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Deletion of *MCD4* Involved in Glycosylphosphatidylinositol (GPI) Anchor Synthesis Leads to an Increase in β-1,6-Glucan Level and a Decrease in GPI-Anchored Protein and Mannan Levels in the Cell Wall of *Saccharomyces cerevisiae*

JARUWAN MANEESRI,[1§] MASAYUKI AZUMA,[1*] YUMIKO SAKAI,[1] KOICHI IGARASHI,[1] TAKESHI MATSUMOTO,[2] HIDEKI FUKUDA,[2] AKIHIKO KONDO,[3] AND HIROSHI OOSHIMA[1]

Department of Applied and Bioapplied Chemistry, Graduate School of Engineering, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan,[1] Division of Molecular Science, Graduate School of Science and Technology, Kobe University, 1-1 Rokkodaicho, Nada-ku, Kobe 657-8501, Japan,[2] and Department of Chemical Science and Engineering, Faculty of Engineering, Kobe University, 1-1 Rokkodaicho, Nada-ku, Kobe 657-8501, Japan[3]

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Most proteins involved in the synthesis of the GPI core structure of *Saccharomyces cerevisiae* are essential for growth. To explore the relationship between the GPI anchor structure and β-1,6-glucan synthesis, we screened deletion mutants in genes involved in GPI synthesis for osmotic remedial growth. Heterozygous diploid strains were dissected on medium with osmotic support and slow growth of the *mcd4* deletion mutant was observed. The *mcd4* mutant showed abnormal morphology and cell aggregation, and was hypersensitive to SDS, hygromycin B and K1 killer toxin. Incorporation of GPI cell wall proteins was examined using a GPI-Flo1 fusion protein. The result suggested that the *mcd4* deletion causes a decrease in GPI cell wall proteins levels. The mutation also caused a decrease in mannan levels and an increase in alkali-insoluble β-1,6-glucan and chitin levels in the cell wall.

[**Key words:** glycosylphosphatidylinositol anchor, *MCD4*, cell wall, β-1,6-glucan, *Saccharomyces cerevisiae*]

Understanding the structure and the biosynthesis of the cell wall of *Saccharomyces cerevisiae* should contribute to developments in the field of cell-surface display of a new catalytic function by cell surface engineering (1), application of the cell wall β-glucan that has the ability to stimulate the human immune system and screening of new antifungal agents that target the cell wall. The cell wall of the yeast is composed of mannoproteins, β-1,3-glucan, β-1,6-glucan and chitin. β-1,6-Glucan connects mannoproteins to β-1,3-glucan and chitin and plays a critical role in maintaining normal wall structure (2, 3). Two binding sites have been proposed for the attachment of β-1,6-glucan to mannoproteins that have a glycosylphosphatidylinositol (GPI) anchor in the endoplasmic reticulum (Fig. 1) (3). Most β-1,6-glucan is thought to be attached to the GPI core structure. A second possible site is thought to be to protein *N*-glycan chains. The binding site of β-1,6-glucan in the GPI core structure is not yet determined, but analysis of Tip1p, a cell wall protein of *S. cerevisiae*, indicates that the GPI anchor is cleaved at the glycosyl moiety and β-1,6-glucan is probably attached to the resultant mannose reducing end (4). Analyses of GPI mutants may provide information on the attachment of β-1,6-glucan to the GPI core structure. The known functions of proteins related to GPI anchor synthesis are shown in Fig. 1. For example, Gpi10p transfers the third core mannose to the GPI core structure (5). Smp3p (stable maintenance of plasmid) is required for addition of the fourth mannose to the GPI core structure (6). Gpi7p, Gpi13p and Mcd4p (morphogenesis checkpoint dependent) are involved in adding phosphoethanolamine (EtN-P) to the mannose chain (7–11). Gpi8p is a subunit of a GPI transamidase and is involved in the attachment of GPI anchors to proteins (12). Apart from Gpi1p (13) and Gpi7p, genes encoding these proteins are essential for growth, and finding permissive growth conditions for these mutants may lead to a better understanding of their function.

Genetic analysis of β-1,6-glucan synthesis suggests that synthesis of the polymer occurs via a series of secretory pathway events and that the structure is completed at the cell surface (3). Previously, we screened for essential genes which were related to β-1,6-glucan synthesis and a haploid *big1* (bad in glucose) deletion strain was obtained on me-

* Corresponding author. e-mail: azuma@bioa.eng.osaka-cu.ac.jp
phone: +81-(0)6-6605-3092 fax: +81-(0)6-6605-2701
§ Present address: Department of Agro-Industry, Faculty of Agriculture, Nakhonsrithammarat, Rajamanagala Institute of Technology (RIT), Thungyai, Nakhonsrithammarat 80240, Thailand.

Abbreviations: CFW, calcofluor white; ConA, Concanavalin A; EtN-P, phosphoethanolamine; FITC, fluorescenin isothiocyanate; GPI, glycosylphosphatidylinositol; MAT, mating type; WT, wild-type.

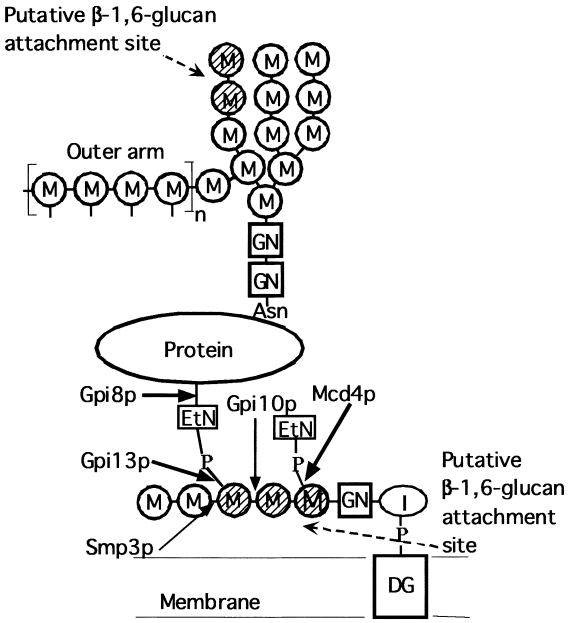

FIG. 1. Schematic representation of GPI biosynthesis and the putative β-1,6-glucan attachment site. M, Mannose; GN, glucosamine; EtN-P, phosphoethanolamine; I, inositol; DG, diacylglycerol.

dium with osmotic support after dissection of a *big1*/*BIG1* heterozygote. The deletion causes a severe reduction in the cell wall β-1,6-glucan (14).

Here, we examined haploid mutants deleted in *mcd4* for growth on YPD with osmotic support, and found that the *mcd4* mutant is partially rescued by the addition of sorbitol. We analyzed cell wall components and phenotype such as cell morphology of the *mcd4* mutant.

MATERIALS AND METHODS

Strains and culture conditions Yeast strains used in this study were BY4743 (MAT**a**/α *his3Δ1*/*his3Δ1 leu2Δ0*/*leu2Δ0 Lys2*/*lys2Δ0 Met15*/*met15Δ0 ura3Δ0*/*ura3Δ0*) (15) and the heterozygous mutant (as BY4743 *orf*Δ::*kanMX4*/*ORF*) obtained from the *Saccharomyces* Genome Deletion Consortium. These deletion mutants were checked for a PCR product of the correct size using the primers flanking the gene. At least four PCR reactions were carried out (16). The haploid strain deleted for the *mcd4* gene was obtained following dissection and growth of ascospores from the heterozygous diploid strain obtained from the Consortium. The deletion mutation in the *mcd4* strain was verified by resistance to G418 (geneticin; 200 μg/ml) and through amplification of the expected PCR fragment. In the PCR analysis, Ex *Taq* (Takara Bio, Otsu) and primers (*MCD4*: 5′-GTACTAGCGTGATGTTAATGTT ACT-3′ and *KANB*: 5′-CTGCAGCGAGGAGCCGTAAT-3′) were used and amplification of a 600-bp fragment was observed. YPD medium (1% yeast extract, 2% peptone, and 2% glucose) was used to culture yeast. Furthermore, to verify the deletion, complementation of the growth was examined using the plasmid pYES2.0-*MCD4*. The plamid was constructed by the insertion of the PCR fragment containing the *MCD4* gene into the *Bam*HI-*Xba*I site of the plasmid pYES2.0 (Invitrogen Corp., Tokyo; multiple-copy-number plasmid containing *URA3*, *GAL1* promoter and 2 μ origin). Primers (5′-CGCGGATCCATGTGGAACAAAACCAG-3′ and 5′-CGCTCTAGAGGTATGTGAATCTATTGTG-3′) were used for the amplification of the fragment. Sorbitol (0.6–1.0 M) was added to YPD when osmotic support was needed. Yeast mating, sporulation, and tetrad analysis were performed as described previously (17). To determine mating type, the tester strains MC75 (MATα *thr5 met*) and MC76 (MAT**a** *lys1 cry1*) were used.

Drug phenotype assay Assays for K1 killer toxin sensitivity were carried out as previously described (18). Yeast strains were grown on YPD+0.6 M sorbitol for 18 h at 30°C. Cells were suspended at approximately 1×10^7 cells/ml in 100 μl of a sterilized solution of 1.0 M sorbitol. Five μl of this suspension was inoculated in 5 ml of medium (1% Difco yeast extract, 2% peptone, 1% agar, 0.001% methylene blue, 0.6 M sorbitol, and 1×Halvorson medium buffered at pH 4.7) and kept at 45°C. The medium was quickly poured into petri dishes (60×15 mm). After the agar gelled and attained room temperature, 5 μl of K1 killer toxin (1000×stock diluted 1:10) was spotted onto the center of the medium. The plate was incubated overnight at 18°C, followed by 24–48 h at 30°C, when the death zone was measured and photographed.

Drug sensitivity was determined by spotting diluted yeast cultures onto agar media containing various drugs. Cells were cultured in liquid YPD+1.0 M sorbitol overnight at 30°C. The cell density was adjusted to 0.5 (optical density at 600 nm), and 2 μl of a set of 1:10 serial dilutions were spotted onto agar plates. We used YPD+1.0 M sorbitol agar medium containing the following drug concentrations: hygromycin B (between 1 and 100 μg/ml); sodium dodecyl sulfate (SDS; between 0.0005% and 0.005%); nystatin (between 1 and 30 μg/ml). Cells were cultured at 30°C for 72 h. To test for temperature sensitivity, cells were cultured on YPD+1.0 M sorbitol at room temperature (between 20°C and 25°C), 18°C, 30°C, 37°C and 42°C for 72 h.

Cell wall analysis Alkali-soluble glucan levels in the cell wall were measured as follows. Yeast strains were pre-grown on YPD+0.6 M sorbitol plates for 2 or 3 d, and cells were transferred to YPD+0.6 M sorbitol liquid medium (25 ml) with a toothpick, and cultured overnight. The cells were harvested by centrifugation for 10 min at 1860×*g*, washed with 1.0 M sorbitol (10 ml), and resuspended in 500 μl of water. Glass beads were added to the cell suspension, and the mixture vortexed 5 times for 30 s, with intervals (at least 5 min) on ice, and lysates removed from the beads. The protein levels in lysates were determined using the Bradford assay. Lysates containing 8 μg of total cell wall protein were bought up to 50 μl with water, and 50 μl NaOH (1.5 N) were added to each, and incubated for 1 h at 75°C. After the alkali-insoluble components were removed by centrifugation (5 min and 11,000×*g*), a 1:2 serial dilution of the alkali-soluble fractions was spotted onto nitrocellulose membrane. The immunoblotting was carried out in TBST (10 mM Tris–HCl, pH 8.0/150 mM NaCl/0.05% Tween 20) containing 5.0% non-fat dried milk powder using a 1:2000 dilution of the affinity-purified rabbit anti β-1,6-glucan primary antibody (19) and a 1:2000 dilution of horseradish peroxidase goat anti-rabbit secondary antibody (Amersham Biosciences, Piscataway, NJ, USA). The glucan signal on the membrane was visualized following development with a chemiluminescence detection kit.

Alkali-insoluble glucan levels were determined as described previously (20). Cells were cultured in 50 ml flasks containing 10 ml YPD+1.0 M sorbitol and harvested by centrifugation (10 min and 2600×*g*) and washed with 1.0 M sorbitol (25 ml). The cells were broken with glass beads on ice, and the cell wall fraction was collected by centrifugation (15 min and 2600×*g*). Alkali-insoluble glucans were extracted from isolated cell walls. After β-1,3-glucanase digestion (Zymolyase 100T) and dialysis, the β-1,6-glucan was collected and quantified. The total alkali-insoluble glucan was measured as the hexose content before dialysis and the β-1,3-glucan level was calculated by subtraction of the β-1,6-glucan content from total glucan.

Photomicroscopy Cells grown on solid medium (YPD+1.0 M