GWT1 Gene Is Required for Inositol Acylation of Glycosylphosphatidylinositol Anchors in Yeast*

Received for publication, January 30, 2003, and in revised form, April 15, 2003
Published, JBC Papers in Press, April 24, 2003, DOI 10.1074/jbc.M301044200

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Glycosylphosphatidylinositol (GPI) is a conserved post-translational modification to anchor cell surface proteins to plasma membrane in all eukaryotes. In yeast, GPI mediates cross-linking of cell wall mannoproteins to β1,6-glucan. We reported previously that the GWT1 gene product is a target of the novel anti-fungal compound, 1-[4-butylnbenzyl]isoquinoline, that inhibits cell wall localization of GPI-anchored mannoproteins in Saccharomyces cerevisiae (Tsukahara, K., Hata, K., Sagane, K., Watanabe, N., Kuromitsu, J., Kai, J., Tsuchiya, M., Ohba, F., Jigami, Y., Yoshimatsu, K., and Nagasu, T. (2003) Mol. Microbiol. 48, 1029–1042). In the present study, to analyze the function of the GWT1 protein, we isolated temperature-sensitive gwt1 mutants. The gwt1 cells were normal in transport of invertase and carboxypeptidase Y but were delayed in transport of GPI-anchored protein, Gas1p, and were defective in its maturation from the endoplasmic reticulum to the Golgi. The incorporation of inositol into GPI-anchored proteins was reduced in gwt1 mutant, indicating involvement of GWT1 in GPI biosynthesis. We analyzed the early steps of GPI biosynthesis in vitro by using membranes prepared from gwt1 and Δgwt1 cells. The synthetic activity of GlcN-(acyl)PI from GlcN-PI was defective in these cells, whereas Δgwt1 cells harboring GWT1 gene restored the activity, indicating that GWT1 is required for acylation of inositol during the GPI synthetic pathway. We further cloned GWT1 homologues in other yeasts, Cryptococcus neoformans and Schizosaccharomyces pombe, and confirmed that the specificity of acyl-CoA in inositol acylation, as reported in studies of endogenous membranes (Franzot, S. P., and Doering, T. L. (1999) Biochem. J. 340, 25–32), is due to the properties of Gwt1p itself and not to other membrane components.

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‡ The abbreviations used are: GPI, glycosylphosphatidylinositol; Te°, temperature-sensitive; PI-PLC, phosphatidylinositol-specific phospholipase C; CoA, coenzyme A; Btq, 1-[4-butylnbenzyl]isoquinoline; ORF, open reading frame; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; ER, endoplasmic reticulum; CPY, carboxypeptidase Y; ConA, concanavalin A.

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between yeast and mammals. However, in the GPI biosynthetic pathway, the gene responsible for inositol acylation of GlcN-PI has not yet been identified, and it is unclear whether acylation precedes the “flip-flop” involving GPI translocation from the cytoplasmic side to luminal side of ER membranes (26). It is reported that de-acetylation, which precedes acylation, occurs on the cytoplasmic side, whereas the first mannosylation, which follows acylation, occurs on the luminal side of ER membranes (31, 32, 42). This raises the question whether inositol acylation occurs on the cytoplasmic or luminal side.

We recently reported (43) a novel compound, 1-(4-butylnaphthalenyl)-2-methoxy-3-(6-bromopyridin-3-yl)isoquinoline (BIQ), that inhibits cell wall localization of GPI-anchored proteins. We also identified a novel uncharacterized gene, GWT1, as a target of this compound. GWT1 gene-deleted (Δgwt1) cells showed a defect in cell wall assembly and extremely slow growth. In the present study, we isolated temperature-sensitive mutants of GWT1 (gwt1) to investigate the function of Gwt1p. Maturation and/or transport of the GPI-anchored protein, Gas1p, judged by the fluorescence microscopy using an Olympus microscope system. Images were acquired by a digital charged couple device camera and processed with CoolSNAP software.

Inositol Acylation of Glycosylphosphatidylinositol in Yeast

In Vitro Mutagenesis of GWT1 and Isolation of Temperature-sensitive gwt1 Mutants—Temperature-sensitive (Ts) alleles of GWT1 were generated by PCR mutagenesis as described by Muhlrad et al. (45) and Mati et al. (46). GWT1 was amplified under mutagenic PCR conditions by using forward primer (5′-GGCCGCTGAGACC-ACCATGGTGAACAGGGGAG-3′) and reverse primer (5′-TGAACATGAGCTTATGATATGACGAG-3′). These PCRs contained the following components: 1× reaction buffer (Takara Shuzo), 50–100 ng of plasmid pRS315-GWT1 as template plasmid; 0.8 mM each primer; 2.5 mM MgCl2; 0.5 mM each of dCTP, dGTP, and dTTP; 1.6 μg/ml of LA polymerase (Takara Shuzo), 5 μl of each DNA fragment. Mutagenized GWT1 plasmid on potential Ts+ mutants were determined by sequencing the coding region of GWT1 gene.

Mutagenized GWT1 was subcloned into an integration vector pRS304 or pRS306 and integrated into the trp1 or ura3 locus on the chromosome of WD20, which is a GWT1Δgwt1 diploid strain (Table I). Trp+ or Ura+ transformants were selected, and His−, Trp+ or Ura+ and Ts− segregants were identified after tetrad analysis.

Plasmid Construction of pGAP-GFP-ScGWT1—GFP-ScGwt1-His chimera cDNA was constructed by connecting each DNA fragment sequentially. The ORF of green fluorescent protein (GFP) in pEGFP-N2 (Clontech) was amplified by PCR with primers 5′-GGCTGCTGAGACC-ACCATGGTGAACAGGGGAG-3′ and 5′-GGTTAATTTCTGTTAC- GCTGCTCCATGC3′. S. cerevisiae GWT1 ORF was amplified using the primers 5′-ACGAGATTATGATCTTATGATATGACGAG-3′ and 5′-CTGGTTAATTTCTGTTAC- GCTGCTCCATGC3′. The His epitope tag 5′-GAGCTTCTAGAGAGG3′ was added to the 3′-end of the GWT1 fusion construct. The resulting GFP-ScGwt1-His cassette was inserted into the low copy expression vector pRS314 or the multicopy expression vector Yep352GAP2GPI under the control of GAPDH promoter, yielding pRS314-GFP-ScGwt1 and pGAP-GFP-ScGwt1-His.

Fluorescence Microscopy—For imaging of GFW-ScGwt1-His fusion protein, cells grown to exponential phase were collected by centrifugation, fixed by 70% ethanol for 30 min, washed twice with distilled water, stained with 100 ng/ml 4′,6-diamidino-2-phenylindole for 15 min, and viewed by fluorescence microscopy using an Olympus microscope system. Images were acquired by a digital charged couple device camera and processed with CoolSNAP software.

Invertase Activity Staining—Cells were pre-cultured in 3 ml of YPAD medium at 24 or 37 °C and then transferred to YPA medium containing 0.2% sucrose to induce invertase expression. After 3 h of incubation at the indicated temperatures, the cells were collected, washed, and suspended in sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 6% mercaptoethanol, 10% glycerol, bromophenol blue). Cells were broken by mixing with glass beads (425–600 μm, Sigma), and supernatants were recovered by centrifugation (13,000 × g). Then the samples were boiled and separated by SDS-PAGE. Mobility of invertase was detected by activity staining.

Radiolabeling and Immunoprecipitation of Gas1p and CPY—Radiolabeling and immunoprecipitation were performed as described by Sutterlin et al. (17). Cells were grown to exponential phase in SD medium with low SO42− concentration as described above, then collected by centrifugation, and replaced SD-SO42− medium (0.17% yeast nitrogen base without amino acid and ammonium sulfate, 2% glucose and required nutrients without methionine and cysteine) by low sulfate medium. Samples were preincubated for 15 min at the indicated temperatures and then pulse-labeled with 3.7 MBq of [35S]Express label (PerkinElmer Life Sciences) and chased. The chase was initiated by adding a 1:100 volume of chase liquid (0.5 μM NH4SO4, 0.3% methio- nine, 0.2% yeast extract). Samples were chased for various periods, washed with sample buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, protease inhibitors (Complete, Roche Applied Science)), and broken by mixing with glass beads (425–600 μm, Sigma) for 15 min at 4 °C. Cell lysates were solubilized by boiling with 1% SDS, combined with 1 ml of TNET buffer (100 mM Tris-HCl, pH 8, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100), and centrifuged at 13,000 × g. Supernatants were incubated with anti-Gs1p (kindly provided by Dr. Howard Riezman) or anti-CPY (Molecular Probes) antibodies and protein G-agarose (Roche Applied Science) for 3 h. The beads were washed with TNET buffer and resuspended in SDS sample buffer. Immunoprecipitated samples were separated by SDS-PAGE and analyzed by Molecular Imager FX (Bio-Rad).

[1H]Hinositol Labeling of Lipids and Proteins—Labeling of proteins and lipids with [1H]Hinositol was performed after cells were grown to exponential phase. Washed cells were resuspended in SD inositol-free medium containing 0.67% yeast nitrogen base without inositol and adenine sulfate (Bio101), 5% glucose, and nutrient broth preincubated at the indicated temperature for 20 min. For labeling of lipids, myo-[2-1H]Hinositol (PerkinElmer Life Sciences) was added to the cell suspension and incubated for 1.5 h. Labeled lipids were extracted by shaking with glass beads (425–600 μm, Sigma) in CHCl3/CH3OH/ water (10:10:3 v/v), and purified by butyl alcohol wash. The lipids were analyzed by TLC with solvent system CHCl3/CH3OH/H2O (10:10:3 v/v). For labeling of proteins, myo-[1,2-1H]Hinositol (PerkinElmer Life Sciences) was added and incubated at the indicated temperature for 1.5 h, and the labeling reaction was stopped by adding Na2SO4 and NaF. Labeled proteins were extracted by shaking with glass beads.
beads in TEPI buffer, and solubilized by boiling with 1% SDS. Samples were combined with ConA buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, and 1% Triton X-100 and centrifuged for 20 min at 4 °C at 13,000 × g. ConA-Sepharose (Amersham Bioscience) was added to the supernatants and incubated at 4 °C for 3 h. The Sepharose was washed with ConA buffer and then resuspended in SDS sample buffer. Samples were separated by SDS-PAGE and analyzed by Molecular Imager FX (Bio-Rad).

**In Vitro Assay for the Early Steps of GPI Biosynthesis**—To prepare ER-enriched membranes, cells were grown in YPMD medium at 24 °C overnight. The cell pellet was washed with TM buffer (50 mM Tris-HCl, pH 7.5, 2 mM MgCl₂). After centrifugation, the cells were homogenized by mixing with glass beads (425–600 μm, Sigma), and cell lysates were prepared by centrifugation at 1,000 × g to remove cell debris, nuclei, and intact cells. ER-enriched membrane fraction was prepared by centrifugation of cell lysates at 13,000 × g and used for the in vitro assay for GPI biosynthetic pathway, as described by Costello and Orlean (22). Mixed membranes were incubated in TM buffer containing 2 mM MnCl₂, 21 μg/ml tunicamycin, 10 μM nikkomycin, and 0.5 M dithiothreitol, in the presence of 0.5–1 μM coenzyme A (CoA) and 1 mM ATP or 0.25 mM acetyl-CoA. 4.44 kBq of UDP-[14C]GlcNAc (PerkinElmer Life Sciences) was added to start the reaction. After incubation for 1 h at 24 or 37 °C, the reaction was stopped by adding 1 ml of CHCl₃/CH₃OH (1:1 v/v), and the supernatant was separated and saved. The pellet was re-extracted with CHCl₃/CH₃OH/water (10:10:3 v/v). The lipid extracts were pooled, dried, and desalted by n-butyl alcohol extraction. Labeled lipid extracts were separated by TLC using silica Gel 60 plate (Merck), with the solvent system CHCl₃/CH₃OH 1× NH₄OH (10:10:3 v/v). The action products separated on TLC plates were detected by autoradiography and analyzed by Molecular Imager FX (Bio-Rad).

**Enzyme Treatment of Radiolabeled Lipids**—Radiolabeled lipids were treated with 0.5 units of PI-PLC prepared from *Bacillus thuringiensis* (ICN) in buffer containing 100 mM Tris-HCl, pH 7.5, and 0.2% Triton X-100. After incubation overnight at 37 °C, lipids were extracted with n-butyl alcohol and analyzed by TLC as described above.

**RESULTS**

**GFP-Gwt1 Is Localized in the ER**—We recently reported a novel anti-fungal compound, 1-[4-butyrylbenzyl]isoquinoline (Biq), that blocks surface expression of GPI-anchored mannoproteins in *S. cerevisiae* (43) and identified the uncharacterized gene *GWT1* as a direct target of Biq. To obtain information about Gwt1p function, we first tried using a low copy plasmid of the *GFP-ScGWT-His* fusion gene to analyze its cellular localization, but this was unsuccessful because of the low expression level of the fusion gene. Instead, we used a multicopy plasmid of the *GFP-ScGWT1* fusion gene (pGAP-GFP-ScGWT-His) to visualize Gwt1p by fluorescence microscopy. The expression plasmid was transformed into wild type cells, and transformed cells were grown to exponential phase.

As shown in Fig. 1, the GFP-ScGWT1-His fusion protein was detected at the peri-nuclear region characteristic of ER staining (50). We confirmed that the majority of the fusion protein (GFP-ScGWT1-His) was almost intact by immunoblot analysis using anti-GFP and anti-His antibodies (data not shown). Moreover, Gwt1 protein is a multiple transmembrane protein (43) and contains charged amino acid residues, such as aspartic acids, in its transmembrane regions, which is consistent with the prediction for ER retention or Golgi-ER retrieval of ER-localized membrane proteins (51). These results suggest that Gwt1p may be localized and may function in the ER.

**Isolation of Temperature-sensitive gwt1 Mutants**—To address the function of Gwt1p, we attempted to isolate temperature-sensitive *gwt1* mutants from W303 background strain (Table I). We generated the mutant alleles by *in vitro* error-prone PCR of the *GWT1* fragment, followed by plasmid shuffling of generated *GWT1* gene, and we identified multiple missense mutations in each mutant allele. We determined which missense mutations caused the temperature-sensitive (Ts) growth at 37 °C by replacing the fragment containing mutations with the corresponding fragment harboring wild type *GWT1* gene. Finally, we isolated three Ts mutant alleles (*gwt1*-16, *gwt1*-20, and *gwt1*-28), which contained only a few mutation points in the *GWT1* gene (Fig. 2A). Three amino acid substitutions (N330S, L362P, and V479A) and two amino acid substitutions (L209P and V259D) were identified as conferring Ts phenotype in *gwt1*-16 and *gwt1*-28, respectively. The indi-
vidual single amino acid substitutions in the \( gwt1-16 \) were not responsible for the Ts\(^\circ\) phenotype, suggesting the possibility that two or three independent amino acids may be responsible for the Ts\(^\circ\) phenotype. The revertant cells showing Ts\(^\circ\) phenotype appeared frequently at 37 °C in \( gwt1-28 \), probably due to a spontaneous mutation. These mutants were not suitable for further experiments. In \( gwt1-20 \), two amino acid substitutions (W63R and V64A) were responsible for Ts\(^\circ\) phenotype, whereas each single amino acid substitution did not show Ts\(^\circ\) phenotype (data not shown). Because the \( gwt1-20 \) mutant contained only two vicinal amino acid substitutions, we used \( gwt1-20 \) mutant for further analysis. The Ts\(^\circ\) phenotype of \( gwt1-20 \) mutant was suppressed by the introduction of \( GWT1 \) expression plasmid, pRS315-GWT1 (data not shown), indicating that the mutation of \( gwt1-20 \) caused the \( gwt1 \) loss of function.

To investigate the \( gwt1-20 \) phenotype, we first checked the morphology of \( gwt1-20 \) mutant at the non-permissive temperature (37 °C). Unlike wild type cells, \( gwt1-20 \) mutants showed swelling and in some cases cell lysis (Fig. 2B). \( gwt1-20 \) cells could not grow on YPD medium at 37 °C but did grow on YPD medium containing 1 M sorbitol, as osmotic stabilizer, at 37 °C. The growth defect of \( gwt1-20 \) cells at 37 °C was also suppressed by the addition of 0.3 M KCl to the medium (data not shown). Because \( \Delta gwt1 \) cells showed a cell wall defect (43), we also further checked whether \( gwt1-20 \) mutant cells were sensitive to SDS (52) or Calcofluor White (53), as another indication of cell wall defect. Fig. 2C shows that they were more sensitive to both compounds than were the wild type cells, indicating a cell wall defect. There Are No General Defects in Secretory Pathway—Because the \( gwt1-20 \) mutant showed a cell wall defect, it was most likely to show an alternation of protein secretion. To investigate whether protein secretion is altered in the \( gwt1-20 \), we analyzed invertase secretion, whose stage can be monitored based on molecular size due to the extent of glycosylation. ER-specific core-glycosylated invertase gives a small discrete band on SDS-PAGE. In Golgi, invertase is further glycosylated by outer chain mannosylation, giving a large diffuse band at a higher molecular weight on SDS-PAGE (48). Wild type, \( gwt1-20 \), and \( sec18 \) mutant cells (Table I) were incubated at permissive (24 °C) or non-permissive (37 °C) temperatures, and invertase expression was induced. In \( sec18 \) cells, core-glycosylated invertase was detected as a small discrete band at 37 °C due to the defect in protein transport from the ER to the Golgi. In contrast, invertase migrated at a higher molecular weight as diffuse in wild type and \( gwt1-20 \) cells even at 37 °C (data not shown), indicating normal transport of invertase from ER to Golgi in these cells.

A soluble yeast vacuolar hydrolase, carboxypeptidase Y (CPY), is core-glycosylated in ER to generate the 67-kDa form (P1), and after transport to the Golgi, it is further glycosylated to generate the 69-kDa form (P2). Finally, after reaching the vacuole, CPY is processed to give a 61-kDa mature form (M) (54). Wild type and \( gwt1-20 \) mutant cells were incubated at 24 or 37 °C, pulse-labeled with a mixture of \([\text{35}S]\)methionine and

### Table I

| Strain name | Plasmid | Genotype |
|-------------|---------|----------|
| G2–10       |         |          |
| \( \Delta gwt1 \) |         |          |
| W303–1A     |         |          |
| W303–1B     |         |          |
| W303D       |         |          |
| WDG2        |         |          |
| W303-v      | pRS315  |          |
| GFP-GWT1    | pGAP-GFP-ScGWT1-His |          |
| gwt1-16     |         |          |
| gwt1-20     |         |          |
| gwt1-28     |         |          |
| gwt1-pG     | pRS315  |          |
| HMSF176     |         |          |
| \( \Delta YG \) | YEpl552-GWT1 (YG) |          |
| \( \Delta g6 \) | pRS15-GWT1 (pG) |          |
| YScGWT1     | pGAP-ScGWT1 |          |
| YCnGWT1     | pGAP-CnGWT1 |          |
| YSpGWT1     | pGAP-ScGWT1 |          |
| MAT \( \Delta gwt1 \) |         |          |
| W303-1A     |         |          |
| \( gwt1-20 \) |         |          |
| \( gwt1-28 \) |         |          |
| MAT \( gwt1-20 \) |         |          |
| MAT \( gwt1-28 \) |         |          |

Fig. 2. Isolation and phenotype of \( gwt1 \) mutants. A, mutational change in \( gwt1 \) alleles. Details of isolation are described under “Experimental Procedures.” B, morphology of \( gwt1-20 \) mutant at 37 °C. Wild type (WT) and \( gwt1-20 \) cells were grown to exponential phase at 25 °C in YPAD medium and then incubated at 37 °C for 8 h. Cell morphology was analyzed by microscopy. C, phenotype of \( gwt1-20 \). Wild type and \( gwt1-20 \) cells were grown in YPAD medium. Diluted cells were spotted onto YPAD plate alone or with 1 M sorbitol (+Sor.), 0.065% SDS (+SDS), or 4 μg/ml Calcofluor White (+CFW). The plates were incubated for 2 days at the indicated temperatures.
Inositol Acylation of Glycosylphosphatidylinositol in Yeast

We investigated whether incorporation of radiolabeled inositol into proteins in gwt1-20 mutant was blocked at 37°C. All detectable protein-bound inositol isomers are present as the GPI-attached form in yeast (59). We labeled wild type, gwt1-20, and sec18 cells with myo-[1,2-3H]inositol at 24 or 37°C, and we prepared total cell lysates as described under "Experimental Procedures." Radiolabeled glycoproteins were affinity-purified by ConA-Sepharose to enrich mannoproteins and separated by SDS-PAGE. The loaded amounts of total proteins were confirmed by Coomassie Brilliant Blue staining of the SDS-PAGE gel (Fig. 3, A and B). Labeled inositol was incorporated into proteins in sec18 cells at the permissive temperature (24°C), and the incorporation was not blocked at the non-permissive temperature (37°C) despite the defect in protein transport from ER to Golgi, indicating that GPI transfer to proteins occurred normally in sec18 even at 37°C (data not shown). In contrast, in gwt1-20 cells harboring a control vector pRS315 (gwt1-v), incorporation of labeled inositol into proteins was reduced slightly at 24°C and was hardly detected at 37°C (Fig. 3A). The gwt1-20 cells harboring the GWT1 expression plasmid (gwt1-pG) restored the incorporation signals into protein even at 37°C (Fig. 3A), indicating that the defect is due to the loss of gwt1 function. Moreover, the incorporation was reduced drastically in Δgwt1 (Δ), as compared with wild type (G2-10 WT) cells at 24°C (Fig. 3C). These results indicate that the gwt1-20 and Δgwt1 cells have a defect in either GPI biosynthesis or GPI anchor attachment to proteins.

In Vitro Assay of Early Steps in GPI Biosynthesis in gwt1-20 Mutant—To determine whether GPI biosynthesis is defective in gwt1-20 cells, we checked enzyme activities of the early steps of the GPI biosynthetic pathway in vitro. Membrane fractions, prepared from wild type and gwt1-20 cells grown to exponential phase, were incubated with UDP-[14C]GlcNac at 24°C, and lipid extracts containing the reaction products were separated by TLC. Membranes from wild type cells generated the three GPI intermediates, GlcNAc-P1, GlcN-P1, and GlcN-(acyl)P1, in the presence of CoA and ATP (Fig. 4A, lane 1). We treated the lipid extracts with PI-PLC at 37°C overnight and confirmed that GlcN-(acyl)P1 was resistant to the cleavage by PI-PLC, whereas the non-acylated intermediates, GlcNAc-P1 and GlcN-P1, were not (Fig. 4A, lane 4). GlcN-(acyl)P1 was not detected in the absence of CoA and ATP (Fig. 4A, lane 3), consistent with the previous finding that generation of acyl-CoA as a donor of

[^55]Cysteine, and then chased for various periods, as described under "Experimental Procedures." The cell lysates were subjected to immunoprecipitation with anti-CPY antibody and analyzed by SDS-PAGE. At both 24 and 37°C, the extent of glycosylation and maturation of CPY was almost the same for gwt1-20 cells as for wild type (data not shown), indicating that transport of CPY from ER to vacuole via Golgi was not altered in gwt1-20 cells.

Gas1p is a hyperglycosylated GPI-anchored protein localized in the plasma membrane (7, 55) and has a core-glycosylated form of 105 kDa in the ER. After cleavage of the C-terminal peptide and subsequent attachment of GPI to the C-terminal processed polypeptide, GPI-anchored Gas1p is transported to Golgi, where Gas1p is converted to an extensively glycosylated 125-kDa mature form (56, 57). To investigate the transport of GPI-anchored proteins in the gwt1-20 mutant, the cells were labeled with a mixture of [35S]methionine and [35S]cysteine, and then chased for various periods, as described under "Experimental Procedures." Radiolabeled glycoproteins were affinity-purified by ConA-Sepharose to enrich mannoproteins and separated by SDS-PAGE. The loaded amounts of total proteins were confirmed by Coomassie Brilliant Blue staining of the SDS-PAGE gel (Fig. 3, A and B). Labeled inositol was incorporated into proteins in sec18 cells at the permissive temperature (24°C), and the incorporation was not blocked at the non-permissive temperature (37°C) despite the defect in protein transport from ER to Golgi, indicating that GPI transfer to proteins occurred normally in sec18 even at 37°C (data not shown). In contrast, in gwt1-20 cells harboring a control vector pRS315 (gwt1-v), incorporation of labeled inositol into proteins was reduced slightly at 24°C and was hardly detected at 37°C (Fig. 3A). The gwt1-20 cells harboring the GWT1 expression plasmid (gwt1-pG) restored the incorporation signals into protein even at 37°C (Fig. 3A), indicating that the defect is due to the loss of gwt1 function. Moreover, the incorporation was reduced drastically in Δgwt1 (Δ), as compared with wild type (G2-10 WT) cells at 24°C (Fig. 3C). These results indicate that the gwt1-20 and Δgwt1 cells have a defect in either GPI biosynthesis or GPI anchor attachment to proteins.

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Inositol acylation from endogenous fatty acid requires CoA and ATP in *S. cerevisiae* (22). Interestingly, membranes prepared from *gwt1-20* cells generated much smaller amounts of GlcN-(acyl)PI than those from wild type cells, when measured at the permissive temperature (24 °C) (Fig. 4A, lane 2). In *gwt1-20* membranes measured at the non-permissive temperature (37 °C), GlcNAc-PI and GlcN-PI were detected, but the acylated product GlcN-(acyl)PI was not (Fig. 4A, lane 6). These results indicate that *gwt1-20* cells are defective in acylation of inositol during the GPI biosynthetic pathway. Moreover, we performed *in vitro* assay using membranes prepared from *Δgwt1* cells. Although these membranes did not generate the acylated product GlcN-(acyl)PI (Fig. 4B, lane 2), such membranes prepared from *Δgwt1* cells harboring the *GWT1* expression plasmid (Δ/pG, as shown in Table I) showed normal inositol acyltransferase activity (Fig. 4B, lane 3). These results clearly indicate that *GWT1* is required for inositol acylation during GPI biosynthesis.

Palmitoyl-CoA is the major donor substrate for inositol acylation in GPI biosynthesis (11, 22). To address the possibility that the inositol acylation defect in *gwt1-20* cells may result from a defect in palmitoyl-CoA synthesis, we performed the above *in vitro* assay in the presence of palmitoyl-CoA as an exogenous substrate for acylation. Inositol acylation was still defective in membranes from *gwt1-20* cells, even when palmitoyl-CoA was added to the reaction mixture (Fig. 4C, lane 2). This finding indicates that *gwt1-20* mutation caused the defect in inositol acylation of GPI biosynthesis but not in acyl-CoA synthesis.

Cloning of *GWT1* Homologues from *S. pombe* and *C. neoformans*—The putative *GWT1* homologous genes of *S. pombe* and *C. neoformans* were identified in the respective genome project data bases. The *GWT1* homologue of *S. pombe* (*SpGWT1*) is termed *Spac144.10c*. This homologue consists of 459 amino acids and shows 35% homology with *GWT1* of *S. cerevisiae* (*ScGWT1*) (Fig. 6). The gene was obtained by PCR using *S. pombe* genome as the template, and primers were designed from the genome (see "Experimental Procedures"). The PCR product of *GWT1* homologue was sequenced and confirmed to contain 1.38-kb ORF of *SpGWT1*.

Nucleotide sequences encoding the entire *C. neoformans* Gwt1 protein were obtained by combination of genomic PCRs and reverse transcriptase-PCRs. Sequence analysis showed that the *C. neoformans* *GWT1* gene (*CnGWT1*) has three introns, and the deduced transcript encodes a protein with 598 amino acids. *C. neoformans* Gwt1 protein has several additional regions—30 amino acids in length, which are not observed in *S. cerevisiae* and *S. pombe* (Fig. 5). The nucleotide sequence data of *CnGWT1* reported in this paper were deposited in the DDBJ/EMBL/GenBank Data Libraries under the accession number AB092505.

These ORFs were cloned into YEp352GAPII vector to construct expression plasmids, pGAP-CnGWT1 and pGAP-SpGWT1, that were introduced into the *Δgwt1* cells of *S. cerevisiae*. These genes suppressed the growth defect of *Δgwt1* cells (data not shown). These cells also showed restoration of inositol acylation activity, which was defective in *Δgwt1* cells of *S. cerevisiae* (Fig. 6, B and C), suggesting that they are the functional homologues of *ScGWT1*.

**Substrate Specificity of Acyl-CoA in Inositol Acylation**—Fatty acid from exogenous acyl-CoA can be directly transferred to inositol during *in vitro* inositol acylation of *S. cerevisiae* and *C. neoformans* using endogenous membranes (29). In contrast, it is considered that the transfer of fatty acid to inositol is not dependent on exogenous acyl-CoA in mammalian cells (30). Efficiency of inositol acylation differed for various fatty acids of acyl-CoA as the donor substrate between *S. cerevisiae* and *C. neoformans* (29), indicating that *C. neoformans* has stricter donor specificity than *S. cerevisiae*.

To investigate whether *GWT1* directly affects the above specificity, we performed *in vitro* inositol acylation assay in GPI biosynthesis and compared activities using membranes pre-
pared from *S. cerevisiae* Δgwt1 cells harboring pGAP-ScGWT1, pGAP-CnGWT1, and pGAP-SpGWT1 plasmids, respectively (YSgWT1, YCnGWT1, and YSpGWT1 strain, as shown in Table I). Addition of various acyl-CoAs stimulated the reaction and directly affected efficiency of GlcN-(acyl)PI formation in three yeast membranes (Fig. 6). Mobility of the acylation product GlcN-(acyl)PI on TLC differed slightly in response to polarity of the fatty acids, indicating that fatty acid of exogenous acyl-CoA was transferred directly to the inositol portion of GlcN-(acyl)PI.

To examine the substrate specificity of acyl-CoA, we compared the efficiency of inositol acylation by analyzing amounts of acylated product GlcN-(acyl)PI on TLC (Fig. 6). Membranes of YScGWT1 or YSpGWT1 cells showed no significant difference in production of GlcN-(acyl)PI, regardless of which acyl-CoA or CoA and ATP as indicated (see "Experimental Procedures"). The no lane means no addition, and the PI-PLC lane means treatment by PI-PLC in the presence of CoA and ATP. Typical results from three independent experiments are shown.

*FIG. 5.* Alignment of the putative amino acid sequence of Gwt1p from *S. cerevisiae*, *C. neoformans*, and *S. pombe*. Alignment of the Gwt1 proteins was generated using the ClustalW program (62). Black and gray boxes indicate identical and conserved amino acids, respectively. Underline indicates the additional sequences specific to *C. neoformans* Gwt1p.

*FIG. 6.* Substrate specificity of acyl-CoA. Membranes prepared from *S. cerevisiae* gwt1 disrupted cells harboring pGAP-ScGWT1 (A, YScGWT1), pGAP-CnGWT1 (B, YCnGWT1), and pGAP-SpGWT1 (C, YSpGWT1) plasmids were assayed for inositol acylation in the presence or absence of acyl-CoA or CoA and ATP as indicated (see "Experimental Procedures"). The no lane means no addition, and the PI-PLC lane means treatment by PI-PLC in the presence of CoA and ATP. Typical results from three independent experiments are shown.

of acylated product GlcN-(acyl)PI on TLC (Fig. 6). Membranes of YScGWT1 or YSpGWT1 cells showed no significant difference in production of GlcN-(acyl)PI, regardless of which acyl-CoA was used as a donor (Fig. 6, A and C). In contrast, for the membrane of YCnGWT1 cells, myristoyl-CoA (C14:0) and linoleyl-CoA (C18:2) were good substrates, whereas stearoyl-CoA (C18:0), oleoyl-CoA (C18:1), and arachidonoyl-CoA (C20:4) were poor substrates (Fig. 6B). These findings on acyl-CoA specificity were largely consistent with previous results on endogenous membranes (29) and indicate that the Gwt1 protein itself, not the other membrane components, determines acyl-CoA specificity in inositol acylation.
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DISCUSSION

A novel uncharacterized gene, GWT1, was identified as a target of a novel compound that inhibits cell wall localization of GPI-anchored mannoproteins in *S. cerevisiae* (43). To address its function, we have analyzed a newly isolated *gwt1* Ts mutant that contains two vicinal amino acid substitutions (W63R and V64A) in Gwt1p. The cell wall defect was suggested by two observations, Calcofluor White sensitivity and suppression of Ts phenotype by the osmotic stabilizer (1 M sorbitol) (Fig. 2C), because these phenotypes were reported for many cells defective in various aspects of cell wall biosynthesis (48, 60). We showed here that the *gwt1* mutant was delayed in the ER-Golgi transport of the GPI-anchored protein, Gas1p, and drastically reduced the incorporation of radiolabeled inositol into proteins. Consequently, the observed delay of Gas1p transport in *gwt1* mutant may be due to the defects of GPI transfer to proteins. Our results suggest that in the *gwt1* mutant, other GPI-anchored proteins, such as cell wall localized mannoproteins, may also show defects of GPI anchor attachment to proteins, impaired assembly to cell wall, and cause a cell wall defect.

The *gwt1* mutant is defective in inositol acylation in GPI biosynthesis, providing a possible explanation for a defect of GPI-anchored proteins in the *gwt1* mutant. Although incorporation of labeled inositol to protein was greatly reduced in *gwt1* mutant, the labeled signals were not completely diminished at 37 °C (Fig. 3), suggesting that GPI biosynthesis and GPI anchor attachment may not be blocked completely in the *gwt1*-20 mutant at the non-permissive temperature. Although inositol acylation products were not detected at 37 °C in the assay of early steps of GPI biosynthesis pathway (Fig. 4), the GPI biosynthesis may still function in *gwt1* living cells.

The *gwt1*-disrupted (*Δgwt1*) cells are viable in the G2-10 strain background, despite extremely slow growth. The *Δgwt1* cells have defects in GPI transfer to proteins and GPI biosynthesis (Figs. 3 and 4B). We initially thought that blocking of inositol acylation might cause a total block of GPI biosynthesis and GPI anchor attachment to proteins. Recently, it was also reported that in the prototrophic *P. falciparum*, GlcN inhibits inositol acylation of GPI, blocking GPI biosynthesis and cell growth (61). In contrast, because *Δgwt1* cells are viable in the G2-10 strain background and lethal in the W303 strain background, we hypothesized that the GPI biosynthesis may still function without inositol acylation. It was reported that mannosylation of GPI core was not completely stopped in the absence of inositol acylation of GlcN-PI (28). Furthermore, the results that the acyl moiety on inositol ring is removed during the lipid remodeling (20, 21) suggest that *GWT1* may be different from other essential genes involved in GPI core structure synthesis in its function for cell viability. Further studies on the structural analysis of the accumulated GPI intermediates in *gwt1* mutant will provide the answer in the future.

No genes have been cloned and characterized so far for the unique step of inositol acylation during GPI biosynthesis in any eukaryotes from yeasts to mammals. We have shown here that *GWT1* is involved in this step, using *gwt1* mutant and *Δgwt1* cells harboring *GWT1* expression plasmid (Fig. 4). The result on GFP- Gwt1p protein localization in the ER (Fig. 1), which is detectable only in the multicopy expression but not in the single copy expression due to the lack of sensitivity, supports the above conclusion, because the proteins involved in the synthesis of GPI core structure are known to reside in the ER (26). Our finding that addition of exogenous acyl-CoA did not suppress the defect of acylation activity in *gwt1* mutant (Fig. 4C) excludes the possibility that *GWT1* might encode acyl-CoA synthetase. We confirmed that *GWT1* itself determines the substrate specificity of inositol acylation of GPI, differences of which were reported in *S. cerevisiae* and *C. neoformans* (29).

The substrate specificity of inositol acylation examined by using the membranes prepared from Sc*GWT1* - and *CnGWT1*-transformed cells (Fig. 5, A and B) was substantially the same as that reported previously (29) in studies of endogenous microsomal membranes prepared from various yeast species. We also tested substrate specificity of Sp*GWT1* (Fig. 5C), which was mostly the same as that of Sc*GWT1*. The differences in substrate specificity between *S. cerevisiae* and *C. neoformans* reflect the different properties of *GWT1* itself, because specificity was compared under the same cell background except for the origin of the *GWT1* gene. Interestingly, *C. neoformans* Gwt1p has several additional regions —30 amino acids in length, in contrast to other Gwt1 proteins (Fig. 5). This insert may define the unique substrate specificity of inositol acylation in *C. neoformans*, which poorly utilizes stearoyl-CoA (C18:0), oleoyl-CoA (C18:1), and arachidonoyl-CoA (C20:4) (Fig. 6B).

The substrate specificity of mammalian acylation enzyme is not reported, because it cannot utilize exogenous acyl-CoA as a donor (30). These differences in the inositol acylation of GPI in yeast may provide a good target for drugs directed against the pathogenic yeasts, such as *Candida albicans* and *C. neoformans*, that do not impair the inositol acylation in human. Because the GPI protein (i.e. inositol acyltransferase) was a direct target for BIQ that was screened for anti-fungal compound, further study will be necessary to elucidate the molecular mechanism on the inhibition of inositol acylation by BIQ.

In conclusion, we have demonstrated that the *GWT1* gene is involved in inositol acylation of GPI biosynthesis, and Gwt1p is responsible for the substrate specificity of acylation. The function of inositol acylation in GPI biosynthetic pathway, especially in flip-flop of GPI and assembly of GPI-anchored proteins to plasma membrane and cell wall, is an important topic of future study. Because the acyl moiety on inositol is removed during the course of lipid remodeling (20, 21), it is also interesting to examine the role of inositol acylation in this process.

Acknowledgments —We are grateful to Dr. Howard Riezman (Biozentrum of the University of Basel) for providing anti-Gas1p antibody. We thank Drs. Sen-itiroh Hakomori and Stephen Anderson (Pacific Northwest Research Institute) for critical reading of the manuscript and helpful discussions. We also thank Drs. Naoki Watanabe and Yasutaka Takase (Eisai Co., Ltd.) for constructing the plasmids and PCR mutagenesis method, respectively. We are grateful to the *C. neoformans* cDNA Sequencing Project (Bruce A. Roe, Doris Kupfer, Heather Bell, Sun So, Yang Tung, Jennifer Lewis, Sola Yu, Ken-ichiro Hanzawa, Dave Dyere, and Junemann Murphy), supported by NIAID Grant AI147079 from the National Institutes of Health; the *C. neoformans* Genome Project at Stanford Genome Technology Center, supported by NIAID Grant U01 AI47087 from the National Institutes of Health; and the Institute for Genomic Research, supported by NIAID Grant U01 AI14594 from the National Institutes of Health.

REFERENCES

1. Leidich, S. D., Drapp, D. A., and Orlean, P. (1994) *J. Biol. Chem.* 269, 10193–10196
2. Hyman, R. (1988) Trends Genet. 4, 5–8
3. Hirose, S., Mohney, R. P., Mutka, S. C., Ravi, L., Singleton, D. R., Perry, G., Tartakoff, A. M., and Medof, M. E. (1992) *J. Biol. Chem.* 267, 5272–5278
4. Caro, L. H., Tetelis, H., Vossen, J. H., Ram, A. F., van den Ende, H., and Kils, F. M. (1997) *Appl. Microbiol.* 52, 1477–1489
5. Smit, G. J., van den Ende, H., and Kils, F. M. (2001) *Microbiology* 147, 781–794
6. Shahinian, S., and Bussey, H. (2000) *Mol. Microbiol.* 35, 477–489
7. Nudoff, C., Jeno, P., Conzelmann, A., and Riezman, H. (1991) *Mol. Cell. Biol.* 11, 27–37
8. Udendruz, S., and Kodukula, K. (1995) *Annu. Rev. Biochem.* 64, 583–591
9. Puoti, A., and Conzelmann, A. (1992) *J. Biol. Chem.* 267, 22673–22680
10. McCauley, M. J., and Ferguson, M. A. (1993) *Biochem. J.* 304, 305–324
11. Roberts, W. L., Myher, J. J., Kuksis, A., Low, M. G., and Rosenberry, T. L. (1988) *J. Biol. Chem.* 263, 18766–18775
12. Hirose, S., Prince, G. M., Sevlever, D., Ravi, L., Rosenberry, T. L., Ueda, E., and Medof, M. E. (1992) *J. Biol. Chem.* 267, 16988–16997
13. Kamitani, T., Menon, A. K., Hallay, Y., Warren, C. D., and Yeh, E. T. (1992) *J. Biol. Chem.* 267, 24611–24619
14. Sipos, G., Puoti, A., and Conzelmann, A. (1994) *EMBO J.* 13, 2789–2796
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15. Grimme, S. J., Westfall, B. A., Wiedman, J. M., Taron, C. H., and Orlean, P. (2001) J. Biol. Chem. 276, 27731–27739
16. Gerold, P., Dieckmann-Schuppert, A., and Schwarz, R. T. (1994) J. Biol. Chem. 269, 2597–2606
17. Sutterlin, C., Doering, T. L., Schimmoller, F., Schroder, S., and Riezman, H. (1997) J. Cell Sci. 110, 2703–2714
18. Muniz, M., Morosonne, P., and Riezman, H. (2001) Cell 104, 313–320
19. Friedmann, E., Salzberg, Y., Weinberger, A., Shaltiel, S., and Gerst, J. E. (2002) J. Biol. Chem. 277, 35274–35281
20. Reggiori, F., Canivenc-Gansel, E., and Conzelmann, A. (1997) EMBO J. 16, 3396–3398
21. Chen, R., Walter, E. I., Parker, G., Lapurga, J. P., Millan, J. L., Ikehara, Y., Udendrieth, S., and Medof, M. E. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9512–9517
22. Costello, L. C., and Orlean, P. (1992) J. Biol. Chem. 267, 8609–8603
23. Guther, M. L., and Ferguson, M. A. (1995) EMBO J. 14, 3080–3083
24. Smith, T. K., Sharma, D. K., Crossman, A., Dix, A., Brimacombe, J. S., and Ferguson, M. A. (1997) EMBO J. 16, 6667–6675
25. Gerold, P., Jung, N., Azzouz, N., Freiberg, N., Kobe, S., and Schwarz, R. T. (1999) Biochem. J. 344, 731–738
26. Kinoshita, T., and Inoue, N. (2000) Curr. Opin. Chem. Biol. 4, 632–638
27. Urakaze, M., Kaminati, T., DeGasperi, R., Sugiyama, E., Chang, H. M., Warren, C. D., and Yeh, E. T. (1992) J. Biol. Chem. 267, 6459–6462
28. Doerrler, W. T. Ye, J., Faeck, J. R., and Lehrman, M. A. (1996) J. Biol. Chem. 271, 27031–27038
29. Franzot, S. P., and Doering, T. L. (1999) Biochem. J. 340, 25–32
30. Stevens, V. L., and Zhang, H. (1991) J. Biol. Chem. 269, 31397–31403
31. Watanabe, R., Ohishi, K., Maeda, Y., Nakamura, N., and Kinoshita, T. (1999) Biochem. J. 339, 185–192
32. Gaynor, R. E., Monroest, G., Grimmer, S. J., Reed, S. I., Orlean, P., and Emr, S. D. (1999) Mol. Biol. Cell 10, 627–648
33. Maeda, Y., Watanabe, R., Harris, C. L., Hong, Y., Ohishi, K., Kinoshita, K., and Kinoshita, T. (2001) EMBO J. 20, 250–261
34. Ban, B. C., Westfall, B. A., and Orlean, P. (2001) Yeast 18, 1383–1389
35. Canivenc-Gansel, E., Imhof, I., Reggiori, F., Burda, P., Conzelmann, A., and Benachour, A. (1998) Glycobiology 8, 761–770
36. Hamburger, D., Egerton, M., and Riezman, H. (1995) J. Cell Biol. 129, 629–639
37. Benghezal, M., Benachour, A., Rusconi, S., Abei, M., and Conzelmann, A. (1996) EMBO J. 15, 6575–6583
38. Hiri, Y., Komuro, I., Chen, R., Hosoda, T., Mizuno, T., Kudoh, S., Georgescu, S. P., Medof, M. E., and Yarzaki, Y. (1998) FEBS Lett. 421, 252–258
39. Ohishi, K., Inoue, N., Maeda, Y., Takeda, J., Riezman, H., and Kinoshita, T. (2000) Mol. Biol. Cell 11, 1523–1533
40. Fraczing, P., Imhof, I., Meyer, U., Strub, J. M., van Dorselaer, A., Vionnet, C., and Conzelmann, A. (2001) Mol. Biol. Cell 12, 3295–3306
41. Ohishi, K., Inoue, N., and Kinoshita, T. (2001) EMBO J. 20, 4088–4098
42. Nakamura, N., Inoue, N., Watanabe, R., Takahashi, M., Takeda, J., Stevens, V. L., and Kinoshita, T. (1997) J. Biol. Chem. 272, 15834–15840
43. Tsukahara, K., Hata, K., Sagane, K., Watanabe, N., Kuromitsu, J., Kai, J., Tsuchiya, M., Ohba, F., Jigami, Y., Yoshimatsu, K., and Nagasu, T. (2003) Mol. Microbiol. 48, 1029–1042
44. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1999) Short Protocols in Molecular Biology, 4th Ed., John Wiley & Sons, Inc., New York
45. Mahdavi, D., Hunter, R., and Parker, R. (1999) Yeast 8, 79–82
46. Maiti, T., Das, S., and Maitra, U. (2000) Gene (Amst.) 244, 109–118
47. Gabriel, O., and Wang, S. F. (1969) Anal. Biochem. 27, 545–554
48. Nagasu, T., Shimma, Y., Nakashima, Y., Kuromitsu, J., Iwama, K., Nakayama, K., Suzuki, K., and Jigami, Y. (1992) Yeast 8, 535–547
49. Hata, K., Kimura, J., Miki, H., Toyosawa, T., Nakamura, T., and Katsu, K. (1996) Antimicrob. Agents Chemother. 40, 2237–2242
50. Sato, K., Sato, M., and Nakano, A. (2001) J. Cell Biol. 152, 935–944
51. Delorenzi, M., Sexton, A., Shams-Eldin, H., Schwarz, R. T., Speed, T., and Schofield, L. (2002) Infect. Immun. 70, 4510–4522
52. Shimizu, J., Yoda, K., and Yamasaki, M. (1994) Mol. Gen. Genet. 242, 641–648
53. Lussier, M., White, A. M., Sheraton, J., di Paolo, T., Treadwell, J., Southard, S. B., Horenstein, C. I., Chen-Weiner, J., Ram, A. F., Kapteyn, J. C., Roemer, T. W., Ye, D. H., Bondoc, D. C., Hall, J., Zhong, W. W., Sida, A. M., Davies, J., Klu, F. M., Robbins, P. W., and Bussey, H. (1997) Genetics 147, 435–450
54. Stevens, T., Esonn, B., and Schehman, R. (1982) Cell 30, 439–448
55. Conzelmann, A., Riezman, H., Desponds, C., and Brun, C. (1988) EMBO J. 7, 2233–2240
56. Fankhauser, C., and Conzelmann, A. (1991) Eur. J. Biochem. 195, 439–448
57. Nuddler, C., Horvath, A., and Riezman, H. (1993) J. Biol. Chem. 268, 10558–10563
58. Horvath, A., Sutterlin, C., Manning-Krieg, U., Movva, N. R., and Riezman, H. (1994) EMBO J. 13, 3687–3695
59. Conzelmann, A., Fankhauser, C., and Desponds, C. (1999) EMBO J. 9, 653–661
60. Ram, A. F., Wolters, A., Ten Hoopen, R., and Klis, F. M. (1994) Yeast 10, 1029–1030
61. Naik, R. S., Krishnegowda, G., and Gowda, C. D. (2002) J. Biol. Chem. 278, 2036–2042
62. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673–4680