Glycosylphosphatidylinositol-anchored Proteins Are Required for the Transport of Detergent-resistant Microdomain-associated Membrane Proteins Tat2p and Fur4p*

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In eukaryotic cells many cell surface proteins are attached to the membrane via the glycosylphosphatidylinositol (GPI) moiety. In yeast, GPI also plays important roles in the production of mannoproteins in the cell wall. We previously isolated a new gwt1 mutant and found that GWT1 is required for inositol acylation in the GPI biosynthetic pathway. In this study we isolated a new gwt1 mutant allele, gwt1-10, that shows not only high temperature sensitivity but also low temperature sensitivity. The gwt1-10 cells show impaired acyltransferase activity and attachment of GPI to proteins even at the permissive temperature. We identified TAT2, which encodes a high affinity tryptophan permease, as a multicopy suppressor of cold sensitivity in gwt1-10 cells. The gwt1-10 cells were also defective in the import of tryptophan, and a lack of tryptophan caused low temperature sensitivity. Microscopic observation revealed that Tat2p is not transported to the plasma membrane but is retained in the endoplasmic reticulum in gwt1-10 cells grown under tryptophan-poor conditions. We found that Tat2p was not associated with detergent-resistant membranes (DRMs), which are required for the recruitment of Tat2p to the plasma membrane. A similar result was obtained for Fur4p, a uracil permease localized in the DRMs of the plasma membrane. These results indicate that GPI-anchored proteins are required for the recruitment of membrane proteins Tat2p and Fur4p to the plasma membrane via DRMs, suggesting that some membrane proteins are redistributed in the cell in response to environmental and nutritional conditions due to an association with DRMs that is dependent on GPI-anchored proteins.

Glycosylphosphatidylinositol (GPI) anchoring is a mechanism by which proteins are attached to the cell surface in all eukaryotic cells (1, 2). GPI-anchored proteins have various physiological roles contributing to transmembrane signaling, cell surface protection, cell adhesion, and cell wall synthesis (3, 4). In yeast, GPI-anchored proteins are major components of the cell wall, and they are essential for cell wall integrity (5, 6) and cell viability (7–9).

The GPI anchor is synthesized from phosphatidylinositol (PI) through multiple steps in the endoplasmic reticulum (ER). It is then transferred to the C terminus of proteins bearing a GPI signal sequence. The GPI anchor has a conserved core structure, NH2-CH2-CH2-PO4-6Man. GPI anchors are typically Triton X-100 (TX-100). Therefore, they are referred to as detergent-resistant membranes (DRMs). Some DRM-associated proteins (16). In mammalian cells, GPI-anchored proteins are concentrated in sphingolipid- and cholesterol-rich domains (17, 18). A distinctive feature of these domains is their resistance to extraction with detergent, typically Triton X-100 (TX-100). Therefore, they are referred to as detergent-resistant membranes (DRMs). Some DRM-associated proteins are sensitive to TX-100 but resistant to other detergents such as CHAPS (19). These sphingolipid- and cholesterol-rich domains, also referred to as lipid microdomains or lipid rafts, play key roles in signal transduction and membrane trafficking (17, 20–24).

In Saccharomyces cerevisiae, DRMs are composed of sphingolipids and ergosterol, which is slightly different from cholesterol, and they contain GPI-anchored proteins similar to those found in mammalian cells (25, 26). Although the physiological functions of GPI-anchored proteins in DRMs of yeast cells are still unknown, lipid microdomains are known to deliver a GPI-anchored protein, Gsp1p, from the ER to the plasma membrane (25). The recent results obtained in sec18 cells, in which ER-to-Golgi transport is blocked in a secretory pathway, suggest that the formation of DRMs and their association with certain proteins, including GPI-anchored proteins, are initiated in the ER (25, 27). Presumably such domains contain ceramide as their sphingolipids because GPI-anchored proteins cannot associate with DRMs in the absence of ceramide (25) and because ceramide is required for the specific transport of GPI-anchored proteins from the ER to the Golgi (28–30). Ceramide may help drive the incorporation of GPI-anchored proteins into ER-derived vesicles that are specific to the GPI-anchored proteins by participating in the formation of DRM structure in the ER.
Besides GPI-anchored proteins, many plasma membrane transporters, such as Pma1p, Tat2p, Fur4p, and Can1p, have been reported to be associated with DRMs, which are required for the precise delivery of these proteins to the plasma membrane (25, 27, 31–33). Tat2p is a high affinity tryptophan permease whose cellular location is affected by the external tryptophan concentration. At high external tryptophan concentrations, Tat2p membrane proteins are sorted to the early endosomes and subsequently to the vacuole through the late endosomes, whereas at low tryptophan concentrations they are sorted to the plasma membrane (31). In erg6 cells, which are defective in the synthesis of ergosterol, Tat2p cannot associate with DRMs and is inappropriately polyubiquitinated and mislocalized to the vacuole even at low tryptophan concentrations (31). These results indicate that ubiquitination and association with DRMs may be involved in the regulation of Tat2p sorting and that sterol may participate in this mechanism at the post-Golgi stage.

In this report we found that gwt1-10 cells show reduced tryptophan uptake similar to erg6 cells and that this defect is caused by a failure of Tat2p to be recruited to the plasma membrane due to a lack of association with DRMs. Moreover, we show that GPI-anchored proteins may play an important role in the transport of membrane proteins that change their cellular localization in response to nutritional conditions.

### EXPERIMENTAL PROCEDURES

#### Yeast Strains and Media—The strains used in this study are listed in Table 1. Except for the strains derived from RH401-7C, RH401-7D, and sec18-1, the mutant strains were all derivatives of W303. Cells were grown in YPAD or synthetic complete (SC) medium (34). Geneticin-resistant yeast colonies were selected on YPAD plates containing 200 μg/ml Geneticin (G418).

#### Gene Disruption and Gene Tagging—Deletions of BUL1 and ERG6 were carried out by a PCR-based method using disruption cassettes derived from the plasmid pFA6a-HIS3MX6 or pFA6a-kanMX6 (35) and the following primers: for deletion of BUL1, BUL1-F (5'-CGAAAAG-AGACTTGTCTGTTGTGTCACACAGTATATCGTACGTAACGGATCCCCGGGTTAATATA-3') and BUL1-R (5'-ATCTATATTC-TATAAGAAAAAGTACAGGATTTTTTTCTATGGTTTGAATTCGACCTTTAACC-3'); and for deletion of ERG6, ERG6-F (5'-TTAAAAAACAAGAATAAATATATATAGGACGCA- TAAGCGATCCCGGGTTAATATA-3') and ERG6-R (5'-TATA-TCGTCGCTTTATTGTTTTGATCTAGATGGAAAGTTTTCGACCTTTAACC-3'). Deletion of Tat2 was carried out by using pKU41 (kindly provided by K. Umebayashi, National Institute of Genetics) as described previously (31). C-terminal green fluorescent protein (GFP)-tagging of Hxt1p was carried out by a PCR-based method.
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using an integrative cassette derived from the plasmid pFA6a-GFP(S65T)-TRP1 and primers HXT1-F (5′-ACCTAATGCTAGATGACCAAACCATTTACAAGAGTTGTTAGCAGGAAACGATCCCGGGTTAATTAA-3′) and HXT1-R (5′- TAAATACGTTAAGTCTCATTAAATATCGATATGCGCTTTGATTGTTTGAATTCAAGATTCGGCGCTTGAATAC-3′).

Construction of Plasmids—The plasmid to produce the monomeric red fluorescent protein (mRFP)-tagged Tat2p was constructed as follows. The Spel-Spel fragment containing the open reading frame of mRFP was prepared from pBlues-mRFP (kindly provided by T. Sumita, National Institute of Advanced Industrial Science and Technology) and subcloned into the XbaI site of pKU42 (31) containing the TAT2 open reading frame to generate pMO9 (pUC-TAT2-mRFP). The PstI-HindIII fragment containing TAT2-mRFP was cloned into the PstI-EcoT22I site of pRS315 (CEN, LEU2) to generate pMO10 (YcpTAT2-mRFP). The plasmid for the expression of TAT2 fused with three copies of the hemagglutinin (HA) epitope was derived from pKU46 (31). The EcoRI-Pstl fragment containing TAT2-3HA was blunt-ended using a DNA blunting kit (Takara, Shiga, Japan) and then inserted into the Smal site of pRS315 to generate pMO12 (YcpTAT2-3HA).

The plasmid to produce the GFP-HDEL fusion was constructed as follows. The Kar2p signal-peptide sequence (the first 135 nucleotides of the KAR2 gene), which bears a Sall site upstream of the initiation codon of KAR2 and a ClaI site at the C terminus, was amplified by PCR and inserted into pBluescript SK+ (+). Next, the GFP gene modified to encode a C-terminal HDEL tetrapeptide, which bears a ClaI site at the C terminus, was amplified by PCR and inserted into this plasmid. The Sall-XbaI fragment containing KAR2-GFP-HDEL was cloned into the Ycp50 expression vector (CEN, URA3), which contains the TDH3 (which encodes glyceraldehyde-3-phosphate dehydrogenase) promoter and the actin terminator, to generate pMO13 (which encodes glyceraldehyde-3-phosphate dehydrogenase) promoter and the actin terminator, to generate pMO13 (CEN, TRP1) to generate pMO14 (YcpGFP-HDEL).

The plasmid used to produce the Fur4p-GFP was constructed as follows. The promoter and open reading frame region of FLR4, which has the Spel site just before the stop codon at the C terminus, was amplified by PCR and inserted into pUC19-GFP and then inserted into the Smal site of pRS314 (CEN, TRP1) to generate pMO24 (YEpFUR4-GFP).

Isolation of gwt1-10 Mutant Cells—Temperature-sensitive alleles of GWT1 were screened by PCR mutagenesis as described previously (15). The multiple missense mutations were separated by replacing the homologous fragments of wild-type GWT1, and a gwt1-10 allele containing a single mutation that generates temperature sensitivity was identified. The gwt1-10 allele was subcloned into integration vector pRS306 and then integrated into the ura3 locus on the chromosome of WDG2 cells (36). Ura+ transformants were selected, and gwt1-10 cells were identified by segregating with His+ Ura−/− mutants starting from the top. Each fraction was mixed with sample buffer, incubated at 37 °C for 10 min, and resolved by 10% SDS-PAGE. Proteins were transferred to a polyvinylidene fluoride membrane for Western blotting (Millipore, Billerica, MA). DRMs for the Fur4p analysis were isolated essentially as described by Umebayashi and Nakano (31) with a slight modification. After incubation with 20 mM CHAPS (Sigma) for 30 min on ice, the lysates were subjected to Optiprep density gradient fractionation by centrifuging at 5.5 h at 37,000 rpm in a SW55 Ti rotor (Beckman Instruments) at 4 °C. After centrifugation, nine fractions of equal volume were collected starting from the top. Each fraction was mixed with sample buffer, incubated at 37 °C for 10 min, and resolved by 10% SDS-PAGE. Proteins were transferred to a polyvinylidene fluoride membrane for Western blotting (Millipore, Billerica, MA). DRMs for the Fur4p analysis were isolated as described by Dupre and Hauwenauer-Taspis (27). Western blotting was carried out using anti-β-actin monoclonal antibody 16B12 (Berkeley Antibody Co., Berkeley, CA), anti-GFP monoclonal antibody IL-8 (BD Biosciences Clontech, Palo Alto, CA), anti-Pma1p monoclonal antibody MCA-40B7 (Molecular Probes, Eugene, OR), or anti-vacuolar alkali phosphatase monoclonal antibody 1D3-A10 (Molecular Probes) followed by horseradish peroxidase-conjugated goat anti-mouse IgG (Cell Signaling Technology, Beverly, MA). An ECL Plus kit (Amersham Biosciences) was used to visualize the immunoreactive proteins.

Metabolic Labeling and Immunoprecipitation—Cells were grown to logarithmic phase at 24 °C in SD medium with low SO42− concentration described previously (15). Lipids were separated by thin-layer chromatography (TLC) in 6:2:5:4 (v/v) CHCl3/CH3OH/H2O, and proteins were separated by SDS-PAGE (10% acrylamide gel). Labeled proteins were detected after TLC and electrophoresis using a Molecular Imager FX (Bio-Rad).

Biosynthesis of GPI Precursors in Vitro—ER-enriched membranes were prepared from the cells grown in YPAD medium at 24 °C as described previously (37). Membrane proteins (300 μg) were incubated in TM buffer (50 mM Tris·HCl, pH 7.5, and 2 mM MgCl2) containing 21 μg/ml tunicamycin, 10 μM nikkimycin, and 0.5 mM dithiothreitol in the presence of 1 mM coenzyme A (CoA), and 1 mM ATP. The reaction was initiated by adding 3.7 kBq of UDP-[3H]GlcNac (10.7 GBq/mmol; PerkinElmer Life Sciences) and then incubated for 1 h at 24 or 16 °C. The reaction was stopped by adding 1 ml of 1:1 (v/v) CHCl3/CH3OH to yield a final mixture of 10:10:3 (v/v) CHCl3/CH3OH/H2O. Radioabeled lipids were separated by centrifugation, and the supernatants were pooled. The pellet was re-extracted with 10:10:3 (v/v) CHCl3/CH3OH/H2O. The confined lipid extracts were dried and desalted by n-butyl alcohol extraction. Radioabeled lipids were separated by TLC in 10:10:3 (v/v) CHCl3/CH3OH/H2O and detected by autoradiography with a Molecular Imager FX.
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(0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% casamino acids, 5% glucose, nutrient supplements, and 200 μM ammonium sulfate). Cells were centrifuged and resuspended in SD-SO\textsubscript{4} medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% casamino acids, 2% glucose, nutrient supplements) lacking tryptophan at 10 A\textsubscript{600} eq/ml. Cells were incubated at 24 or 37 °C for 10 min and then labeled with 925 kBq of [\textsuperscript{35}S]methionine/ cysteine (37 TBq/mmol; Amersham Biosciences) per A\textsubscript{600} equivalent of cells for 40 min at each temperature. Radiolabeled proteins were subjected to CHAPS extraction and density gradient floatation as described above (see “Isolation of DRMs and Western Immunoblotting”). Fractions 2 and 3 were separated as insoluble fractions, and fractions 7 and 8 were soluble fractions. For immunoprecipitation of Tat2-3HAp and Gas1p, each fraction was divided in two halves, one of which was used for immunoprecipitation of Tat2-3HAp and the other for immunoprecipitation of Gas1p. After adding SDS (final 1%), the samples for immunoprecipitation of Tat2-3HAp were incubated at 37 °C for 10 min, and those of Gas1p were incubated at 95 °C for 5 min. The samples were diluted in IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 1% TX-100) and precleared with Sepharose CL-4B (Sigma) at 4 °C for 30 min. After removal of Sepharose CL-4B by centrifugation, the supernatants were incubated overnight at 4 °C with 30 l of anti-HA antibody-bound affinity matrix (Roche Applied Science) or 1:150 rabbit anti-Gas1p antibody (kindly provided by K. Hata, Eisai co., Ltd.) and protein G-agarose (Roche Applied Science). Immunoprecipitates were washed three times with IP buffer and once with 0.2 mM Tris-HCl, pH 7.5, and then resuspended in SDS sample buffer. After incubating at 37 °C for 10 min (for Tat2-3HAp) or 95 °C for 5 min (for Gas1p), the samples were separated by SDS-PAGE and analyzed using a Molecular Imager FX.

Fluorescence Microscopy—To obtain fluorescence images of Tat2p-mRFP or Fur4p-GFP fusion proteins, cells were grown to the logarithmic phase at 25 °C in tryptophan-free or uracil-free SC medium. The cellular localization of Tat2p was observed using a microscope (Olympus, Tokyo, Japan) equipped with a confocal laser scanning unit (Yokogawa Electric, Tokyo, Japan). Images were obtained with a CCD camera (Andor Technology, Belfast, UK) or a High-gain Avalanche Rushing Amorphous Photoconductor camera (Hitachi Kosuki Electric and NHK, Tokyo, Japan) and processed by IPLab software (Scanalytics, Fairfax, VA). The cellular localization of Fur4p was observed using a fluorescence microscope (Olympus).

RESULTS

Isolation of gwt1-10 Cells That Show Cold Sensitivity—We previously isolated three gwt1 mutants, gwt1-16, gwt1-20, gwt1-28, that show high temperature sensitivity (Ts\textsuperscript{+}) at 37 °C, and we showed that GWT1 encodes an inositol acyltransferase involved in the biosynthesis of the GPI anchor (15). To further investigate the physiological role of the GPI anchor in yeast, we attempted to isolate other gwt1 mutants that cannot survive at low temperatures. Using in vitro error-prone PCR, we generated one mutant, gwt1-10, that has a single amino acid substitution conferring a growth defect at 16 and 37 °C. In gwt1-10 cells, the eighth lysine of Gwt1p is replaced with glutamic acid (K8E). This lysine residue is located in an N-terminal region conserved among many species from yeast to human (Fig. 1A). Unlike other gwt1 mutants, the gwt1-10 cells exhibited not only the Ts\textsuperscript{−} phenotype but also cold sensitivity (Cs\textsuperscript{−}) at 16 °C (Fig. 1B). The gwt1-10 cells grow slower than wild-type cells even at the permissive temperature (30 °C). They also show sensitivity to Calcofluor White, a drug that affects the cell wall architecture, and their growth defect at 37 °C was suppressed by adding an osmotic stabilizer, 0.3 M KCl, to the medium (Fig. 1B). Moreover, gwt1-10 cells were prone to lysis at 37 °C (data not shown). These observations indicate that this mutant has defects in the cell wall similar to other mutants related to the biosynthesis of the GPI anchor (6, 39).

gwt1-10 Cells Show Decreased Inositol Acrlytransferase Activity—The GWT1 gene is required for the acylation of inositol in glucosaminyl-PI to form GlcN-(acyl)PI, an intermediate in the biosynthesis of GPI anchors (15). We measured the activity of inositol acylation in gwt1-10 cells in vitro to determine whether the K8E mutation affects the acyltransferase activity. Membranes from wild-type and gwt1-10 cells were incubated with UDP-[\textsuperscript{14}C]GlcNAc at 24 and 16 °C, and radiolabeled lipids were extracted and separated by TLC. Membranes prepared from wild-type cells synthesized the acylated product, GlcN-(acyl)PI, in the presence of CoA and ATP at 24 and 16 °C (Fig. 2A). Although membranes from gwt1-10 cells also synthesized GlcN-(acyl)PI at both temperatures, the amount produced by gwt1-10 membranes was much lower than that produced by wild-type membranes even at the permissive temperature (24 °C (Fig. 2A)). There was no significant difference between the acyltransferase activities at 16 and 24 °C in gwt1-10 cells. At 37 °C, GlcN-(acyl)PI was not synthesized from gwt1-10 membranes (data not shown).

Inositol acylation is essential for the transfer of GPI anchors to proteins (16). We, therefore, examined whether the incorporation of radiolabeled inositol into proteins is impaired in gwt1-10 cells. Because all detectable protein-bound inositols are present as the GPI-attached form in yeast (40), cells were labeled with myo-[\textsuperscript{1,2-3H}]inositol at 24 or 16 °C for 2 h. Labeled proteins were prepared and subjected to SDS-PAGE. The gwt1-10 cells showed a decrease in inositol labeling of proteins compared with the wild-type cells at both 24 and 16 °C, although no significant difference was observed between the quantities of inositol-labeled proteins at each temperature (Fig. 2B). This result indicated that gwt1-10 cells are defective in the transfer of GPI anchors to proteins even at the permissive temperature (24 °C).

![Figure 1. gwt1-10 mutation and phenotype. A, alignment of the amino acid sequences of GWT1 orthologs of S. cerevisiae, Schizosaccharomyces pombe, Candida albicans, human, mouse, and rat. Black and gray boxes indicate identical and similar amino acids, respectively. In gwt1-10 mutant cells, the eighth residue, lysine (K), which is conserved among many organisms, is replaced with glutamic acid (E). B, phenotypes of various gwt1 alleles. The sites of mutation in each gwt1 mutant were as follows: gwt1-10 (K8E), gwt1-16 (N330S, L362P, and V479A), and gwt1-20 (W63R and V64A). Cells were grown at the indicated temperatures on YPAD, YPAD supplemented with 7 μg/ml Calcofluor White (CFW), or YPAD supplemented with 0.3 M KCl.](image)
Isolation of a Multicopy Suppressor Gene for Cold Sensitivity of gwt1-10 Cells—Most mutants defective in the biosynthesis of GPI anchors show a loss of cell wall integrity (6, 39). This was also observed in gwt1-10 cells (Fig. 1B). First, we screened for genes that suppress the Ts” phenotype at 37 °C in gwt1-10 cells, and we obtained GFA1, EXG1, and RH02, which are involved in the biosynthesis of the cell wall (data not shown). Because these suppressor genes did not suppress the Cs” phenotype at 16 °C, it is conceivable that the cold sensitivity of gwt1-10 cells was not caused by a defect in the integrity of the cell wall.

To investigate what causes the death of gwt1-10 cells at low temperature, we isolated multicopy suppressors for the cold sensitivity of the cells at 16 °C. We obtained five suppressor genes, TAT2, UBP5, EAF3, SSB1, and YDR266C (Fig. 3A). Tat2p is a high affinity tryptophan permease (41), and Ubp5p is a putative ubiquitin-specific protease whose function remains unknown (42). Eaf3p is a component of the Nua4 histone acetyltransferase complex (43), and Ssb1p is a pair of Hsp70 molecular chaperones that associate with ribosomes to fold nascent polypeptide chains (44). Ydr266cp is a protein of unknown function that contains a RING finger domain. These genes did not suppress the growth defect of gwt1-10 cells at 37 °C (Fig. 3A).

The Cold Sensitivity of gwt1-10 Cells Is Caused by an Impaired Uptake of Tryptophan—A multicopy suppressor of TAT2 was the most effective at suppressing the cold sensitivity of gwt1-10 cells (data not shown). Therefore, we focused on TAT2 to understand why gwt1-10 cells have a Cs” phenotype. First, we measured the uptake of a radiolabeled tryptophan into gwt1-10 cells (Fig. 3B). The relative rate of the uptake was lower than that in wild-type cells even at the permissive temperature (28 °C); the uptake by gwt1-10 cells at 45 min was ~62% that of the wild-type cells. The difference in the uptake of tryptophan between gwt1-10 and wild-type cells was much greater at 16 °C than at 28 °C; the uptake by gwt1-10 cells at 45 min was 30% that of the wild-type cells. These results suggest that the growth defect of gwt1-10 cells at 16 °C may be caused by impaired tryptophan uptake.

Recently, it was reported that the localization of Tat2p is regulated by the external tryptophan concentration. At low concentrations of tryptophan, Tat2p is sorted to the plasma membrane to import tryptophan into the cells, whereas at high concentrations of tryptophan, it is sorted to the vacuole to be degraded (31). erg6 cells, which are defective in a late step of ergosterol biosynthesis, show decreased tryptophan import activity and, consequently, a growth defect in synthetic medium containing even a standard concentration of tryptophan (20 µg/ml) (45). This growth defect was suppressed by the overexpression of TAT2, indicating that it was caused by the impaired function of Tat2p (31). These results suggested that the Cs” phenotype of gwt1-10 cells might also be caused by the impaired function of Tat2p. To examine this possibility further, we determined whether the phenotype of gwt1-10 cells is restored at a high concentration of tryptophan (200 µg/ml). Under such conditions, Tat2p is dispensable for cell growth, presum-
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FIGURE 4. The cold sensitivity of gwt1-10 cells is suppressed by a high concentration of tryptophan and disruption of BUL1. A, the growth defect of gwt1-10 cells at 16 °C was suppressed by the addition of tryptophan to the medium. Wild-type (WT), and gwt1-10 cells were grown in SC medium containing the indicated concentration of tryptophan (200 μg/ml) corresponds to a high concentration and 20 μg/ml to a standard concentration). B, effect of BUL1 deletion on the Cs BUL1 phenotype of gwt1-10 cells. Wild-type, gwt1-10, bul1Δ (YMO20), and bul1Δ gwt1-10 (YMO21) cells were grown on SC plates supplemented with a standard concentration of tryptophan (20 μg/ml) at the indicated temperatures.

It is reported that the severe tryptophan auxotrophy of erg6Δ cells is suppressed by the disruption of BUL1, which encodes a component of the Rsp5p ubiquitin ligase complex (46) that is required for the polyubiquitination of Tat2p (31). In bul1Δ cells, the growth of gwt1-10 cells at 16 °C was restored at a high concentration of tryptophan (200 μg/ml), whereas it did not recover at 37 °C (Fig. 4A), indicating again that the cell death at 16 °C was due to the impaired uptake of tryptophan.

Next, we investigated whether the accumulation of Tat2p in the ER is unique to gwt1-10 cells or is common to other GPI mutants. Tat2p-mRFP fluorescence was observed in gwt1-20 cells, which showed a strong defect in the biosynthesis of GPI-anchored protein at the restrictive temperature but only a weak defect at the permissive temperature (15). In gwt1-20 cells in tryptophan-free medium at the permissive temperature, 25 °C, Tat2p was localized to the plasma membrane, similar
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FIGURE 6. Phenotypes of the mutants with defects in GPI-anchor biosynthesis. A, wild-type (WT) and gwt1-20 cells harboring YCpTAT2-mRFP and YCpGFP-HDEL in the tat2Δ genetic background (YMO24-2 and YMO29-1) were grown in tryptophan-free SC medium at 25 °C and then shifted to 37 °C for 30 min. In gwt1-20 cells Tat2p-mRFP was localized at the plasma membrane at 25 °C, but it was localized in the ER at 37 °C. B, the gaa1-1 cells harboring YCpTAT2-mRFP and YCpGFP-HDEL (YMO46-1) were grown in tryptophan-free SC medium at 25 °C. Tat2p-mRFP was localized in the ER and the vacuole.

to the case in wild-type cells. In contrast, Tat2p was localized in the ER of these cells when they were shifted to 37 °C for 30 min (Fig. 6A). These results indicate that the decrease in inositol acyltransferase activity generally causes an impairment in the export of Tat2p from the ER.

To further examine whether other mutant cells with defects in GPI-anchored biosynthesis have the same phenotype as gwt1-10 cells, we examined the cellular localization of Tat2p in gaa1-1 cells. Gaa1p is an essential component of GPI transamidase, which mediates attachment of GPI anchor to proteins, and gaa1-1 cells are reported to have a greatly reduced incorporation of inositol into proteins even at a permissive temperature (9). In gaa1-1 cells, Tat2p-mRFP was observed in the ER and the vacuole under tryptophan-free conditions at a permissive temperature (25 °C) (Fig. 6B). We also found the same mislocalization of Tat2p in gpi7Δ cells (data not shown), which have defects in the addition of ethanolamine phosphate to the GPI moiety during the biosynthesis of the GPI anchor (48) and in the localization of daughter cell-specific GPI-anchored proteins (49). Moreover, we found that gpi7Δ cells also show cold sensitivity at 16 °C and that the CΔ phenotype of gpi7Δ cells is suppressed by the addition of a high concentration of tryptophan, similar to gwt1-10 cells (data not shown). Taken together, these results imply that GPI-anchored proteins are required for the transport of Tat2p, independent of ergosterol.

Tat2p Is Not Sorted to the DRMs in Mutant Cells Defective in the GPI-Anchored Proteins—It is reported that the association of Tat2p with DRMs is required for the delivery of Tat2p to the plasma membrane (31). In yeast, DRMs are rich in phosphoinositol-based sphingolipids and ergosterol (25). In erg6Δ cells, Tat2p cannot associate with DRMs, probably because their properties are altered by the accumulation of ergosterol intermediates (31). Because GPI-anchored proteins are also contained in DRMs along with sphingolipids and ergosterol, we investigated whether Tat2p associates with DRMs in gwt1-10 cells and gaa1-1 cells.

Wild-type, gwt1-10, and gaa1-1 cells were mechanically disrupted and then extracted with 20 mM CHAPS at 4 °C, and the extracts were fractionated on an Optiprep density gradient. Pma1p, a control DRM-associated protein, was located primarily in fractions 2 and 3 in these strains. In wild-type cells, Tat2p is also found predominantly in fractions 2 and 3, indicating its association with DRMs (Fig. 7, A and B). The quantity of Tat2p associated with DRMs was notably reduced in gwt1-10 cells (Fig. 7A), and the association of Tat2p with DRMs was not maintained in gaa1-1 cells (Fig. 7B). These results indicate that GPI-anchored proteins may be required for the association of Tat2p with DRMs. However, we cannot exclude the possibility that Tat2p is unable to associate with DRMs in gwt1-10 and gaa1-1 cells because it is not transported to the appropriate compartment.

To address this possibility, we performed a pulse experiment with 35S-labeled amino acid in sec18-1 temperature-sensitive mutant cells (50) in which ER-to-Golgi trafficking is blocked at 37 °C (Fig. 8). We incubated the sec18-1 cells at the permissive (24 °C) and restrictive (37 °C) temperatures and examined the detergent insolubility of Tat2p. As reported previously (25), Gas1p was present in the insoluble fraction...
(in Fig. 8) at 37 °C as a 105-kDa ER form (precursor (p)). Tat2p was also present in the insoluble fraction even if the protein transport from the ER to the Golgi was blocked at 37 °C, indicating that the association of Tat2p with DRMs occurs in the ER. Therefore, we concluded that a decrease of GPI-anchored proteins results in the failure of Tat2p to associate with DRMs.

**gwt1-10 Cells Are Defective in the Functions of Other DRM-associated Proteins**—We next examined whether proteins other than Tat2p, which is associated with DRMs, are affected by the defect in GPI-anchored proteins. For these studies we focused on another transmembrane protein, Fur4p, which functions as a uracil permease. Fur4p is also known to be associated with DRMs (27), and in response to exogenous uracil, it is sorted directly from the Golgi to the endosomal system via a process regulated by Rsp5p-dependent ubiquitination and without passage through the plasma membrane (51). To examine the effects on Fur4p, we first investigated whether gwt1-10 and gpi7Δ cells exhibit resistance to 5-fluorouracil (5-FU), a toxic analog of uracil. The fur4Δ cells were resistant to 5-FU because they cannot import it due to a lack of uracil permease. Interestingly, both gwt1-10 and gpi7Δ cells were also resistant to 5-FU (Fig. 9A), indicating that a defect in the biosynthesis of the GPI anchor affects the function of Fur4p.

To address whether the resistance to 5-FU in gwt1-10 and gpi7Δ cells is caused by the mislocalization of Fur4p, we examined the fluorescence in cells transformed with a high copy number plasmid for expressing GFP-tagged Fur4p under the control of the FLR4 promoter. We confirmed that this plasmid is functional because the growth defect of fur4Δ cells in low uracil medium was suppressed by the introduction of the plasmid (data not shown). In exponentially growing wild-type cells, Fur4p-GFP fluorescence was present mainly at the plasma membrane with lesser amounts in the vacuole, whereas the fluorescence in gwt1-10 cells was predominantly localized in the vacuole (Fig. 8B). The same results were obtained for gpi7Δ cells (data not shown).

We also examined the cellular localization of Fur4p in gaa1-1 cells. Unlike wild-type cells, Fur4p-GFP in gaa1-1 cells was mainly present in the intracellular compartments (Fig. 9D). The gaa1-1 cells have been reported to be defective in endocytosis and to accumulate an abnormal organelles that seem to be endosomes (52). Therefore, we speculated that Fur4p-GFP is transported to abnormal endosomes and fragmented vacuoles, which are probably formed as a result of defects in endocytosis in gaa1-1 cells. As shown in Figs. 9, B and D, the recruitment of Fur4p to the plasma membrane was impaired in gwt1-10 and gaa1-1 cells. Thus, it is likely that the transport of Fur4p to the plasma membrane in these mutant cells is defective due to the lack of GPI-anchored proteins required for the transport of Fur4p.
Next, to investigate whether the mislocalization of Fur4p in gwt1-10 and gaa1-1 is caused by a failure of Fur4p to associate with DRMs, we examined the solubility of Fur4p in 1% TX-100 at 4 °C in cells expressing the Fur4p-GFP fusion protein. As reported previously, in wild-type cells, we found that Fur4p is present in the detergent-resistant fraction (fraction 1 in Fig. 9C). This fraction also contained Pma1p, a known raft-associated protein. In gwt1-10 cells, Fur4p was hardly detected in the detergent-soluble fraction (fractions 5 and 6 in Fig. 9C) and was not detected in the detergent-resistant fraction (fraction 1). Rather, we detected low molecular weight proteins corresponding in size to free GFP in the detergent-soluble fraction (fractions 4–6). As described previously (27), it is conceivable that the free GFP arises from the degradation of Fur4p-GFP in the vacuole because GFP tends to be resistant to the vacuolar protease. Although free GFP was detected in both wild-type and gwt1-10 cells, the amount of free GFP was higher in gwt1-10 cells than in wild-type cells (Fig. 9C), indicating that, in gwt1-10 cells, Fur4p-GFP cannot associate with DRMs and is degraded in the vacuole. We also obtained the same results in gaa1-1 cells. Although there was a high level of Fur4p-GFP in the detergent-resistant fraction (fraction 1 in Fig. 9E) of wild-type cells, it was not detected in any of the fractions in gaa1-1 cells; instead, free GFP was detected in the detergent-soluble fraction (fractions 4–6 in Fig. 9E), as observed in gwt1-10 cells (Fig. 9C). Therefore, we concluded that GPI-anchored proteins are required for the transport of both Fur4p and Tat2p via DRMs.

Finally, we ascertained whether the defect of GPI-anchored protein biosynthesis is specific to the DRM-associated membrane proteins. We focused on the hexose transporter, Hxt1p, which is not associated with DRMs (33). We constructed cells carrying chromosomal Hxt1p fused C-terminally to GFP and used them to examine the cellular localization of Hxt1p. In both wild-type and gwt1-10 cells, Hxt1p-GFP was specifically localized at the cell surface (Fig. 10), indicating that GPI-anchored proteins are required for the transport of DRM-associated proteins such as Tat2p and Fur4p but not generally required for the transport of the non-DRM-associated membrane protein, Hxt1p.

**DISCUSSION**

In the current studies we investigated the role of GPI-anchored proteins by analyzing gwt1-10 cells, which show temperature sensitivity at both 37 and 16 °C. In gwt1-10 cells, inositol acyltransferase activity is decreased in vitro, and the transfer of the GPI anchor onto proteins is decreased even at a permissive temperature in vivo. Many kinds of mutant cells known to be defective in the biosynthesis of GPI anchors show a severe defect in the cell wall at their restrictive temperatures (6). Therefore, in such cells, it is difficult to find a new physiological function for GPI-anchored proteins other than the maintenance of cell wall integrity. In this report we showed that the Ts− phenotype of gwt1-10 cells is caused by cell wall defects similar to other mutant cells defective in the biosynthesis of GPI anchors. However, Cs− suppressor genes could not suppress the Ts− phenotype, indicating that the causes of cold and high temperature sensitivity are different. Therefore, we considered that investigating the cause of Cs− phenotype in gwt1-10 cells should help determine what other physiological roles are played by GPI-anchored proteins besides maintenance of cell wall integrity.

The **Reason for the Cold Sensitivity of gwt1-10 Cells**—To understand why gwt1-10 cells are sensitive to the cold, we focused on TAT2, which suppresses the Cs− (16 °C) phenotype most effectively among several isolated Cs− suppressor genes. Tat2p is a high affinity tryptophan permease and is known to be delivered from the trans-Golgi to the early endosomes, where tryptophan-dependent sorting occurs. When there is a low concentration of tryptophan in the medium, Tat2p is sorted to the plasma membrane, whereas at a high tryptophan concentration, Tat2p is polyubiquitinated and sorted to the vacuole through the late endosomes (31). The polyubiquitination of Tat2p is performed by the Rsp5pbu1p ubiquitin ligase complex, and the deletion of **BUL1** is reported to cause a redirection of Tat2p to the plasma membrane even at high tryptophan concentrations (31). We found that the uptake of tryptophan is decreased in gwt1-10 cells and that the Cs− phenotype is suppressed by the addition of tryptophan to the medium, suggesting that the Cs− phenotype is caused by an impaired function of Tat2p. Moreover, we found that, at low tryptophan concentrations, Tat2p is mainly localized to the ER rather than the plasma membrane in gwt1-10 cells. This indicates that a loss of Tat2p function in gwt1-10 cells is due to its mislocalization. Because Tat2p is required for cell growth at a low temperature (15 °C) (53, 54), it is quite conceivable that the Cs− phenotype of gwt1-10 cells is caused by the reduced uptake of tryptophan due to the mislocalization of Tat2p. If Tat2p is accumulated entirely in the ER, it is unexpected that the Cs− phenotype of gwt1-10 cells is suppressed by the **BUL1** deletion. We suspect that Tat2p is not entirely blocked in the ER in gwt1-10 cells and that a small amount of it is sorted to the vacuole. In bul1Δ cells, this Tat2p would be sorted to the plasma membrane.

GPI-anchored Proteins Are Required for the Recruitment to the Plasma Membrane and DRM Association of Tat2p—The association of Tat2p with DRMs is required for its transport to the plasma membrane (31). In yeast, the major components of DRMs are ergosterol and sphingolipids. In erg6Δ cells and erg13Δ cells, which have defects in ergosterol synthesis, Tat2p cannot associate with DRMs, and at a low concentration of tryptophan, it is transported to the vacuole instead of the plasma membrane (31). Ergosterol is thought to function in the post-Golgi sorting of Tat2p. In this study, we found that the phenotype of the gwt1-10 cells was similar to that of erg6Δ cells, including reduced tryptophan uptake and suppression of the Cs− phenotype by a high concentration of tryptophan or the deletion of **BUL1**. We also found that Tat2p cannot associate with DRMs in gwt1-10 or gaa1-1 cells. Gaa1p is required for the attachment of a completed GPI anchor to proteins. It is possible that ergosterol or sphingolipids could be abnormal in gwt1-10 and gaa1-1 cells, resulting in the failure of Tat2p to associate with DRMs. However, we think it unlikely based on the following two points. First, the localization of Tat2p is different between erg6Δ cells and gwt1-10 or gaa1-1 cells; Tat2p was mainly localized to the ER in gwt1-10 and gaa1-1 cells, whereas it is found in the vacuole in erg6Δ cells. These results suggested that GPI-anchored proteins are required for the transport of Tat2p from the ER to the Golgi (Fig. 11, 1a) and that they function independently of ergosterol in the transport of Tat2p (Fig. 11, 1a).
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The finding that Tat2p is localized to the ER in erg6Δ gwt1-10 cells clearly supports this possibility. Second, lipid analysis did not identify any abnormalities of sphingolipids in gaal-1 cells (9, 48) and gwt1-10 cells. From these results we conclude that the dissociation of Tat2p from DRMs found in gwt1-10 and gaal-1 cells is caused by defects in GPI-anchored proteins and not by defects in ergosterol or sphingolipids. Although some GPI-anchored proteins are associated with DRMs, it was not known that GPI-anchored proteins are involved in the association of membrane proteins with DRMs as well as ergosterol and sphingolipids. To our knowledge this is the first report suggesting that GPI-anchored proteins participate in the sorting of membrane proteins.

Why does the dissociation of Tat2p from DRMs cause a delay in the export of Tat2p from the ER in cells that are defective in GPI synthesis? GPI-anchored proteins are expected to be exported from the ER by specific vesicles that are distinct from those for other secretory proteins, such as α-factor precursor and the general amino acid permease, Gap1p (55). On this basis of the following reports, it is predicted that ceramide-rich microdomains are involved in the export of GPI-anchored proteins from the ER (56); (i) in yeast, newly synthesized GPI-anchored protein Gas1p is recruited to lipid rafts in lcb1-100 cells, which have a defect in ceramide biosynthesis (25), and (ii) ceramide is required for the transport of GPI-anchored proteins from the ER to the Golgi (29, 30). Also, all precursors of sphingolipids are synthesized in the ER. Based on these findings, we postulate that some GPI-anchored proteins are selectively recruited to ceramide-rich microdomains in the ER. GPI-anchored proteins are selectively recruited to ceramide-rich microdomains (see Fig. 11) because of the physical properties of the GPI anchors and that they are then sorted to GPI-anchored protein-specific vesicles (Fig. 11, 2). In this report, we showed that Tat2p in the ER is already associated with DRMs (Fig. 8). If Tat2p normally associates with ceramide-rich microdomains composed of GPI-anchored proteins in the ER, in mutant cells defective in GPI synthesis its export from the ER is likely delayed because it is not sorted to GPI-anchored protein-specific vesicles due to a lack of association with ceramide-rich microdomains. In this case Tat2p may not be associated with ceramide-rich microdomains because they are destabilized by the lack of GPI-anchored protein or because a particular GPI-anchored protein involved in the recruitment of Tat2p to microdomains is lacking (Fig. 11, 3). Thus, we suspect that certain GPI-anchored proteins stabilize Tat2p-associated DRMs through their lipid portion or that they recruit Tat2p to DRMs. In the latter case, if only one specific GPI-anchored protein is directly involved in the export of Tat2p from the ER, it should have been possible to isolate it as the gene that suppresses the cold sensitivity of gwt1-10 cells. However, we did not isolate any GPI-anchored proteins in our screens. Thus, another factor might act along with GPI-anchored proteins to recruit Tat2p to the DRMs.

In addition to Tat2p, we found that Fur4p, a uracil permease that is also recruited to DRMs in the ER (27), cannot associate with DRMs in gwt1-10 or gaal-1 cells (Fig. 7, A and B). However, unlike Tat2p, Fur4p is mainly localized in the vacuole in gwt1-10 cells. This difference in the localization may be caused by a difference either in the mechanism of their transport or in the specific GPI-anchored proteins involved in their transport. Alternatively, because we used a multicopy overexpression system for Fur4p-GFP due to the inability to detect natively expressed Fur4p-GFP from single copy plasmid, it is possible that we observed an artificial localization of Fur4p in gwt1-10 cells as a result of its overexpression. Also, Fur4p does not associate with DRMs and is mislocalized to the ER in lcb1-100 cells, indicating that ceramide is required for its transport to the membrane (27). Whatever the reason, these reports combined with our findings strongly suggest that Fur4p, like Tat2p, is transported through ceramide-rich microdomains containing GPI-anchored proteins.

There is no direct evidence linking the formation of ceramide-rich microdomains in the ER with the selective transport of GPI-anchored proteins or indicating that Tat2p or Fur4p is cotransported with GPI-anchored proteins. To clarify the function of GPI-anchored protein in the ER, it will be necessary to identify GPI-anchored proteins involved in the association of Tat2p or Fur4p with DRMs. In yeast, after transfer to protein, the GPI-lipid moieties are remodeled from phosphatidylinositol to ceramide or diacylglycerol carrying C26 fatty acid (57). This remodeling occurs mainly in the ER and partially in the Golgi (58). It will be interesting to determine whether ceramide remodeling of the GPI anchor is involved in the association of Tat2p and Fur4p with DRMs.

GPI-anchored Proteins May Be Required for the Transport of Membrane Proteins That Change Their Cellular Localization According to Environmental Conditions—It is noteworthy that GPI-anchored protein is involved in the association of certain proteins with DRMs, such as Tat2p and Fur4p but not Pma1p. This could be due to the structural features of the different membrane proteins. However, the homology between Tat2p and Fur4p is very low, and the two proteins have different numbers of transmembrane

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domains (Tat2p is predicted to contain the same 12 transmembrane domains as Hxt1p, a non-DRM associated protein, whereas Fur4p and Pma1p contain 10 transmembrane domains). In yeast there are at least two different raft-based membrane compartments on the plasma membrane; they are the Pma1p-rich domain and the Can1p-rich domain (33). Recently, Fur4p was shown to be localized in the Can1p-rich domain but not the Pma1p-rich domain (59). However, homologous regions have not been found within Can1p and Fur4p. Therefore, it appears that the difference in behavior among Tat2p, Fur4p, and Pma1p in cells with defective GPI-anchored protein synthesis cannot be explained by their structural features alone. GPI-anchored proteins might function in the Can1p-rich domain, but whether Tat2p is localized in the Can1p-rich domain remains unknown.

Tat2p and Fur4p change their cellular localization according to the concentration of nutrients, but the localization of Pma1p is extremely stable. At low tryptophan concentrations, Tat2p is transported to the plasma membrane, whereas at high tryptophan concentrations it is polyubiquitinated by the Rsp5p ubiquitin ligase complex in early endosomes and transported to the vacuole (Fig. 11, 4). Like Tat2p, Fur4p is polyubiquitinated by Rsp5p ubiquitin ligase in the late Golgi and transported to the vacuole rather than the plasma membrane in response to uracil (51). The endocytosis of Tat2p and Fur4p is also regulated by Rsp5p-dependent ubiquitination in response to nutrient levels (Fig. 11, 4) (60, 61). Interestingly, Nedd4, the mammalian homolog of Rsp5p has been reported to associate with lipid rafts (62). Therefore, Tat2p and Fur4p may be polyubiquitinated by Rsp5p via DRM’s in response to the presence of certain nutrients. Together, the previous reports and our current data suggest that GPI-anchored proteins participate in the regulatory system that determines the destination of Tat2p and Fur4p through Rsp5p-dependent ubiquitination in DRM’s and, furthermore, that Pma1p acts independently of this system.

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