Rot1p of *Saccharomyces cerevisiae* is a putative membrane protein required for normal levels of the cell wall 1,6- β -glucan

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Although *ROT1* is essential for growth of *Saccharomyces cerevisiae* strain BY4741, the growth of a *rot1* Δ haploid was partially restored by the addition of 0.6 M sorbitol to the growth medium. Rot1p is predicted to contain 256 amino acids, to have a molecular mass of 29 kDa, and to possess a transmembrane domain near its C-terminus. *Candida albicans* and *Schizosaccharomyces pombe* have Rot1p homologues with high identity that also have predicted transmembrane domains. To explore the role of Rot1p, the phenotypes of the *rot1* Δ haploid were analysed. Deletion of *ROT1* caused cell aggregation and an abnormal morphology. Analysis of the cell cycle showed that *rot1* Δ cells are delayed at the G2/M phase. The *rot1* Δ cells were resistant to K1 killer toxin and hypersensitive to SDS and hygromycin B, suggesting that they had cell wall defects. Indeed, greatly reduced levels of alkali-soluble and -insoluble 1,6- β -glucan, and increased levels of chitin and 1,3- β -glucan, were found in *rot1* Δ cells. Furthermore, the phenotypes of *rot1* Δ cells resemble those of disruption mutants of the *KRE5* and *BIG1* genes, which show greatly reduced levels of cell wall 1,6- β -glucan. Incorporation of glycosylphosphatidylinositol (GPI)-dependent cell wall proteins in *big1* Δ and *rot1* Δ cells was examined using a GFP-Flo1 fusion protein. GFP fluorescence was detected both on the cell surface and in the culture medium, suggesting that, in these mutants, mannoproteins may become only weakly bound to the cell wall and some of these proteins are released into the medium. Electron microscopic analyses of *rot1* Δ and *big1* Δ cells showed that the electron-dense mannoprotein rim staining was more diffuse and paler than that in the wild-type, and that the outer boundary of the cell wall was irregular. A *big1* Δ *rot1* Δ double mutant had a growth rate similar to the corresponding single mutants, suggesting that Rot1p and Big1p have related functions in 1,6- β -glucan synthesis.

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INTRODUCTION

The yeast cell wall is a complex extracellular organelle, composed of 1,3- β -glucan, 1,6- β -glucan, mannoproteins and chitin, and it is essential for maintaining cell shape and integrity. The wall structure can dynamically change to adapt to extracellular conditions and different states of the fungal life cycle (Orlean, 1997). 1,3- β -Glucan accounts for about 40 % of the cell wall dry weight; it is synthesized at the cell surface and consists of linear chains with an average

length of 1500 glucose units. 1,6- β -Glucan, which constitutes approximately 10 % of the cell wall dry weight, consists of polymers with an average length of 350 glucose units; it plays a critical role in the cell wall architecture by anchoring mannoproteins to other cell wall components (Kollar *et al.*, 1997; Kapteyn *et al.*, 1996). This linkage of 1,6- β -glucan to mannoproteins is thought to be mainly through mannose residues of the glycosylphosphatidylinositol (GPI) core structure (Shahinian & Bussey, 2000).

Many genes involved in 1,6- β -glucan biosynthesis have been identified based on resistance to K1 killer toxin, which kills yeast following binding to a 1,6- β -glucan-containing cell surface receptor (Al-Aidroos & Bussey, 1978; Boone *et al.*, 1990; Brown *et al.*, 1993), and on hypersensitivity to

Abbreviations: ConA–FITC, concanavalin A–fluorescein isothiocyanate; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; SEM, scanning electron microscopy; SGD, *Saccharomyces* Genome Database; TEM, transmission electron microscopy.

Calcofluor white (Ram *et al.*, 1994; Lussier *et al.*, 1997). Their role in 1,6- β -glucan biosynthesis is supported by the fact that null mutations of these genes lead to a reduction in the level of 1,6- β -glucan. These gene products and their functional homologues are located along the secretory pathway, including the endoplasmic reticulum (ER), the Golgi apparatus, the cytoplasm and the cell surface, suggesting that 1,6- β -glucan is synthesized along the secretory pathway and completed at the cell surface (Shahinian & Bussey, 2000). Among those gene products, Kre5p is particularly worth noting. The Kre5p protein is a soluble N-glycoprotein located in the ER (Meaden *et al.*, 1990; Levinson *et al.*, 2002), and mutation of the Kre5p gene greatly reduces formation of cell wall 1,6- β -glucan, leading to severe growth defects or lethality (Meaden *et al.*, 1990; Levinson *et al.*, 2002; Shahinian *et al.*, 1998; Azuma *et al.*, 2002). The biological activity of this protein is still unknown, but it is reported to have a limited but significant sequence similarity with UDP-glucose: glycoprotein glucosyltransferases (UGGT) from *Drosophila melanogaster* (Parker *et al.*, 1995) and *Schizosaccharomyces pombe* (Fernandez *et al.*, 1996). Recently, we have found that deletion of *BIG1* leads to an approximately 95 % reduction in cell wall 1,6- β -glucan (Page *et al.*, 2003). In addition, Big1p is an N-glycosylated integral membrane protein with a type I topology, it is located in the ER, and some phenotypes of a *big1* Δ mutant resemble those of a *kre5* Δ mutant. Although a Big1p homologue is also found in *Candida albicans* (Azuma *et al.*, 2002), its biochemical function has not yet been defined.

BIG1 was first described as a multicopy suppressor of the synthetic lethality of a *rot1-1 rot2-1* double mutant. Mutations in *ROT1* (reversal of *TOR2*) and *ROT2* cause cell wall defects and suppress the loss of *TOR2*, an essential phosphatidylinositol-kinase-like protein kinase (Bickle *et al.*, 1998). *ROT2* was identified based on the similarity of its protein sequence with that of the α subunit of mammalian

glucosidase II (Trombetta *et al.*, 1996). In addition, a *rot2* Δ mutant has a partial 1,6- β -glucan defect (Simons *et al.*, 1998). However, like Big1p, the functional role of Rot1p remains unknown. *ROT1* is essential for growth, with *rot1* Δ ascospores germinating and arresting growth within one cell cycle (Bickle *et al.*, 1998). In the disruption consortium BY4741 strain series used in this work, *ROT1* is also classified as an essential gene (Winzeler *et al.*, 1999).

Here, to examine whether the presence of Rot1p is necessary for formation of normal levels of cell wall 1,6- β -glucan, we begin an analysis of *rot1* Δ mutants.

METHODS

Strains, plasmids and media. Strains, plasmids and primers used in this study are shown in Tables 1 and 2. Wild-type strains used were *S. cerevisiae* BY4741 (*MATa*), BY4742 (*MAT α*) and BY4743 (*MATa/ α*) (Brachmann *et al.*, 1998). Deletion strains were obtained from the *Saccharomyces* Genome Deletion Consortium (Winzeler *et al.*, 1999). Haploid *rot1* mutants were obtained by dissection of the heterozygous diploid strains from the Consortium on medium containing 0.6 M sorbitol. The mating type and the nutrient requirement of the haploid mutants obtained were analysed, and strains isogenic with the wild-type were taken and used for this study. Media for yeast growth were as described by Bussey *et al.* (1982). YPD is yeast complex medium; YNB is a synthetic medium, which was supplemented with appropriate nutrients. Sorbitol (0.6 or 1.0 M) was added to those media when osmotic support was needed; the resulting media were called YPDS or YNBS. Yeast mating, sporulation, and tetrad analysis were performed as described by Sherman *et al.* (1982). To determine mating type the tester strains MC75 and MC76 were used. Yeast transformation was carried out using the one-step transformation method (Chen *et al.*, 1992).

Plasmid DNA was prepared from *Escherichia coli* strain JM109 via the boiling method. Bacterial cells were cultured and transformed using standard media and methods (Sambrook *et al.*, 1989). Alkaline phosphatase and T4 DNA ligase were purchased from NIPPON GENE. Restriction endonucleases were purchased from NIPPON GENE and Takara Bio. The vector pMB2 (provided by M. N. Hall;

Table 1. Yeast strains used in this study

Strain	Genotype	Source
BY4741	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	Brachmann <i>et al.</i> (1998)
BY4742	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Brachmann <i>et al.</i> (1998)
BY4743	<i>BY4741/BY4742 MATa/α</i>	Brachmann <i>et al.</i> (1998)
Haploid a*	<i>As BY4741, orfΔ::kanMX4</i>	Winzeler <i>et al.</i> (1999)
Haploid α *	<i>As BY4742, orfΔ::kanMX4</i>	Winzeler <i>et al.</i> (1999)
Heterozygous*	<i>As BY4743, orfΔ::kanMX4</i>	Winzeler <i>et al.</i> (1999)
<i>big1</i> Δ haploid a	<i>As BY4741, big1Δ::kanMX4</i>	Azuma <i>et al.</i> (2002)
<i>big1</i> Δ haploid α	<i>As BY4742, big1Δ::kanMX4</i>	Azuma <i>et al.</i> (2002)
<i>rot1</i> Δ haploid a	<i>As BY4741, rot1Δ::kanMX4</i>	This study
<i>rot1</i> Δ haploid α	<i>As BY4742, rot1Δ::kanMX4</i>	This study
MC75	<i>MATα thr5 met</i>	M. W. Clark, McGill University, Montreal, Canada
MC76	<i>MATa lys1 cry1</i>	M. W. Clark

*Strains obtained from the *Saccharomyces cerevisiae* Genome Deletion Consortium.

Table 2. Oligonucleotide primers and plasmids used in this study

Primer	Sequence	
KanB	5'-CTGCAGCGAGGAGCCGTAAT-3'	
ScROT1-A	5'-ATAGAATTCCCAACTCATCATCCGCT-3'	
ScBIG1-A	5'-CTCGATGGGCTGTAAATAGATAAGA-3'	
nGFPfw	5'-CGGTCCATGGAATGGTGAGCAAGGGCGAGGAGCTGTTACAC-3'	
nGFPrv	5'-GCGGCTCGAGAACTTGTACAGCTCGTCCATGCCGAGAGTGAT-3'	
Plasmid	Insert	Source
pRS426	None	Christianson <i>et al.</i> (1992)
pRS426- <i>ROT1</i>	<i>ROT1</i>	This study
pMB2	<i>ROT1</i>	Bickle <i>et al.</i> (1998)
pRS406	None	Christianson <i>et al.</i> (1992)
pRS406-2 μ m	2 μ m	This study
pEGFP	<i>GFP</i>	Clontech
pIF318	Prepro- α -factor and <i>FLO1</i>	Sato <i>et al.</i> (2002)
pWI3 α -GFP-Flo318	Prepro- α -factor, <i>GFP</i> and <i>FLO1</i>	This study
pWI3	None	Kanai <i>et al.</i> (1996)
pMUF-GFP	Prepro- α -factor, <i>GFP</i> and <i>FLO1</i>	This study

Bickle *et al.*, 1998), which is YCplac33 (Gietz *et al.*, 1995) containing a 1.4 kb *SacI*-*EcoRI* fragment bearing *ROT1*, was cut with *SacI* and *EcoRI*. The resulting 1.4 kb *ROT1* fragment was inserted into *SacI*- and *EcoRI*-cut pRS426 (Christianson *et al.*, 1992), generating the construct pRS426-*ROT1*. DNA sequencing of *ROT1* (pRS426-*ROT1*) was performed with an ABI377 DNA sequencer at Takara Bio. The plasmid pMUF-GFP, bearing the gene for the GFP-Flo1 fusion protein, was constructed by first amplifying the GFP gene from pEGFP (Clontech) using PCR with nGFPfw and nGFPrv primers (Table 2). The amplified fragment was inserted into the *NcoI* and *XhoI* sites of pIF318 (Sato *et al.*, 2002), and the resulting plasmid was named pWI3 α -GFP-Flo318. Next, pWI3 α -GFP-Flo318 was cut with *PvuII*. The resulting fragment contained the gene encoding the prepro- α -factor leader region, GFP, and the C-terminal region of Flo1p (318 aa) under the control of the 5'-upstream region of the isocitrate lyase gene from *Candida tropicalis* (*UPR-ICL*), which is induced by glucose exhaustion (Sato *et al.*, 2002). The fragment was inserted into the *PvuII* site of pRS406-2 μ m, which was constructed by introducing part of the 2 μ m ori from pWI3 (Kanai *et al.*, 1996) into the *AraII* site of pRS406 (Sikorski & Hieter, 1989).

Confirmation of the disruption of *ROT1*. The existence of a deletion mutation was verified by slow growth and resistance to G418 (geneticin, 200 μ g ml⁻¹; Gibco-BRL). The mutation was also verified by PCR analysis using Takara Ex Taq and primers KanB and ScROT1-A (Table 2). An amplification of the 910 bp fragment was found. To verify the deletion, the complementation of the growth defect by plasmid pRS426-*ROT1* was examined.

FACS analysis. Cells were pre-cultured at 30 °C in 4 ml YPDS for 1 day, then 0.2 ml of the culture was transferred to 4 ml YPDS and cells were cultured to exponential phase. Hydroxyurea (0.4 ml of 1.0 M solution) was added to the culture solution and incubated for 4 h. The cells were washed with YPDS three times to remove hydroxyurea, resuspended in 10 ml YPDS and cultured at 30 °C for 2–9 h. Cells were fixed in 70 % cold ethanol at –20 °C overnight, washed with 0.2 M Tris/HCl (pH 7.5) and treated with 1 mg RNase A ml⁻¹ (Sigma) at 37 °C overnight. Cells were washed with 0.2 M Tris/HCl (pH 7.5), and resuspended in 100 μ l Na-PI solution (0.05 mg propidium iodide ml⁻¹, 1.0 mg sodium citrate ml⁻¹, 0.58 mg sodium chloride ml⁻¹); 10 μ l of 2.0 mg propidium iodide

ml⁻¹ was then added to the solution. Samples were allowed to stand at room temperature for 30 min and diluted with 900 μ l 0.2 M Tris/HCl buffer and 10 μ l of 2.0 mg propidium iodide ml⁻¹. After the cell suspensions had been briefly sonicated, samples were analysed using a Becton Dickinson FACSCalibur and Cell Quest. Flow cytometric analysis was performed using FACSCalibur (Becton Dickinson). Event rate was maintained at 300 cells s⁻¹ and data for 20 000 events were collected.

Phenotypes of *rot1* Δ cells. Assays for K1 killer toxin sensitivity were carried out as previously described (Bussey, 1991). Yeast strains were grown on YPD + 0.6 M sorbitol at 30 °C for 18 h. Cells were suspended at approximately 1 $\times$ 10⁷ cells ml⁻¹ in 100 μ l of a sterilized solution of 1.0 M sorbitol, and 5 μ l of this suspension was added to 5 ml medium (1 % Difco yeast extract, 2 % peptone, 1 % agar, 0.001 % methylene blue, 0.6 M sorbitol and 1 $\times$ Halvorson medium buffered at pH 4.7) kept at 45 °C. The medium was quickly poured into Petri dishes (60 $\times$ 15 mm). After the agar had gelled and attained room temperature, 5 μ l K1 killer toxin (1000 $\times$ stock diluted 1 : 10) was spotted onto the centre of the medium. The plate was incubated at 18 °C overnight, followed by 24–48 h at 30 °C, after which the death zone was measured and photographed.

Drug sensitivity was determined by spotting diluted yeast cultures onto agar media containing various drugs (Ram *et al.*, 1994; Lussier *et al.*, 1997). 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 μ l drops 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, 1–100 μ g ml⁻¹; or SDS, 0.0005–0.005 %. Cells were cultured at 30 °C for 72 h, and the growth was then observed.

Alkali-soluble 1,6- β -glucan assay. Yeast strains were pre-grown on YPD + 0.6 M sorbitol plates for 2 or 3 days, and then 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 μ l water. Glass beads were added to the cell suspension, and the mixtures were vortexed five 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 total cell wall protein were brought up to 50 µl with water, and 50 µl NaOH (1.5 M) was added to each, followed by incubation for 1 h at 75 °C. After removal of the alkali-insoluble components by centrifugation (5 min, 11 000 g), a 1:2 serial dilution of the alkali-soluble fractions was spotted on Hybrid-C nitrocellulose membrane (Amersham). 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-1,6-β-glucan primary antibody (Kollar *et al.*, 1997) and a 1:2000 dilution of horseradish peroxidase–goat anti-rabbit secondary antibody (Amersham). The glucan signal on the membrane was visualized following development with a chemiluminescence detection kit.

Alkali-insoluble β-glucan assay. Alkali-insoluble β-glucan levels were determined as described by Kapteyn *et al.* (1999). Cells were cultured in 50 ml flasks containing 10 ml YPD+1.0 M sorbitol, harvested by centrifugation (10 min, 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, 2600 g). Alkali-insoluble β-glucans were extracted three times in 3 % (w/v) NaOH at 75 °C for 1 h. The pellet was washed twice in 0.1 M Tris/HCl (pH 7.5), washed in 0.01 M Tris/HCl (pH 7.5), resuspended in 0.01 M Tris/HCl (pH 7.5) containing 1,3-β-glucanase (Zymolyase 100T, 1.0 mg ml⁻¹) and incubated at 37 °C overnight. After dialysis, the 1,6-β-glucan was collected and quantified. The total alkali-insoluble glucan was measured as the hexose content before dialysis. The alkali-insoluble 1,3-β-glucan level was calculated by subtraction of the 1,6-β-glucan content from total glucan.

Fluorescence microscopy. Chitin was visualized by Calcofluor white staining. Cells grown on solid medium (YPD+1.0 M sorbitol) for 48 h were suspended in 1.0 M sorbitol, stained with Calcofluor white M2R (Sigma) at 0.1 mg ml⁻¹, washed with 1.0 M sorbitol, and observed with a fluorophotomicroscope (Olympus BX50-34FLAD/PM30). The images were recorded with a Coolsnap camera (Nippon Roper). Mannan was visualized by staining with concanavalin A–fluorescein isothiocyanate (ConA–FITC, Sigma). Cells grown on YPD+0.6 M sorbitol for 48 h were fixed with 3.7 % formaldehyde for 30 min and washed with PBS. ConA–FITC (0.1 mg) was added to the cell suspension (1.0 ml). The suspension was incubated at room temperature for 10 min, washed with PBS, and observed.

Observation of GFP–Flo1p. To evaluate cell surface proteins in *rot1Δ* cells, the fluorescence of a fusion protein of GFP and the C-terminal part of Flo1p (a GPI-anchored protein located in cell wall) (Bony *et al.*, 1997) was analysed. Cells with pMUF–GFP, bearing the gene encoding the fusion protein, were cultured in YNB (without uracil)+0.6 M sorbitol containing 50 mM HEPES (pH 7.2) at 30 °C. Cells were washed, resuspended in the same medium and observed with a fluorophotomicroscope. The cultures were also centrifuged, and the fluorescence intensities of the supernatant and the pellet (cells) resuspended in a volume of the same medium equal to the culture were measured (excitation, 488 nm; emission, 510 nm) with a Shimadzu RF-5000 fluorometer.

SEM and TEM images. Cells were cultured on YPDS plates at 30 °C for 2 days. Specimens were fixed by the freeze-substitution method with minor modifications (Baba & Osumi, 1987). The cells were mounted on the copper meshes to form a thin layer and plunged into liquid propane cooled with liquid N₂. Frozen cells were transferred to anhydrous acetone containing 2.5 % OsO₄, cooled in a solid CO₂/acetone bath, and kept in the bath for 48 h. The solution including cells was incubated at –20 °C for 2 h, at 4 °C for 2 h, and subsequently at room temperature for 2 h. After washing the cells with anhydrous acetone three times, cells for SEM were

incubated in anhydrous acetone/isoamyl acetate (1:1, v/v) for 20 min, and then in 100 % isoamyl acetate for 20 min. The cells were dried with a critical point dryer (Hitachi), coated with a gold layer and observed with a Hitachi Natural SEM S-3500N scanning electron microscope. Cells for TEM were embedded using ERL4206-Quentol 653 (Nissin EM). Ultrathin sections were prepared with an ULTRACUT N microtome (Reichert-Nisei); they were stained with uranyl acetate and lead citrate and viewed with a Hitachi H-7000 electron microscope.

Genetic interaction. Crossing of *rot1Δ* and *big1Δ* cells was carried out. The *rot1Δ* and *big1Δ* cells were mixed on a YPDS plate, incubated at 30 °C for 6 h, transferred to a YNBS plate (not containing Met and Lys) to select only heterozygous diploid cells, and cultured at 30 °C for 4 or 5 days. The heterozygous diploid cells that grew on the selective medium were transferred to a GNA plate, cultured for 1 day, transferred to a SPO plate to make spores, and incubated at room temperature for 7 or 9 days. These cells were treated with 0.5 mg Zymolyase 100T ml⁻¹. The spores in individual asci were dissected onto YPDS plates and incubated at 30 °C for 6 days. Genotypes of resulting spores were determined by growth on media containing G418 and by PCR analysis using primers KanB, ScROT1-A and ScBIG1-A (Table 2).

RESULTS AND DISCUSSION

Rot1p bioinformatics

According to the *Saccharomyces* Genome Database (SGD; <http://www.yeastgenome.org/>), Rot1p is predicted to contain 256 amino acid residues and have a molecular mass of 29 kDa. However, in the original description of Rot1p (Bickle *et al.*, 1998), an additional thymidine at position 758 was found that was not reported in the database. We sequenced the 3'-end of *ROT1*, and found that our sequence matched that in the database (data not shown). Therefore, in the current studies, we used the sequences in the SGD database.

The PSORT and Sosui programs predicted the presence of a transmembrane domain between residues 238 and 256 in Rot1p. Although a signal peptide was not found using either of these two programs, another program, SignalP, predicted an N-terminal signal peptide. Rot1p also has a consensus sequence for GPI-modified proteins, suggesting that it remains attached to the plasma membrane (Caro *et al.*, 1997, De Groot *et al.*, 2003). On the other hand, Huh *et al.* (2003) predicted that Rot1p may be localized in the ER, based on analyses of a collection of yeast strains expressing Rot1p tagged with GFP at the C-terminus (Huh *et al.*, 2003). However, further work is required to determine the subcellular location of Rot1p more precisely.

According to a BLASTP search and the *C. albicans* genome sequence at the Stanford University Genome Center (<http://genome-www.stanford.edu/>), Rot1p has homologues in *Sz. pombe* and *C. albicans*. Because these homologues have high identities with Rot1p, we refer to them here as SzRot1p (43 % identity) and CaRot1p (53 % identity), respectively. The presence of a transmembrane domain near the C-terminus and an N-terminal signal peptide was predicted in both SzRot1p and CaRot1p using PSORT and Sosui.

Isolation of a haploid *rot1Δ* mutant

The *rot1-1* mutant has an altered content of chitin (188 % of wild-type), suggesting the participation of the *ROT1* gene product in cell wall synthesis (Bickle *et al.*, 1998). To understand its role, we performed a phenotypic analysis of *ROT1* by isolating a haploid *rot1Δ* mutant. The heterozygous *rot1Δ/ROT1* mutant was sporulated, and spores were dissected on YPD containing 0.6 M sorbitol. After 6 days of culture, the *rot1Δ* mutant produced small colonies on this medium; it was unable to produce colonies on YPD without sorbitol. A representative tetrad from the *rot1Δ/ROT1* heterozygous diploid is shown in Fig. 1. The colony size was similar or slightly smaller than the *big1Δ* colonies (data not shown). To test if the haploid mutants obtained were indeed disruptants with an insertion of the G418 resistance gene, cells were picked and transferred to medium containing G418. The mutants grew, although poorly, on YPD containing sorbitol and G418. The poor growth of the mutants was complemented by transformation with the plasmid pRS426-*ROT1* (data not shown). Confirmation of the disruption of *ROT1* was also obtained by PCR analysis.

Phenotypes of *rot1Δ* cells

We next examined the morphology and cell cycle phenotypes of *rot1Δ* cells. Cell aggregation was observed when *rot1Δ* cells were cultured in liquid YPD + 0.6 M sorbitol medium. The *rot1Δ* cells were round and large compared to the wild-type. This may be due to the cells staying in isotropic growth for a longer period. The cells were sensitive to hypo-osmotic conditions, bursting when they were transferred from a sorbitol solution to distilled water (data not shown). These results suggested that the deletion mutant has a weak cell wall.

Fig. 1. Isolation of *rot1Δ* mutants. Wild-type (WT, diploid) and *rot1Δ/ROT1* cells were sporulated and dissected onto YPD + 0.6 M sorbitol (a) and YPD (b). Cells were cultured at 30 °C for 6 days. Arrows indicate colonies of *rot1Δ* cells.

Bickle *et al.* (1998) described that *rot1*-null spores germinated and arrested growth within one cell cycle as medium- or large-budded cells. To examine cell cycle progression, the DNA content of *rot1Δ* cells was quantified by FACS. After arresting the cells in S phase with hydroxyurea, cell progression was restarted by removing the hydroxyurea. In wild-type cells there was a progression of ($\rightarrow$ G2/M $\rightarrow$ G1) over a 4 h period (Fig. 2a). In both *rot1Δ* and *big1Δ* mutants (Fig. 2b and 2c, 0 h), the cell cycle was not completely arrested in S phase by hydroxyurea. This may be due to slow growth of the mutants. However, after removal of the hydroxyurea most of the cells arrested in G2/M for at least 4 h. These results suggested that *rot1Δ* cells are delayed at the G2/M phase.

We also examined the sensitivity of *rot1Δ* cells to SDS and hygromycin B, known indicators of cell surface defects. Compared to the wild-type, *rot1Δ* was hypersensitive to SDS and hygromycin B, and there was no growth in 0.003 % SDS or in 20 μ g hygromycin B ml^{-1} (Fig. 3a). These phenotypes were similar to those of *big1Δ* and *kre5Δ*, which were also resistant to K1 killer toxin. We therefore examined the sensitivity of *rot1Δ* cells to K1 killer toxin (Fig. 3b). We found that the *rot1Δ/ROT1* heterozygote was as sensitive as the parental strain, while the haploid deletion mutant was resistant to the toxin. Thus, the *rot1Δ* haploid mutant, as well as *big1Δ* and *kre5Δ*, may be defective in cell wall 1,6- β -glucan synthesis.

Fig. 2. Analysis of cell cycle progression in *rot1Δ* and *big1Δ* by flow cytometry. Cells were incubated at 30 °C after removing hydroxyurea. Event rate was maintained at 300 cells s^{-1} and data for 20 000 events collected. (a) Wild-type, (b) *rot1Δ*, (c) *big1Δ*. 1C and 2C denote DNA content.

Fig. 3. (a) Sensitivities to SDS and hygromycin B. Tenfold serial dilutions of precultures of wild-type and *rot1Δ* (haploid **a**) were spotted onto YPDS (A), YPDS containing 0.003% SDS (B) and YPDS containing 20 μg hygromycin B ml⁻¹ (C). (b) Sensitivity to K1 killer toxin. A diploid *rot1Δ* mutant is a *rot1Δ/ROT1* heterozygote.

Cell wall analysis

To further test for wall defects, we examined alkali-soluble and -insoluble 1,6-β-glucan levels in the cell wall. The level of the alkali-soluble glucan was measured by immunodetection (Fig. 4a). We found that the *rot1Δ* mutant, like the *big1Δ* mutant (Azuma *et al.*, 2002), has greatly reduced levels of alkali-soluble 1,6-β-glucan ($\leq 3\%$ of the wild-type). However, a weak signal was detected, indicating that residual 1,6-β-glucan remains. Analysis of alkali-insoluble 1,6-β-glucan levels indicated that the level of *rot1Δ* was 3% or less of that in the wild-type (Fig. 4b). These results indicate that Rot1p is required for maintenance of normal 1,6-β-glucan levels. In contrast, based on Calcofluor white staining, *rot1Δ* has increased levels of alkali-insoluble 1,3-β-glucan (Fig. 4c) and chitin (Fig. 5a).

We further examined the effect of the 1,6-β-glucan defect on mannan in the outer layer of the cell wall because 1,6-β-glucan anchors mannoproteins to other cell wall components (Kollar *et al.*, 1997; Kapteyn *et al.*, 1996). The mannan layer in wild-type, *big1Δ*, *cwh41Δ* and *rot1Δ* cells was examined by staining the cells with ConA-FITC and observing them by fluorescence microscopy. Cwh41p is a homologue of mammalian glucosidase I and has been reported to be an ER protein involved in 1,6-β-glucan synthesis. Disruption of the *CWH41* gene leads to a 50% reduction in the cell wall 1,6-β-glucan level (Jiang *et al.*, 1996). We found no differences in the fluorescence intensities of wild-type, *big1Δ*, *cwh41Δ*, and *rot1Δ* cells (Fig. 5b).

Recently, a system to display proteins on the cell surface of

S. cerevisiae was developed using the prepro-α-factor leader region and the C-terminal GPI-anchor attachment signal sequences of Flo1p, a native cell wall protein (Sato *et al.*, 2002). To examine the effect of the 1,6-β-glucan defect on cell surface proteins, wild-type, *big1Δ*, *cwh41Δ* and *rot1Δ* cells were transformed with pMUF-GFP (bearing the gene encoding the GFP-Flo1p fusion protein) and observed by fluorescence microscopy. We found that the fluorescence of GFP in *big1Δ*, *cwh41Δ*, and *rot1Δ* cells was similar to that in the wild-type (Fig. 5c).

Because of the role of 1,6-β-glucan in anchoring mannoproteins to the cell wall, we expected that disruption of Big1p or Rot1p would cause a release of the GFP-Flo1p fusion protein into the medium. We therefore examined the intensity of GFP fluorescence in the culture supernatant of the *rot1Δ*, *big1Δ*, *cwh41Δ*, and wild-type cells (Fig. 6). After 72 h of culture, the fluorescence intensities of the supernatants from *rot1Δ* and *big1Δ* cells were approximately fivefold stronger than those in wild-type and the *cwh41Δ* cells. These results indicate that mannoproteins in *rot1Δ* and *big1Δ* cells fail to be correctly linked to the cell wall and are released to the medium.

Microscopic observation showed no difference in the levels of mannan or GFP-Flo1p between the wild-type strain and *rot1Δ*. However, considering the role of 1,6-β-glucan, we predicted that disruption of *ROT1* would cause some changes in the outer mannoprotein layer. In support of this possibility, *KRE5* mutants with a defect in 1,6-β-glucan synthesis have rough cell walls lacking the outer mannoprotein layer (Simons *et al.*, 1998). To investigate the

Fig. 4. (a) Alkali-soluble 1,6-β-glucan level in *rot1Δ* cells. The alkali-soluble fractions were extracted and spotted onto nitrocellulose membranes. The immunoblotting was carried out using 1,6-β-glucan primary antibody. Arrows indicate the spot positions. (b, c) Alkali-insoluble 1,6-β-glucan (b) and alkali-insoluble 1,3-β-glucan (c) in *rot1Δ* cells, measured as mg alkali-insoluble glucan × 100 per mg dry weight of cell wall. The data shown represent the results of at least three independent experiments. Error bars represent standard deviations.

structure of the mannoprotein layer in *rot1Δ*, we carried out electron microscopic analyses of *rot1Δ* and *big1Δ* cells. SEM images showed that the cell surface structures of *rot1Δ* and *big1Δ* are very rough and substantially different from those of wild-type cells (Fig. 7). TEM revealed that the outer layer of the *S. cerevisiae* cell wall is composed of mannoproteins, which have an electron-dense appearance in ultrathin sections on TEM images. The underlying layer, which is amorphous, is composed of 1,6-β-glucan and 1,3-β-glucan (Cid *et al.*, 1995). In wild-type cells, the

thickness of the wall around the cell was almost uniform, and the darkly stained outer layer of the mannoproteins was clearly observed. However, in many of the *rot1Δ* and *big1Δ* cells, the electron-dense mannoprotein rim staining was more diffuse and paler than that in the wild-type. Also, the outer boundary of the cell wall was irregular and the outer part of the cell wall was rough.

Genetic interaction of *rot1* with *big1*

Because some phenotypes of *rot1Δ* cells resembled those of *big1Δ* cells, we tested whether a *big1 rot1* double mutant would have a more severe phenotype than the corresponding single mutants. Growth of double mutants was compared to that of the single mutants (Fig. 8). Two tetrads of nonparental ditype (NPD) and 14 tetrads of tetrad ditype (TT) were analysed. In all colonies, the genotypes were verified by PCR analysis. Double mutant colonies were obtained in two NPD tetrads and nine TT tetrads. Although the colony size of the double mutant was a little variable compared to that of the single mutants, the average growth rate of the double mutant was similar to that of the single mutants. These results suggest that Rot1p and Big1p may have similar effects on the synthesis of 1,6-β-glucan.

Conclusions

Although disruption of *ROT1* is lethal, growth of *rot1Δ* cells can be achieved with an osmotic support. In the current studies, we examined the function of Rot1p using a haploid *rot1Δ* mutant. This haploid mutant had greatly reduced levels of 1,6-β-glucan, suggesting that, like Big1p and Kre5p, Rot1p is required for 1,6-β-glucan synthesis. Clarification of the functions of these proteins will be necessary for a full understanding of the mechanism of 1,6-β-glucan synthesis. Previously, we reported that Big1p and Kre5p appear to have similar effects on 1,6-β-glucan synthesis (Azuma *et al.*, 2002), and in the current studies, we show that *rot1Δ* mutants have properties similar to *big1Δ* mutants, with *big1Δrot1Δ* double mutants showing a similar growth rate to the single mutants. Therefore Rot1p, Big1p and Kre5p may have related effects on the synthesis of 1,6-β-glucan. Furthermore, it seems that Rot1p and Big1p not only have a similar function, but they may also reside in the same compartment or at the same location and might form a complex. In the near future, the subcellular location of Rot1p must be determined. Although *rot1Δ*, *big1Δ* and *kre5Δ* mutants all have an osmoremedial phenotype, the restorations were partial, and growth, even on medium with osmotic support, was very slow compared to the wild-type (see Fig. 3a, A). Our analysis of the cell cycle also suggested that *rot1Δ* and *big1Δ* cells are delayed at the G2/M phase. Therefore, 1,6-β-glucan may play a significant role in maintaining not only the physical strength of the cell wall but also normal cell division.

Considering the role of 1,6-β-glucan in the cell wall, it was expected that *big1Δ* and *rot1Δ* mutants would have defects in anchoring cell surface mannoproteins. However,

Fig. 5. Fluorescence micrographs of (a) wild-type and *rot1Δ* cells stained with Calcofluor white, (b) wild-type, *cwh41Δ*, *big1Δ* and *rot1Δ* cells stained with ConA-FITC, and (c) wild-type, *cwh41Δ*, *big1Δ* and *rot1Δ* cells carrying pMUF-GFP. In each observation, the same exposure time was used for the images. Bars, 5 μm (a, b); 10 μm (c).

Fig. 6. Fluorescence intensities of GFP-Flo1p released to the medium. Cultures (1 ml) of wild-type (▼), *cwh41Δ* (◇), *big1Δ* (■) and *rot1Δ* (○) cells carrying pMUF-GFP were centrifuged, and fresh medium (0.6 ml) was added to the supernatant (0.9 ml). Fluorescence intensities of the solutions (1.5 ml) were measured. Values were expressed as percentages relative to the intensity of the solution from the culture of *rot1Δ* at 72 h. The data represent the results of at least two independent experiments.

microscopic analyses using ConA-FITC and GFP-Flo1p showed no differences relative to the wild-type cells. In contrast, when a strain with defects in GPI anchor synthesis (*mcd4* mutant) was observed as a negative control, there was little ConA-FITC and GFP-Flo1p fluorescence (unpublished results). In addition, the quantity of cell wall proteins obtained from *rot1Δ* cells was similar to or slightly more than that from the wild-type when the two cell types were cultivated to the same culture volume (data not shown). These results suggest that, even with this large defect in 1,6-β-glucan level, mannoproteins were transported to the cell surface and anchored to 1,3-β-glucan and chitin. In *rot1Δ* cells expressing pMUF-GFP, the fluorescence intensity in the supernatant was 77 % of that in the cells, suggesting that much of the GFP-Flo1p was released to the medium. Furthermore, electron microscopic observations indicate that the cell walls of *rot1Δ* and *big1Δ* mutants are very rough, that these strains do not have the outer layer with a high density of mannoproteins, and that the outer boundary of the cell wall is irregular. Therefore, in these mutants, the mannoproteins do not localize in the outer layer at a high density, and, instead, may be spread around the whole cell wall.

Fig. 7. SEM images of the surface structure (left) and TEM images of ultrathin sections (right) of wild-type, *big1Δ* and *rot1Δ* cells. Bars, 1 μ m.

Fig. 8. Genetic interactions of *rot1* with *big1*. After crossing of *rot1Δ* and *big1Δ* cells, heterozygous diploids were dissected onto YPD+0.6 M sorbitol and incubated at 30 °C for 6 days. DM, *rot1Δbig1Δ* double mutant; NPD, non-parental ditype; TT, tetatype; WT, wild-type.

Rot1p appears to be a membrane protein required for normal levels of the cell wall 1,6- β -glucan, and has homologues with high identity in *Sz. pombe* and *C. albicans*. Although in both SzRot1p and CaRot1p the presence of a transmembrane domain is predicted, the functional role of those proteins has not yet been defined. Analysis of Rot1p function should contribute to an understanding of fungal 1,6- β -glucan biosynthesis. Rot1p is also potentially interesting as a target for antifungal drugs because the protein is essential for growth and has a homologue in the pathogenic yeast *C. albicans*.

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