWH43*, but redistributed over two different open reading frames, which together have about the same length as *CWH43*. In view of the pronounced conservation of several aspects of GPI biosynthesis between yeast and mammals, including GPI protein remodelling, it seems to be legitimate to ask if the mammalian homologues of the C-terminal part of *CWH43* have some role in GPI remodelling. There is no known mammalian counterpart of the exchange of pG1 for an IPC but a variable fraction of mammalian anchors contains mild base-resistant alkylglycerols. Indeed, 70% of the early GPI lipid intermediate glucosamine-acyl-PI accumulating in murine class E BW5147 Thy1$^-$ mutants were found to be mild base resistant and the same intermediate accumulating in the human HeLa S3 cells was shown to contain 15% of alkylglycerol-based lipid moieties (Puoti and Conzelmann, 1993; Sevlever *et al*., 1995). Moreover, a recent in-depth analysis of all early GPI intermediates shows that glucosamine-PI mostly has 18:0–20:4 diacyl-type lipid moieties, while glucosamine-acyl-PI contains a large fraction of 1-alk(en)yl-2-acyl-glycerol moieties (Houjou *et al*., 2007). In view of the proposed role of the C-terminal part of *CWH43* in a transesterification reaction introducing ceramides into yeast GPI anchors, it seems conceivable that the mammalian homologue of this C-terminal part of *CWH43* would operate a similar reaction, e.g. to introduce alk(en)yl-acyl-glycerol-type lipids into mammalian GPI lipids, but there are presently no data to support this notion.

Acyl exchange occurs also in *Trypanosoma brucei* and other protozoa, but in these organisms it occurs before the GPI lipids are transferred onto proteins (Masterson *et al*., 1990; Bosson and Conzelmann, 2007). A further marked difference between yeast and *T. brucei* resides in the fact that biosynthetic GPI intermediates in *T. brucei* exist in two forms, either with or without a fatty acid attached to their inositol moiety (Guther and Ferguson, 1995). In contrast, in yeast all free GPI intermediates that accumulate in GPI mutants contain an acyl chain linked to the inositol and none of them seems to undergo lipid remodelling or to contain a ceramide moiety. Interestingly, acyl exchange in *T. brucei* can only occur on deacylated GPI lipids (Guther and Ferguson, 1995). Similarly, the findings reported here show that generation of both pG1-type and IPC-type anchor lipids is not possible before the acyl-inositol is deacylated by Bst1p. It is possible that remodelling in yeast only occurs after GPI lipids have been added to proteins, and not, as in *T. brucei*, already before, because yeast Bst1p is not able to deacylate free GPI lipids but only GPI-anchored proteins. Further studies to confirm this notion are under way.

Experimental procedures

Strains, media and materials

Strains with single deletions of non-essential genes (*CWH43*, *GUP1*) in BY4741, *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* and BY4742, *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* were obtained from EUROSCARF (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/col_index.html). Further deletions in those strains yielded the following mutants: FBY4186 = Δ^+ = Δ+pCWH43, *cwh43*Δ from EUROSCARF harbouring pCWH43; FBY4184 = *cwh43*Δ from EUROSCARF harbouring pD713A; FBY4185 = *cwh43*Δ from EUROSCARF harbouring pH802A. FBY972 = *bst1*Δ *gup1*Δ, *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bst1::natMX3 gup1::kanMX4; FBY4175 = bst1*Δ *per1*Δ, *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bst1::kanMX4 per1::hphNT1; FBY4176 = bst1*Δ *cwh43*Δ, *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bst1::kanMX4 cwh43::hphNT1*. Strains were cultured at 30°C in YPD medium or in minimal media supplemented with glucose (SD), galactose (SG) or raffinose (SR) and amino acids (aa) (Sherman, 2002). Selection for integration of KanMX4-, hphNT1- or natMX4-containing deletion cassettes was performed on YPD plates containing 200 μg ml^{-1} G418 (CALBIOCHEM) or 300 μg ml^{-1} hygromycin respectively. NHS-LC-Biotin reagent [sulphosuccinimidyl-6-(biotinamido)hexanoate] was from Soltec Ventures, Beverly, USA. Unless specified otherwise, chemicals were purchased from Sigma. Pepstatin was obtained from Alexis, octyl-Sepharose and concanavalin A-Sepharose from Amersham Biosciences. Rabbit antibodies against Yps1p and Cwp1p were kind gifts from Dr Niahm Cawley and Dr Hitoshi Shimoi respectively.

Construction of strains and plasmids

HphNT1 cassettes conferring resistance to hygromycin for the replacement of *PER1* and *CWH43* were generated by PCR using primers *Cwh43* hphNT1 F and *Cwh43* hphNT1 R (Table S1A) and plasmid pFA6a-hphNT1 as a template

(Janke *et al*., 2004). The success of gene deletions was assessed by PCR. FBY972 was generated by crossing a *bst1::natMX3* strain obtained from Maya Schuldiner with the *gup1*Δ::*kanMX4* strain of EUROSCARF (Schuldiner *et al*., 2005). The plasmids harbouring *CWH43* and mutant alleles thereof were generated as follows: the open reading frame of *CWH43* was amplified by PCR using genomic DNA of BY4741 as a template and primers *Cwh43* rec1 and *Cwh43* rec2 (Table S1B). The PCR fragment was purified by a DNA purification kit (Qiagen, Chatsworth, CA) and introduced into pGREG536 by co-transfection into *cwh43*Δ yeast cells, thus generating pCWH43 (Jansen *et al*., 2005). Point mutations to change Asp 713 and His 802 to Ala were introduced into pCWH43 by means of the quick change site-directed mutagenesis kit (Stratagene) using primers D713A F, D713A R, H802A F, and H802A R and pCWH43 as a template (Table S1C). The open reading frames of the generated mutant alleles were verified by DNA sequencing.

Metabolic labelling of cells and analysis of proteins, lipids and anchor lipids

Ten A_{600} units of exponentially growing cells were harvested, cells were re-suspended in 1 ml of SDaa, SRaa or SGaa medium and labelled at 30°C in a shaking water bath by adding 40 μCi of [^{3}H]inositol or 30 μCi of [^{3}H]DHS. After 40 min, the samples were diluted with 4 ml of fresh medium and were incubated for a further 90 min. Labelling was terminated by adding NaF and NaN_3 (10 mM final concentration). Cells were washed, re-suspended in 0.6 ml of $CHCl_3/CH_3OH$ (1:1), broken with glass beads at 4°C for 1 h and the lysate was sedimented at 10 000 *g* for 3 min. The protein pellet was delipidated twice with $CHCl_3/CH_3OH/H_2O$ (10:10:3), dried and analysed by SDS-PAGE/fluorography. To detect tagged Cwh43p in Western blots, cells were frozen and thawed re-suspended in reducing sample buffer for SDS-PAGE, cells were broken by vortexing with glass beads on ice, insoluble material was removed by centrifugation (12 000 $g \times 15$ min, 4°C), the supernatant was left at 4°C for 16 h and finally loaded on the gel for SDS-PAGE.

Isolation of the lipid moieties of GPI anchors was performed as described (Guillas *et al*., 2000). Unless indicated otherwise, lipids generated from the anchor peptides eluting from octyl-Sepharose with 50% propanol are shown. Lipids were analysed by TLC on 20×20 cm silica gel 60 plates using $CHCl_3/CH_3OH$/0.25% KCl, 55:45:5 as a solvent. Free lipids extracted from [^{3}H]inositol- or [^{3}H]DHS-labelled cells were desalted by butanol/water partitioning and analysed by TLC. Alkaline hydrolysis was performed using 0.1 M NaOH in $CHCl_3/CH_3OH/H_2O$ (10:10:3) for 1 h at 37°C.

Assessment of the secretion and cell wall incorporation of GPI proteins

Proteins were precipitated from the culture medium using 10% trichloroacetic acid and cellular proteins were extracted as described (Kushnirov, 2000). Cell walls were prepared and proteins released from them by boiling in SDS and digestion with β1,3 glucanase (Quantazyme, Qbiogene) exactly as described before (Frieman and Cormack, 2003). Cell wall proteins of intact cells were biotinylated using a described method (Mrsa *et al*., 1997) with the following modifications: 500 A_{600} units of cells in 2 ml of buffer containing 1 mg of NHS-LC-Biotin reagent were incubated for 90 min on ice. SDS-extracted cell walls were washed, one-half was further treated by incubation in 30 mM NaOH during 16 h at 4°C, the other half was re-suspended in 0.6 ml of 33.5 mM K^+ phosphate pH 7.5, 60 mM β-mercaptoethanol, to which 20 units of Quantazyme were added. After 2 h at 30°C, another 20 units of Quantazyme were added and the incubation at 30°C was continued for another 16 h. Proteins were precipitated by acetone, subject to SDS-PAGE, blotted onto PVDF membranes and probed with streptavidin-HRP conjugate as described.

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