Saccharomyces cerevisiae CWH43 Is Involved in the Remodeling of the Lipid Moiety of GPI Anchors to Ceramides

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The glycosylphosphatidylinositol (GPI)-anchored proteins are subjected to lipid remodeling during their biosynthesis. In the yeast Saccharomyces cerevisiae, the mature GPI-anchored proteins contain mainly ceramide or diacylglycerol with a saturated long-fatty acid, whereas conventional phosphatidylinositol (PI) used for GPI biosynthesis contains an unsaturated fatty acid. Here, we report that S. cerevisiae Cwh43p, whose N-terminal region contains a sequence homologous to mammalian PGAP2, is involved in the remodeling of the lipid moiety of GPI anchors to ceramides. In cwh43 disruptant cells, the PI moiety of the GPI-anchored protein contains a saturated long fatty acid and lyso-PI but not inositolphosphorylceramides, which are the main lipid moieties of GPI-anchored proteins from wild-type cells. Moreover, the C-terminal region of Cwh43p (Cwh43-C), which is not present in PGAP2, is essential for the ability to remodel GPI lipids to ceramides. The N-terminal region of Cwh43p (Cwh43-N) is associated with Cwh43-C, and it enhanced the lipid remodeling to ceramides by Cwh43-C. Our results also indicate that mouse FRAG1 and C130090K23, which are homologous to Cwh43-N and -C, respectively, share these activities.

INTRODUCTION

Many proteins are attached to the cell surface through glycosylphosphatidylinositol (GPI). GPI modification is conserved among mammalian cells, yeast, and protozoa (Ferguson, 1999; Kinoshita and Inoue, 2000; Pittet and Conzelmann, 2007). In the budding yeast Saccharomyces cerevisiae, ~60 proteins have been predicted to be GPI-anchored (Caro et al., 1997). Some of these proteins, such as Gas1p, seem to remain anchored to the plasma membrane, whereas the others become mainly cell wall-linked (Caro et al., 1997; Hamada et al., 1999; Kapteyn et al., 1999). Thus, yeast GPI-anchored proteins are mainly involved in cell wall integrity.

The biosynthesis of GPI and its attachment to the target protein are carried out on the endoplasmic reticulum (ER) membrane. GPIs, which have conserved core structures, are preassembled in the ER in a multistep pathway before their transfer to target proteins. GPIs are assembled by the sequential addition to PI of glucosamine, an acyl chain, mannoses, and ethanolamine phosphates. The complete GPIs are then attached to ER-translocated proproteins bearing a C-terminal signal sequence for GPI attachment. More than 20 genes involved in GPI biosynthesis and attachment to proteins have been identified, and, in most cases, these genes are conserved in yeast and mammalian cells (Kinoshita and Inoue, 2000; Orlean and Menon, 2007; Pittet and Conzelmann, 2007).

After the transfer of GPI to a protein, the acyl chain is removed from the inositol portion of GPI-anchored proteins (Tanaka et al., 2004; Fujita et al., 2006b). The inositol-deacylated GPI-anchored proteins are then transported to the plasma membrane by vesicular trafficking (Mayor and Riezman, 2004). GPI-anchored proteins are concentrated at the microdomains of plasma membrane, generally called lipid rafts, that are enriched with sterols and sphingolipids, and they can be isolated biochemically as detergent-resistant membrane (DRM) fractions (Simons and Ikonen, 1997). The lipid rafts are thought to play an important role in signal transduction and membrane trafficking.

Some mature GPI-anchored proteins have saturated fatty acid chains in the lipid moiety (McConville et al., 1993; Brewis et al., 1995; Benting et al., 1999), whereas GPI is synthesized from conventional PI, which usually has an unsaturated fatty acid chain. This conversion of the lipid...
moiety is called “lipid remodeling” of GPI. In mammalian cells, after exchanging an unsaturated chain at the sn-2 position of the lipid moieties with a saturated chain, the lipid moieties of mature GPI-anchored proteins contain PI with two saturated fatty chains (Maeda et al., 2007). In contrast, mature GPI-anchored proteins in yeast contain two different types of lipid moieties (Conzelmann et al., 1992; Fankhauser et al., 1993; Sipos et al., 1994, 1997; Reggiori et al., 1997). One is diacylglycerol with a C26 fatty acid at the sn-2 position. The other type of lipid moiety is ceramide consisting mainly of C18:0 phytosphingosine (PHS) and C26:0 fatty acid (Sipos et al., 1997). In both types of lipid moieties, the C26 fatty acid may be hydroxylated (Sipos et al., 1997). However, GPI-anchored proteins contain a ceramide-type lipid moiety in the GPI anchor remains unclear, although the ceramide type represents a major fraction of the total cells and Gas1p, a major GPI-anchored protein, is an example of protein containing diacylglycerol type of lipid moiety (Fankhauser et al., 1993).

The remodeling of the yeast lipid starts in the ER with the removal of the unsaturated acyl chain at the sn-2 position of diacylglycerol to form lyso-GPI. This GPI-phospholipase A₂ (GPI-PLA₂) activity requires Per1p (Fujita et al., 2006a). Next, a C26 saturated acyl chain is transferred to the sn-2 position by the O-acyltransferase Gup1p, generating diacylglycerol with a C26 fatty acid (Bosson et al., 2006). Recent studies suggest that these lipid remodeling events are required for both the efficient transport of GPI-anchored proteins from the ER to the Golgi and their association with lipid rafts (Bosson et al., 2006; Fujita et al., 2006a; Maeda et al., 2007). The diacylglycerol of the lipid moiety is replaced by ceramide consisting of PHS and a C26 fatty acid, a process that seems to occur in the ER (Sipos et al., 1997). In the Golgi, the ceramide of the lipid moiety is further changed to ceramide consisting of PHS with a hydroxy-C26 fatty acid (Reggiori et al., 1997; Sipos et al., 1997). The pathway for replacement by ceramide has not been elucidated, and proteins involved in this event have not been identified. Gpi7p and Mcd4p, which transfer ethanolamine phosphate to mannose during GPI biosynthesis, are reported to be indirectly involved in the remodeling of diacylglycerol to ceramides (Benachour et al., 1999; Zhu et al., 2006), but it remains unclear what proteins are directly responsible for this function.

In this study, we found that yeast CWH43 is required for the remodeling of the lipid moiety of GPI anchors to ceramides and that its C-terminal domain is especially important for this function. The N-terminal region of CWH43 associated with C-terminal region is able to enhance the lipid remodeling to ceramides by C-terminal region. Furthermore, in yeast cwh43-disruptant cells, the mouse FRAG1 and C130090k23 gene product, which are homologues of the N- and C-terminal regions of CWH43p, respectively, can share these activities, indicating that the GPI lipid is remodeled to ceramides by mouse C130090k23 protein, whose function is enhanced when FRAG1 protein is introduced.

MATERIALS AND METHODS

Media and Strains

YPAD and synthetic complete (SC) media were described previously (Sherman, 1991). For [3H]dihydrosphingosine (DHS) labeling, cells were cultured at 30°C in SC medium. The yeast strains used in this study included the following: CWH43Δ (MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cwh43Δ::KanMX4; EUROSCARF), BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 wild-type strain), and gpl7Δ (MATa his3Δ1 leu2Δ0 met15Δ0 gpl7Δ::KanMX4; EUROSCARF).

Plasmids

Plasmids used in this report are listed in Table 1.

Construction of CWH43-HA, CWH43-C, and CWH43-mRFP Plasmids

The promoter region and open reading frame of CWH43 were amplified by PCR (polymerase chain reaction) from genomic DNA by using primers pRS316-CWH43-F18 (5'-GGGCTGATATTGCTAGCTAGCTACGATGATGCA-3') and HANheXba-R17 (5'-GCTCTAGATTCGACTAGCTAGCTACGATGATGCA-3'). The amplified fragment was digested with NheI and XbaI and inserted into the NheI and XbaI sites of pRS316T-CWH43 to generate pRS316T-CWH43-HA. The DNA fragment encoding monomeric red fluorescent protein (mRFP; kindly provided by Dr. Roger Tsien, University of California, San Diego, La Jolla, CA) was also amplified and inserted into the NheI site of pRS316T-CWH43 to generate pRS316T-CWH43-mRFP.

Construction of Plasmid for Overexpression of CWH43-HA

The open reading frame of CWH43 with a sequence encoding three copies of the HA epitope at the C terminus of the protein was amplified from pRS316T-CWH43-HA. The fragment encoding the HA epitope was amplified and inserted into the NheI site of pRS316T-CWH43-HA to generate pRS316T-CWH43-HA-HA. This fragment was inserted into pYEp352GAPII to generate pYEp352GAPII-CWH43-HA-HA.

Construction of CWH43-N and CWH43-N-HA Plasmids

The short promoter region and the open reading frame encoding the N-terminal region (229 amino acids) of CWH43p (CWH43-N) were amplified from pRS316T-CWH43 by using primers Xho-CWH43-P-C1-F11 (5'-TGGCTGATATTGCTAGCTAGCTACGATGATGCA-3') and HANheStpXba-R17 (5'-GCTCTAGATTCGACTAGCTACGATGATGCA-3'). The amplified fragment was digested with XhoI and XbaI and inserted into the NheI site of pRS316T-CWH43-N to generate pRS316T-CWH43-N-HA. The open reading frame of CWH43-N and the sequence encoding three copies of the HA epitope (CWH43-N-HA) were amplified from pRS316T-CWH43-N-HA by using primers Kpn-CWH43-F18 (5'-GGGCTGATATTGCTAGCTACGATGATGCA-3') and HANheStpXba-R17 (5'-GCTCTAGATTCGACTAGCTACGATGATGCA-3'). The amplified fragment was digested with KpnI and XbaI. The fragment was inserted into KpnI/XbaI-digested Yep352GAPII to generate Yep352GAPII-CWH43-N-HA.

Construction of CWH43-C and CWH43-C-HA Plasmids

The short promoter region and open reading frame encoding the C-terminal region (756 amino acids) of CWH43p (CWH43-C) were amplified from pRS316T-CWH43 by using primers Xho-CWH43-P-C1-F11 (5'-TGGCTGATATTGCTAGCTAGCTACGATGATGCA-3') and HANheStpXba-R17 (5'-GCTCTAGATTCGACTAGCTACGATGATGCA-3'). The amplified fragment was digested with XhoI. The fragment was inserted into pRS316T-CWH43, which contains the entire CWH43 promoter region to generate pRS316T-CWH43-C. A sequence encoding three copies of the HA epitope (CWH43-C-HA) was also amplified and inserted into the NheI site of pRS316T-CWH43-C to generate pRS316T-CWH43-C-HA. The sequence encoding the open reading frame of CWH43-C and three copies of the HA epitope (CWH43-C-HA) were amplified from pRS316T-CWH43-C-HA by using primers Kpn-CWH43-F19 (5'-GGGCTGATATTGCTAGCTACGATGATGCA-3') and HANheStpXba-R17 (5'-GCTCTAGATTCGACTAGCTACGATGATGCA-3'). The amplified fragment was digested with KpnI/XbaI. The fragment was inserted into KpnI/XbaI-digested Yep352GAPII to generate Yep352GAPII-CWH43-C-HA.

Construction of Mutated cwh43-HA and cwh43-C-HA Plasmids

We substituted G57 with arginine by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and pRS316T-CWH43-HA, generating the plasmid pRS316T-CWH43-G57R-HA. We also substituted H472, D693, H770/H771, E807, D862, and R882 with Glu by using pRS316T-CWH43-C-HA, generating plasmids pRS316T-CWH43-H472E-HA, pRS316T-CWH43-D693A-HA, pRS316T-CWH43-H770A/H771A-HA, pRS316T-CWH43-E807A-HA, pRS316T-CWH43-C-D862A-HA, and pRS316T-CWH43-C-R882A-HA, respectively.

Construction of FRAG1, FRAG1-HA, and FRAG1-FLAG Plasmids

The open reading frame of the mouse FRAG1 gene was amplified from the plasmid of the National Institutes of Health mammalian gene collection clone (no. 3588752) by using primers Em-mFRAG1-F24 (5'-GGCGGTGACATGGTGAATCT-3') and HANheFr1 (5'-GCTCTAGATTCGACTAGCTACGATGATGCA-3'). The amplified fragment was digested with HaeIII and inserted into the NheI site of pRS316T-CWH43 to generate pRS316T-CWH43-mRFP.
CAGGTGACACTGCA-G3′) and mmFRAG1-NotI-R33 (5′-CTGTTTGATGAGGAAGCAG-3′). The amplified fragment was digested with SmaI and SalI. The fragment was ligated into the NheI site of YEp352GAPII-C130090K23 to generate YEp352GAPII-C130090K23-HA or YEp352GAPII-C130090K23-FLAG.

Construction of C130090K23, C130090K23-HA, and C130090K23-FLAG Plasmids

The open reading frame of the mouse cwh43-C gene amplified from genomic DNA by using primers Sma-mmFLJ21511-F30 (5′-CTGCCAGCGCTTGAGAG-3′) and mmFLJ21511-N90ΔR27 (5′-CTGCGTGCTACGCTAGCTAAGAAGATTTGCGGTATTCA-3′). The amplified fragment was digested with SmaI and SalI. The fragment was ligated into the NotI/SalI-digested YEp352GAPII-HA, which contains three copies of the HA epitope, to generate YEp352GAPII-ALG13-HA.

**Fluorescence Microscopy**

For imaging of Cwh43p-mRFP fusion protein, cells were grown to the exponential phase at 30°C in SC–uracil medium and washed twice. The cellular localization of Cwh43p-mRFP was visualized by fluorescence microscopy by using an Olympus IX-71 fluorescence microscope system (Olympus, Tokyo, Japan) and processed using IPLab software (Scionetics, Fairfax, VA).

**Analysis of PI Moieties of GPI-anchored Protein**

Isolation of the radiolabeled lipid moieties of GPI anchor and lipid analysis were performed as described previously (Guillas et al., 2000; Fujita et al., 2006a).

**Lipid Analysis**

The lipids were analyzed by thin-layer chromatography (TLC) on a Silica gel 60 plate (Merck, Darmstadt, Germany) by using solvent system 1 (55:45:10, vol/vol CHCl3:CH3OH:0.25% KCl) or solvent system 2 (10:10:3, vol/vol CHCl3:CH3OH:H2O). Labeling of ceramide-containing proteins with [3H]DHS was performed after cells were grown to the exponential phase in SC medium. Washed cells were resuspended in fresh SC medium containing 80 μg/ml mycophenolic acid (Sigma-Aldrich, St. Louis, MO), incubated at 30°C for 20 min, and incubated with [3H]DHS for 2 h. The labeling reaction was stopped by adding cycloheximide, and cells were washed with 10 mM NaN3. Labeled proteins were extracted by boiling in 1% SDS. Samples were combined with concanavalin A (Con A) buffer (50 mM Tris-HCl, pH 7.5, containing protease inhibitor cocktail; Roche, Mannheim, Germany), and solubilized by boiling in 1% SDS. Samples were run on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was incubated with a polyclonal antiphosphatidyl inositol antibody (Calbiochem, La Jolla, CA), followed by a secondary antibody labeled with [125I]protein A. Autoradiography was performed using Hyperfilm (Amersham, Arlington Heights, IL).

**Radiolabeling of Proteins**

Radiolabeling of proteins with [3H]DHS (American Radiolabeled Chemicals, St. Louis, MO) was performed as described previously with some modifications (Guillas et al., 2000; Umemura et al., 2003).

Labeling of ceramide-containing proteins with [3H]DHS was performed after cells were grown to the exponential phase in SC medium. Washed cells were resuspended in fresh SC medium containing 80 μg/ml mycophenolic acid (Sigma-Aldrich, St. Louis, MO), incubated at 30°C for 20 min, and incubated with [3H]DHS for 2 h. The labeling reaction was stopped by adding cycloheximide, and cells were washed with 10 mM NaN3. Labeled proteins were extracted by boiling with glass beads in TEB buffer (100 mM Tris-HCl, pH 7.5, containing protease inhibitor cocktail; Roche, Mannheim, Germany), and solubilized by boiling in 1% SDS. Samples were run on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was incubated with a polyclonal antiphosphatidyl inositol antibody (Calbiochem, La Jolla, CA), followed by a secondary antibody labeled with [125I]protein A. Autoradiography was performed using Hyperfilm (Amersham, Arlington Heights, IL).
Preparation of Cell Lysate and Immunoblotting

Cells were grown to the exponential phase and collected by centrifugation. The cells were washed and broken using glass beads in TNPI buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, protease inhibitor cocktail [Roche], and 1 mM phenylmethylsulfonyl fluoride) at 4°C. Cell debris was removed by centrifugation. The cell extracts were then centrifuged at 13,000 × g to sediment the ER-rich fraction. The ER-rich pellet was solubilized in 1% Triton X-100 at 4°C for 30 min, and then it was centrifuged again at 13,000 × g. The supernatant was collected, mixed with SDS sample buffer, separated by SDS-PAGE, and analyzed by immunoblotting by using one of the following antibodies: anti-HA (PAGE) and analyzed using a Molecular Imager FX (Bio-Rad). The samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed using a Molecular Imager FX (Bio-Rad).

Isolation of DRMs

Cells were grown at 30°C in YPAD to the exponential phase, and 3 × 10^9 cells were collected. DRMs for the Gas1p analysis were isolated as described previously with a slight modification (Bagnat et al., 2000; Okamoto et al., 2006). After incubation with 1% Triton X-100 for 30 min on ice, the lysates were subjected to OptiPrep density gradient fractionation by centrifugation for 2 h at 40,000 rpm in a SW55 Ti rotor (Beckman Coulter, Fullerton, CA). After centrifugation, six fractions of equal volume were collected starting from the top. Each fraction was mixed with sample buffer and subjected to SDS-PAGE and immunoblotting.

Immunoprecipitation

Immunoprecipitation was performed as described previously with some modifications (Rayner and Munro, 1998). The cells were grown to the exponential phase in YPAD, and then they were collected by centrifugation and washed with water. The washed cells were resuspended in Tri-Sorb buffer (1 M sucrose, 50 mM Tris-HCl, pH 7.5) containing Zymolyase 100T (Seikagaku, Tokyo, Japan), and then they were incubated at 30°C for 30 min. The resulting spheroplasts were washed in Tri-Sorb buffer and lysed at 4°C for 30 min by gentle mixing in TNPI buffer. Next, the lysate was solubilized with 1% digitonin. Cell debris was removed by centrifugation, and the supernatant was incubated overnight with anti-FLAG M2 affinity gel (Sigma-Aldrich) or anti-HA affinity matrix (Roche). The beads were washed with TNPI buffer containing 1% digitonin and suspended into TNPI buffer containing 3xFLAG peptide (Sigma-Aldrich) or HA peptide (Roche) to elute the protein. The eluted proteins were denatured with SDS sample buffer, separated by SDS-PAGE, and analyzed by immunoblotting.

RESULTS

CWH43 Is a Homologue of Mammalian PGAP2 That Is Involved in GPI Lipid Remodeling

Recently, rat PGAP2 was reported to be a Golgi/ER resident membrane protein that seems to participate in the transfer of a saturated acyl chain to the sn-2 position of lyso-PI of the GPI anchor after the removal of the unsaturated acyl chain (Tashima et al., 2006). To further investigate lipid remodeling in yeast, we searched for the homologue of PGAP2 in S. cerevisiae by using BLAST (Altschul et al., 1997). The BLAST search revealed that the yeast homologue of PGAP2 is Cwh43p, a 953-amino acid protein that contains 18 potential membrane-spanning regions according to SOSUI analysis (Hirokawa et al., 1998). The amino acid sequence of human PGAP2 shares 24% homology with yeast Cwh43p; however, Cwh43p has an extra ~700-amino acid C terminus containing several membrane-spanning regions. This additional C-terminal extra part of Cwh43p is 31% identical to the product of an uncharacterized human gene, FLJ21511 (Pittet and Conzelmann, 2007). Furthermore, it has been reported that CWH43 is involved in the cell wall integrity (Martin-Yken et al., 2001). Therefore, we expected that Cwh43p participates in GPI lipid remodeling but that it also has some additional functions that are not shared by mammalian PGAP2.

CWH43 Is Involved in Lipid Remodeling of the GPI Anchor from a Diacylglycerol- to a Ceramide Type

To address whether CWH43 is also involved in the lipid remodeling of GPI anchor, we examined PI moieties obtained from GPI-anchored proteins of cwh43Δ cells. Cells were labeled with [3H]inositol, and the protein pellet was delipidated. The solubilized glycoproteins were then concentrated by Con A-Sepharose and digested with pronase. The PI moieties were liberated from GPI anchors by nitrilotriphosphorycceramide/B (IPC/B), which consists of PHS and C26:0 fatty acid, and pG1, which consists of PHS containing a C26:0 fatty acid at the sn-2 position. The PI moieties of the GPI anchor in gup1Δ, per1Δ, and gup1Δ cells were used as controls to identify the PI species. As reported previously (Bossen et al., 2006), the
The incorporation of [3H]DHS into proteins. When endogenous DHS biosynthesis is blocked by myriocin (Horvath et al., 1999), the amount of [3H] incorporation was reduced in cwh43Δ cells. First, we compared the amount of Gas1p in the wild-type and cwh43Δ cells. Total cell lysates were examined by immunoblotting with an anti-Gas1 antibody. In contrast to wild-type cells, Gas1p was drastically reduced in per1Δ and gup1Δ cells, and, in turn, released to the medium (Supplemental Figure S1A), as reported previously (Fujita et al., 2006a). Alternatively, in cwh43Δ cells, the level of Gas1p in cell lysates was not decreased, and it was not released into the medium, indicating that Gas1p is almost retained normally in cwh43Δ cells. Next, we analyzed the cellular localization of mRFP-tagged Gas1p in wild-type and cwh43Δ cells by fluorescence microscopy. In cwh43Δ cells, mRFP-Gas1p was localized at the cell surface in the same manner as in wild-type cells (Supplemental Figure S1B), indicating that the localization of Gas1p is not affected in cwh43Δ cells.

It is reported that Gas1p is incorporated into lipid rafts at the plasma membrane (Bagnat et al., 2000). Therefore, we checked whether Gas1p is sorted to the DRM in cwh43Δ cells. In cwh43Δ cells, Pma1p, a marker for DRM-associated membrane protein, was associated with the DRMs, indicating that raft formation itself was normal (Supplemental Figure S1C). In the wild-type and cwh43Δ cells, Gas1p was also located predominantly in the DRM fraction, suggesting that Gas1p in cwh43Δ cells is located in lipid rafts as in wild-type cells, different from that in per1Δ or gup1Δ cells. It has been reported that the lipid moiety of GPI anchor of Gas1p is a diacylglycerol type containing a C26 fatty acid (Fankhauser et al., 1993). Therefore, it is possible that the lipid moiety of Gas1p could be normal and localization of Gas1p is not affected in cwh43Δ cells, consisting with the aforementioned results that CWH43 is involved in the lipid remodeling of GPI anchor to ceramides.

The C-Terminal Domain of Cwh43p Is Essential for Lipid Remodeling to Ceramide

Although the N-terminal region of Cwh43p (Cwh43-N) is homologous to mammalian PAGAP2, Cwh43p has a large C-terminal region (Cwh43-C) that is similar to an uncharacterized gene product encoded by human FLJ21511. Therefore, Cwh43p corresponds to a fusion of PAGAP2 and FLJ21511 proteins. To investigate which region of Cwh43p is important for its function, we constructed plasmids for expressing both CWH43-N (encoding amino acids 1-229; 229 amino acids) and CWH43-C (encoding amino acids 218-953; 736 amino acids). The proteins produced by these expression plasmids, Cwh43-N and Cwh43-C, are tagged with three copies of the HA epitope at their C termini. Furthermore, these expression plasmids were under control of the CWH43 promoter. These two plasmids were referred to as pRS316T-CWH43-N-HA and pRS316T-CWH43-C-HA, respectively. The expression plasmids were introduced into cwh43Δ cells, and the phenotypes of the cwh43Δ cells harboring them were characterized (Figure 3). The cwh43Δ cells harboring the empty vector were CFU and temperature sensitive, and these sensitivities were rescued by introduction of CWH43.
and then incubated for 2 days at 30°C. CWH43-HA and CWH43-C-HA expression plasmids (pCwh43 and CWH43-C-HA) respectively, cwh43 was confirmed by immunoblotting by using N-HA CWH43-HA expression of essential for lipid remodeling to ceramides. (A) Expression of CWH43-C and CWH43-N-HA plasmids. Cells were labeled with [3H]inositol. PI moieties obtained from delipidated glycoproteins by deamination were analyzed by TLC as described in Methods. (B) Analysis of PI moieties obtained from GPI anchor in wild-type (WT), gpi7Δ, and cwh43Δ cells harboring CWH43, CWH43-N (N), CWH43-C (C), and both CWH43-N and CWH43-C (N+C) expression plasmids. Cells were labeled with [3H]inositol. PI moieties obtained from delipidated glycoproteins by deamination were analyzed by TLC as described in Methods. CWH43-C; CWH43-N: CWH43-C-HA expression plasmid, [3H]labeled DHS derivatives were incorporated into proteins, whereas the incorporation of [3H]DHS derivatives into proteins in the same cells as shown in Figure 4B was analyzed as described in methods. The C-terminal domain of Cwh43p is important for its ability to remodel lipids to ceramides. In contrast, the cwh43Δ cells harboring the CWH43-C expression plasmid (CWH43-C cells) accumulated a small but significant amount of IPC/B in addition to pG1 and lyso-PI (Figure 4B, lane 4), suggesting that the C-terminal region of Cwh43p is important for its ability to remodel lipids to ceramides. In addition, the cwh43Δ cells coexpressed both CWH43-N and CWH43-C (CWH43-N/C cells) accumulated more IPC/B than the cells expressed CWH43-C alone (Figure 4B, lane 6), indicating that CWH43-N is able to enhance the lipid remodeling to ceramides by CWH43-C.

To confirm that CWH43-C is important for the remodeling of GPI anchors to ceramides, we further examined the incorporation of [3H]DHS derivatives into proteins in CWH43-C–expressing cells. The cells were labeled with [3H]DHS, and cell lysates were prepared. Glycoproteins were concentrated with Con A-Sepharose, and they were separated by SDS-PAGE. In cwh43Δ cells harboring the CWH43 expression plasmid, [3H]labeled DHS derivatives were incorporated into proteins, whereas the incorporation of [3H]labeled
DHS derivatives was scarcely detected in cwh43Δ cells harboring the empty vector (Figure 4C). In contrast, in CWH43-C cells but not CWH43-N cells, 3H-labeled DHS derivatives were incorporated into proteins. Moreover, in CWH43-N/C cells, there was a higher level of incorporation than in CWH43-C cells. These results suggest that the C-terminal region of Cwh43p is important for GPI lipid remodeling from the diacylglycerol type to the ceramide type and that the N-terminal region of Cwh43p enhances ceramide formation by the C-terminal region. Therefore, we designated these N- and C-terminal regions of Cwh43p as Cwh43-N and Cwh43-C domains, respectively.

The Cwh43-N Domain Associates with the Cwh43-C Domain

Because CWH43-N enhanced the function of CWH43-C, we suspected that Cwh43-N protein associates with Cwh43-C protein. To test this possibility, we performed a coimmunoprecipitation assay. Cwh43-N was tagged at its C terminus with three copies of FLAG epitope (encoded by CWH43-N-FLAG gene). CWH43-N-FLAG and CWH43-C-FLAG were coexpressed from the CWH43 promoter in yeast cells, and the products were immunoprecipitated from total lysates solubilized with digitonin. Cwh43-N-FLAG was immunoprecipitated with FLAG-beads, and Cwh43-C-FLAG was immunoprecipitated with HA-beads. The immunoprecipitated samples were separated by SDS-PAGE and analyzed by immunoblotting using an anti-FLAG antibody or an anti-HA antibody.

A Highly Conserved Region of Cwh43-C Domain Is Important for the Lipid Remodeling Function

As described previously (Martin-Yka et al., 2001), we found that the cwh43Δ-2 mutant, in which a conserved glycine at amino acid 57 was replaced with arginine (G57R), is CFW sensitive and that it reduced cell wall integrity (Figure 6A), but why the mutant is sensitive to CFW and whether G57 is essential for the remodeling activity of Cwh43p has not been determined. To examine the GPI phenotypes in the cwh43Δ-2 mutant, we constructed a plasmid for expressing G57R-introduced Cwh43p (Figure 6C, lanes 1 and 2). The amount of Cwh43p was confirmed by immunoblotting by using an anti-HA antibody. The amounts of these mutant Cwh43-C proteins were similar to that of wild-type Cwh43p (Figure 6C).

Next, we characterized the PI moieties obtained from the GPI-anchored proteins produced by these mutant cells (Figure 6D). Wild-type CWH43-C-introduced cwh43Δ cells accumulated a small amount of IPC/B in addition to pG1 and lyso-PI, whereas cwh43Δ cells harboring the empty vector accumulated pG1 and lyso-PI but not IPC/B (Figure 6D), as described above. Interestingly, in R882A mutant cwh43Δ introduced cwh43Δ cells, IPC/B lipid moieties were not detected in GPI anchor (Figure 6D, lane 9). Also, the amount of IPC/B was decreased in D862A mutant cwh43Δ introduced cwh43Δ cells (Figure 6D, lane 8), whereas the amount of IPC/B was not decreased in the other mutant cwh43Δ introduced cwh43Δ cells. Furthermore, D862A and R882A mutants of CWH43-C did not complement the CFW sensitivity of cwh43Δ cells, whereas the sensitivity was complemented by the wild type and H472A, D693A, H770A/H717A, and E807A mutants of CWH43-C (Figure 6E). We also checked whether GPI-anchored proteins are remodeled to ceramide, which can be detected by incorporation of 3H-labeled DHS derivatives into proteins in these mutant cells. In the cwh43Δ cells introduced with the R882A mutant CWH43-C, 3H-labeled signals were not detected in proteins, whereas 3H-labeled signals were observed in the other mutant and wild-type CWH43-C-introduced cwh43Δ cells (Figure 6F).

The Mouse CWH43 Counterparts FRAG1 and C130090K23 Complement CWH43-deficient Yeast Phenotypes

According to a BLAST search, the mouse homologues of human PGAP2 and FLJ21511 are FRAG1 and C130090K23, respectively. The predicted amino acid sequences of mouse FRAG1 and C130090K23 are 29 and 31% identical to yeast Cwh43p, respectively. To address whether mouse C130090K23 is a functional homologue of CWH43-C, we introduced expression plasmids of FRAG1 and C130090K23 into the cwh43Δ cells and examined CFW sensitivity and PI moieties obtained from GPI-anchored proteins. The C130090K23 gene rescued the CFW sensitivity of cwh43Δ, but the FRAG1 gene did not (Figure 7A). This is similar to the results for CWH43-C and CWH43-N-introduced cwh43Δ cells, respectively (Figure 3). These results suggest that C130090K23 but not FRAG1 rescues the cell wall defect in cwh43Δ cells.
Figure 6. The highly conserved region containing D862 and R882 is essential for the function of Cwh43-C. (A) Alignment of the amino acid sequences of the highly conserved N-terminal region of Cwh43p containing G57. The amino acid sequences of *S. cerevisiae*, *Aspergillus fumigatus*, *Schizosaccharomyces pombe*, *Homo sapiens*, and *Mus musculus* proteins were aligned using ClustalW. Asterisks (*) and colons (:) indicate identical and conserved amino acids, respectively. (B) Alignment of the amino acid sequences of the highly conserved C-terminal region of Cwh43p containing H472A, D693A, H770A/H771A, E807A, D862A, and R882A. The amino acid sequences of *S. cerevisiae*, *A. fumigatus*, *S. pombe*, *H. sapiens*, and *M. musculus* proteins were aligned using ClustalW. (C) The production of Cwh43-HA, G57R mutant Cwh43-HA, Cwh43-C-HA, and H472A, D693A, H770A/H771A, E807A, D862A, and R882A mutants of Cwh43-C-HA in cwh43/H9004 cells was examined by immunoblotting by using anti-HA and anti-Dpm1p antibodies. Cells were cultured at 30°C, and cell lysates were extracted. ER-rich fractions were concentrated by centrifugation at 13,000 × g and separated by SDS-PAGE. Dpm1p was used to confirm equal loading. (D) Analysis of PI moieties obtained from GPI-anchored protein by deamination in the same cell as shown in Figure 6C. Cells were labeled with [3H]inositol, and PI moieties obtained from GPI-anchored proteins by deamination were analyzed as described in Figure 2A. (E) The same cells as in Figure 6C were analyzed for CFW sensitivity. The cells were cultured on YPAD and YPAD containing 10 μg/ml CFW at 30°C for 2 d as described in Figure 3. (F) Analysis of incorporation of [3H]DHS derivatives into proteins in the same cells as in Figure 6C. Cells were labeled with [3H]DHS in the presence of myriocin, and incorporation of [3H]DHS was analyzed as described in Figure 2B.
proteins in was lower than that of the yeast Cwh43-HA protein. The cells were labeled with [3H]inositol, and the labeled glycoproteins were concentrated by Con A-Sepharose and digested with pronase. The PI moieties were obtained by deamination were analyzed in GPI-anchored proteins from diacylglycerol type to ceramides. PI moieties obtained using FRAG1-HA in lysates containing C130090K23-FLAG but not in control lysates (Figure 7A). C130090K23-FLAG also coprecipitated with FRAG1-HA in lysates containing FRAG1-HA. Because interaction of FRAG1-HA and C130090K23-FLAG are shown in low efficiency, we further tried the immunoprecipitation by using FRAG1-FLAG and C130090K23-FLAG. FRAG1-FLAG was coprecipitated with C130090K23-FLAG and C130090K23-FLAG with FRAG1-FLAG when the two were coexpressed but not in control sample. These results indicate that, like Cwvh43-N and Cwvh43-C, FRAG1 and C130090K23 associate with each other.

Moreover, we examined the association of C130090K23-FLAG and FRAG1-FLAG with Alg14-HA to exclude the possibility that the interaction is derived from some artifact. Alg14p, which is an ER-resident membrane protein, is associated with Alg13p and involved in GlcNAc2-PP-dol formation (Bickel et al., 2005; Gao et al., 2005). Alg14-FLAG expressing plasmid was cointroduced with C130090K23-FLAG, FRAG1-FLAG, or ALG13-FLAG-expressing plasmids into cwh43Δ cells. Total lysate was immunoprecipitated using FLAG affinity agarose gels, and immunoprecipitation samples are analyzed by immunoblotting using anti-FLAG, anti-Dpm1p antibodies. Alg13-FLAG was coimmunoprecipitated with Alg14-FLAG, but not with FRAG1-FLAG and C130090K23-FLAG. Moreover, in all three immunoprecipitation samples, Dpm1p was not detected as any distinct bands, although Dpm1p was detected in total lysate of all three samples. These results suggest that C130090K23-FLAG and FRAG1-FLAG are not physically interacted with Alg14p-FLAG and Dpm1p. Together, C130090K23 and FRAG1 are specifically associated together.

**DISCUSSION**

The lipid remodeling of yeast GPI-anchored protein begins with the removal of an unsaturated fatty acyl chain from diacylglycerol by a GPI-PLA activity. Per1p is involved in this reaction (Fujita et al., 2006a). Next, a saturated long fatty
acyl chain is transferred to lyso-GPI by the O-acyltransferase Gup1p (Bosson et al., 2006), generating diacylglycerol containing a C26 fatty acid. Most mature GPI-anchored proteins, however, contain ceramides in their lipid moieties. Our analysis of the lipid moiety obtained from GPI-anchored proteins by deamination showed that cwh43Δ cells accumulate pG1, which is a PI containing a saturated C26 fatty acid, and lyso-PI but not IPCs. Therefore, it is conceivable that CWH43 is involved in the remodeling of GPI-anchored proteins from the diacylglycerol type to the ceramide type.

On the basis of our results and previous reports (Sipos et al., 1994; Bosson et al., 2006; Fujita et al., 2006a), we speculated that there are two possibilities for the remodeling pathway of GPI lipids to ceramides (Figure 9). One possibility may be a sequential pathway and the other may be diverged pathway. In former case [Figure 9, (1)] an unsaturated fatty acid is removed from diacylglycerol of conventional PI to generate lyso-PI-type GPI anchor (lyso-PI). Per1p is involved in this process. Next, a C26 saturated fatty acid is transferred to lyso-PI by the O-acyltransferase Gup1p, generating PI with diacylglycerol containing a C26 saturated fatty acid at the sn-2 position (pG1). Diacylglycerol containing a C26 saturated fatty acid may then be converted to a ceramide moiety (IPC). Cwh43p may be involved in this step because the GPI-anchored proteins from cwh43Δ cells accumulated pG1-type PI. Alternatively, the pathway may diverge. In this case, the lyso-PI generated by Per1p could be used as a substrate for two reactions executed by Gup1p and Cwh43p [Figure 9, (2)]. In one of these reactions, C26 saturated fatty acid is transferred to lyso-PI by Gup1p to generate diacylglycerol containing a C26 fatty acid (pG1). In the other reaction, lyso-PI may be substituted directly for ceramide moiety (IPC), a step that may involve Cwh43p. In this case, the pathway where lyso-PI is transferred with C26 fatty acid to generate diacylglycerol might be enhanced when the conversion to ceramides is defective. A third additional pathway may also exist as an option of both sequential and diverged pathways, as shown by dotted lines in Figure 9 (3). Diacylglycerol with an unsaturated fatty acid in conventional PI is directly exchanged for a ceramide moiety, together with the pathway mediated by Per1p and Gup1p. Cwh43p could be involved in this direct exchange of unsaturated fatty acid for a ceramide moiety because GPI-anchored proteins from per1Δgup1Δ double-mutant cells accumulate IPC/C in addition to PI (Fujita et al., 2006a). This third alternative may operate as a bypass or as an emergency route for exchange when there is a defect in the steps mediated by Per1p and Gup1p. Identification of a direct substrate for Cwh43p should help determine the detailed molecular mechanism of lipid remodeling in GPI anchors to ceramides.

We found that the plasma membrane localization of a major GPI-anchored protein, Gas1p, was unaffected in cwh43Δ cells, whereas other studies have shown that Gas1p...
Gas1 could be a diacylglycerol type even in cwh43Δ cells (Fankhauser et al., 1993). Therefore, it is possible that the lipid moiety of Gas1 could be a diacylglycerol type even in cwh43Δ cells, which is consistent with the finding that the localization of Gas1p was unchanged in cwh43Δ cells. Except for Gas1p, specific GPI-anchored proteins predominantly containing ceramide in the lipid moiety have not been identified in S. cerevisiae, even though they more often contain ceramide-type diacylglycerol-like lipid moieties. Thus, it is possible that in some specific tissues where C130090K23 is produced, FRAG1 and C130090K23 associate with other proteins in the formation of ceramidetype lipid moieties in GPI-anchored proteins. In contrast, in mammalian cells, alkyl/acyl PI moieties are selectively concentrated during the GPI biosynthesis whereas endogenous PI, which is used for the start of GPI biosynthesis, is generally diacyl species (Houjou et al., 2007). Although it is unknown what genes are involved in these events and how reactions occurred, it is also possible that C130090K23 might be involved in the uncharacterized lipid remodeling reaction as described above in mammalian cells.

According to SGD, Cwh43p has a DnaSE I-like region at its C terminus. Isc1p, Inp51p, Inp52p, Inp53p, and Inp54p also have this Dnase I-like region. Isc1p is an inositol phospho-phingolipid phospholipase C (Sawai et al., 2000), and Inp51/52/53/54 proteins are involved in inositol 5-phosphatase activity (Stolz et al., 1998a; Guo et al., 1999; Wiradja et al., 2001). Therefore, this motif may be related to the recognition of inositol phosphate, implying that a Dnase I-like region in the C-terminal domain of Cwh43p is important for the recognition of inositol phosphate in the GPI anchor. However, it is still unclear whether the conserved D862 and R882 residues, which are likely to be important for its function but are not in the Dnase I-like region, are directly involved in the recognition of inositol phosphate.

In this study, we identified how Cwh43p participates in the lipid remodeling of diacylglycerol to ceramides in GPI-anchored proteins; however, how diacylglycerol is replaced with ceramides and what substrate is used for the replacement remains unclear. Future investigations of Cwh43p-mediated lipid remodeling should clarify the molecular mechanism by which the lipid moiety of GPI anchors is replaced with ceramides and its precise biological function.

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