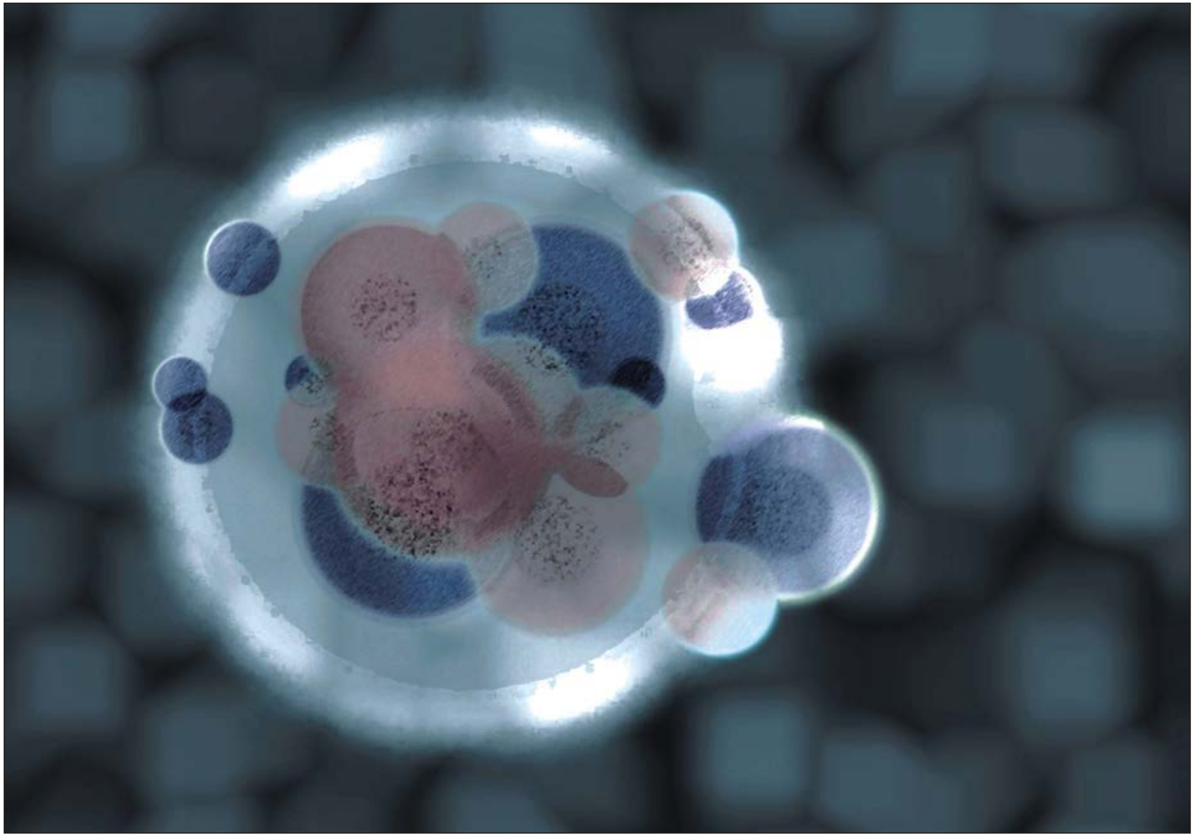

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The GDP-Mannose Transporter is Required for Cell Wall Integrity in *Saccharomyces cerevisiae*

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Abstract: The GDP-mannose transporter gene *VRG4* has been identified from a number of yeasts including *Saccharomyces cerevisiae*. The *vrg4-2* allele of the GDP-mannose transporter of *S. cerevisiae* has been primarily characterized as having general phenotypes affecting glycosylation. In this study, *vrg4-2* was characterized, specifically with regard to defects in cell wall biosynthesis. The cell wall in *vrg4-2* mutants was found to be profoundly compromised, as measured by the spheroplast lysis assay. These mutant cells exhibited increased sensitivity to Congo red which is believed to interfere with chitin-glucan assembly in the cell wall. An increased level of cell wall fluorescence was also observed when the cells were incubated with Calcofluor white suggesting an increased level of chitin in the wall. Despite these apparent major alterations in the cell wall, the *vrg4-2* mutants exhibited near normal growth relative to the wild-type. All together, these results suggest that *vrg4-2* mutation affects the cell wall structure, but less so in general cellular metabolism.

Key words: GDP-mannose transporter, cell wall, Congo red, Zymolyase, Calcofluor white

INTRODUCTION

The cell wall of *Saccharomyces cerevisiae* is primarily composed of an array of mannoproteins (mannan) and glucans linked to small amounts of chitin (Duran and Nombela, 2004). Although this complex structure is often represented as rigid, it is actually highly dynamic, capable of modifying its own structure in response to changing physiological conditions and environmental stresses. If the cell wall's normal structure is altered (stressed) such as through antifungal drug echinocandins treatment or cell wall gene mutation or deletion, cells typically respond by incorporating increased levels of chitin which presumably strengthens the cell wall (Imai *et al*., 2005; Klis *et al*., 2002; Popolo *et al*., 1997; Walker *et al*., 2008).

Mannoproteins are synthesized and processed in the ER and Golgi complex after which they are transported to and incorporated into the cell surface. The core mannose groups are N- and O-linked to the wall proteins in the endoplasmic reticulum and terminal mannosylation is completed in the Golgi apparatus (Gemmill and Trimble, 1999).

The substrate for protein mannosylation in the Golgi is the activated sugar, GDP-mannose. GDP-mannose, which is synthesized in the cytoplasm, must be transported, through a sugar transporter, into the lumen of the organelle where protein mannosylation occurs (Dean *et al*., 1997). The gene which codes for the GDP-mannose transporter (*VRG4*) was first isolated from *S. cerevisiae* by Poster and Dean (1996). Subsequently, her group isolated two further orthologs from two different species of *Candida* (Nishikawa *et al*., 2002a, b). More recently, orthologs of the GDP-mannose transporter have also been isolated from *Pichia pastoris* (Arakawa *et al*., 2006), *Cryptococcus neoformans* (Cottrell *et al*., 2007) and *Aspergillus nidulans* (Jackson-Hayes *et al*., 2008). In non-yeast species, *VRG4* orthologs are found in plants, such as *Arabadopsis thaliana* (Baldwin *et al*., 2001) and two species of rice (*Oryza sativa*) cDNA (Poster and Dean, 1996).

Saccharomyces cerevisiae vrg4-2 mutant cells are defective in the production of mannoproteins. Such cells produce shortened versions of the glycoproteins invertase and chitinase, as evidenced by migration on gel electrophoresis (Dean *et al*., 1997; Poster and Dean, 1996). Presumably, all mannoproteins produced by these cells will be defective. Consequently, we reasoned that this mutant should also have defective cell wall phenotypes. In this study, we investigated the changes in the integrity and structure of the cell wall of *vrg4-2* mutant cells as characterized by a Zymolyase lysis assay and through the binding of two dyes: Congo red and Calcofluor white.

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MATERIALS AND METHODS

Yeast strains, media and general methods: The yeast strains used in this study are listed as follows: *RSY255: MATα ura3-52 leu2-211; NDY5: MATα ura3-52 leu2-211 vrg 4-2. SEY6210: MATα ura3-52 his3-Δ200 trp1-Δ 901 lys2-801 suc2-Δ 9 leu2-3, 112.* All strains were provided as gifts from Dr. Neta Dean of Stony Brook University (SUNY). The strains were maintained on YPAD agar as described by Adams *et al.* (1997). The strains were grown in YPAD liquid media at 30°C with shaking unless otherwise stated.

Measurement of spheroplast formation and cell lysis by Zymolyase: Spheroplast formation assay was as described by Lim *et al.* (1995). Briefly, cells were grown in liquid YPAD until mid-log phase (0.3 OD) Cells were washed and suspended in 3 mL CE buffer (0.1 M sodium citrate, 10 mM EDTA, pH 6.25) in 13×100 mm glass tubes. Three microliters of Zymolyase at a concentration of 5 U μL^{-1} (Zymo Research) was added to each tube and mixed. Every 3 min the OD at 660 nM was measured using a Spectronic 20 spectrophotometer. The decrease of OD value was taken as cell lysis.

Effect of Congo red on cell growth: Sensitivity to Congo red assay has been described by Ram and Klis (2006). Congo red was dissolved in sterile water at 50 mg mL^{-1}. Fifty milliliters of YPAD agar buffered with 100 mM MES-NaOH pH 6.0 was melted and maintained at 50°C in a water bath. An appropriate aliquot of Congo red stock was added to the melted agar to 50 µg mL^{-1}. The Congo red containing agar was poured into an 8.5×12.5 cm sterile plastic plate. The agar was then allowed to solidify at room temperature. Cells were adjusted to 5×10^6 cells mL^{-1}. The stock was further serially diluted by 10 fold four times. Ten microliters of each dilution was spotted onto the Congo red and control plates which were then incubated at 30°C for 48 h. Sensitivity to Congo red was determined by visually comparing colony density between wild type and mutant cells.

Cell wall chitin content: Calcofluor white binding to yeast cell walls was adapted from Pringle (1991). 2×10^7 exponential-phase yeast cells were centrifuged at 3,000 rpm and the pellet was washed once with water. The washed pellet was resuspended in 50 µg mL^{-1} Calcofluor white and incubated for 10 min at room temperature. The cells were again pelleted, washed twice with water and the final pellet was resuspended in water. Cells were observed using brightfield and fluorescent microscopy with an Olympus BX51 microscope. The fluorescence from Calcofluor white was filtered with a DAPI filter.

Growth curves: Cells diluted with YPAD to $OD_{600} \sim= 0.05$ were grown in YPAD liquid culture at 30°C. Growth curves were generated by measuring the absorbance at 600 nm every 1.5 h for 9 h and after 24 h.

RESULTS AND DISCUSSION

***Vrg4-2* mutant cells are hypersensitive to Zymolyase:** Zymolyase has a β-1,3-glucanase activity that hydrolyzes glucose polymers at the β-1,3-glucan linkages, as well as protease activity (Zlotnik *et al.*, 1984). Yeast cells treated with Zymolyase experience a gradual degradation of their cell walls to form cell wall deficient spheroplasts. Unlike intact cells that have lysis resistant cell walls, spheroplasts will undergo lysis in hypoosmotic medium. The sensitivity of the cell wall to Zymolyase will vary depending on the cell wall structure. Cell walls with altered structure (i.e., fewer mannan groups or mannan more accessible to hydrolysis) should be more susceptible to these lytic enzymes and will therefore more readily undergo lysis than normal walls. Cell lysis can be measured as a reduction in optical density at 660 nM and has been used as a sensitive assay for disruption of cell wall integrity by a number of laboratories (Herrero *et al.*, 2002; Lim *et al.*, 1995). As shown in Fig. 1, incubation of *vrg4-2* cells with Zymolyase results in a 25% reduction in absorbance after 3 min, a 50% reduction after 5 min and maximal reduction in absorbance (80%) after 20 min. Wild-type cells formed spheroplasts at a much slower rate, with only a 10% reduction in absorbance after 15 min and a 32% reduction after 30 min. The rate of lysis for *vrg4-2* cells is quite dramatic, not only in comparison to wild type, but even in comparison to published results for other cell wall deficient mutants such as gda1 (Herrero *et al.*, 2002).

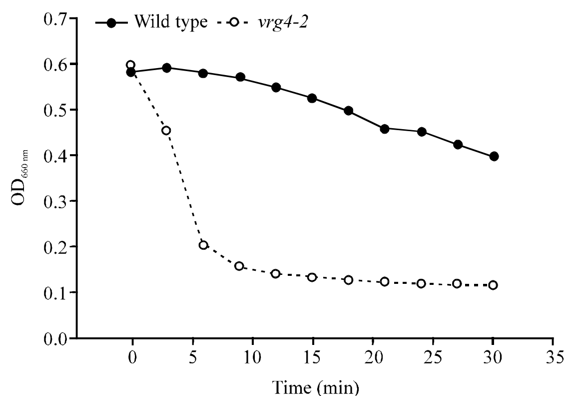

Fig. 1: Sensitivity of *vrg4-2* to Zymolyase. Wild-type and *vrg4-2* cells in midlog phase were incubated with Zymolyase. The optical density at 660 nm (OD_{660}) was measured every 3 min

Clearly *vrg4-2* cells rapidly form spheroplasts in the presence of Zymolyase. The results of this experiment do not identify the cell wall components that play a part in this process nor resolve which cell wall components have been altered or if there is increased accessibility of cell wall components to the lytic enzymes. However, these results, together with the known function of the gene, strongly suggest that hypomannosylation in the cell walls of *vrg4-2* mutants result in a weakened cell wall that is more susceptible to lysis.

Other manifestations of the differential structure of mutants versus wild type of *VRG4* have been published. An important study by Poster and Dean (1996) determined that *vrg4-2* mutant cells exhibit significant differences in the morphology of their internal membranes by both confocal and electron microscopy. Abnormal endomembrane structure would likely interfere with its normal role in the processing, transport and integration of glycoproteins into the cell wall. In the electron micrographs published by this group, one can also observe a difference in the appearance of the cell wall of mutant versus wild-type cells. The outer layer of the cell wall appears thicker in wild-type than in mutant cells. In addition, there is staining of some diffuse material outside of the cell walls of wild-type, but not mutant, cells.

***VRG4* mutant cells exhibit increased sensitivity to Congo red:** Congo red is an anionic dye that interferes with cell wall assembly, likely by binding to cell wall chitin (Imai *et al*., 2005; Ram and Klis, 2006). This binding prevents chitin molecules from forming cross-linkages with other cell wall components, thereby destabilizing the cell wall (Imai *et al*., 2005). Other strains of yeast with cell wall abnormalities exhibit increased levels of chitin in their cell walls (Popolo *et al*., 1997) and cell wall mutants have previously been characterized based on their sensitivity to Congo red (Ram and Klis, 2006).

When incubated with this dye, wild-type cells exhibit a very mild inhibition of growth at a density of 5×10^1 cells while *vrg4-2* cells exhibit inhibition of growth at a cell density of 5×10^3 cells with no growth of colonies at lower cell concentrations (Fig. 2). This sensitivity to Congo red would be expected if *vrg4-2* cells incorporate increased levels of chitin into the cell wall as a compensatory mechanism to mitigate the effects of hypoglycosylation.

***Vrg4-2* mutant cells exhibit increased levels of binding to Calcofluor white:** Calcofluor white is an anionic dye that binds to chitin in yeast cell walls (Ram and Klis, 2006). Although this dye is not specific for chitin, its binding has been used to visualize chitin content and localization in cell walls (Imai *et al*., 2005). In wild-type

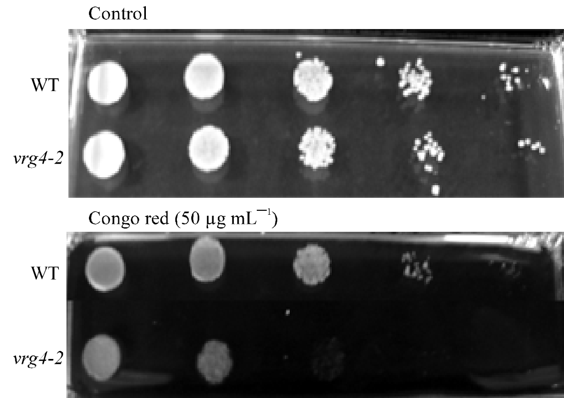

Fig. 2: Inhibition of *vrg4-2* growth in the presence of Congo red. *vrg4-2* and wild-type cells (5×10^6 cells mL^{-1}) were serially diluted (1:10) and plated (10 µL) on YPAD agar plates, with and without Congo red (50 µl mL^{-1}). Plates were incubated for 48 h at 30°C

cells, this dye binds significantly to bud necks, sites of bud emergence, bud scars and to a lesser extent, the lateral cell walls, as shown in Fig. 3A. Figure 3C illustrates that many of the *vrg4-2* cells exhibit an increase in fluorescence of the lateral cell walls, as compared to wild-type cells (Fig. 3A). As reported by Francois (2006), many cell wall defective mutants, such as *fks1*, *mnn9*, *gas1*, *kre6* and *krn4*, increase the chitin content of the cell wall, presumably to increase cross linking as compensation for quantitative and/or qualitative reductions in cell wall glycoproteins.

***Vrg4-2* growth approximates that of wild-type cells:** As shown in Fig. 4, *vrg4-2* cells exhibit a growth rate similar to that of wild type cells. Although we have shown that the *vrg4-2* cells have cell wall defects (Fig. 1, 2) it is interesting that their rate of growth remains unaffected. Normal growth rate in this type of mutant suggests that the most profound effects may be limited to cell wall structure, with relatively little effect on general cellular metabolism.

Indeed, in spite of the profound cell wall effects observed in this study, some significant functions dependent on cell wall structure seem to remain intact. One of the more important functions of the cell wall in yeasts involves mating. These *vrg4-2* mutants are mating type α, which produces a sex-specific cell wall glycoprotein called α-agglutinin. This molecule facilitates mating with the opposite mating type (**a** cells) by promoting adhesion to the corresponding **a**-agglutinin on the surface of **a** cells (Terrance *et al*., 1987). An increase in the concentration of these agglutinins at the cell surface is induced in each cell type under the influence of

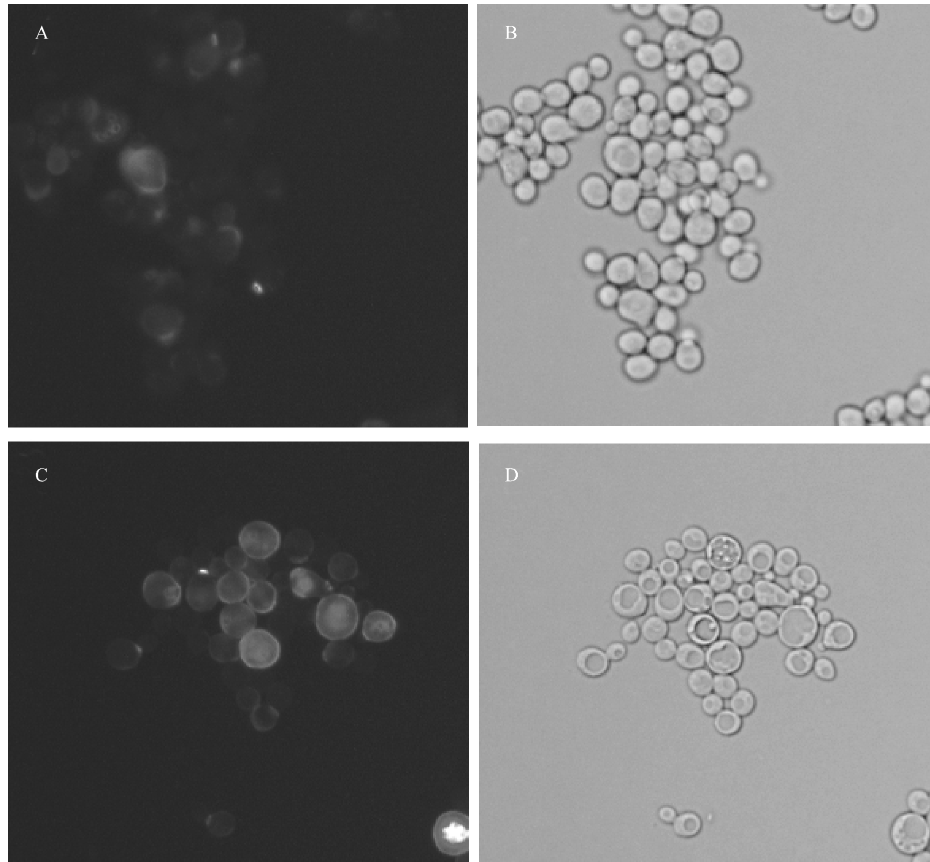

Fig. 3: Fluorescent staining of chitin by Calcofluor white in wild type and *vrg4-2* cells. Mutant and wild-type cells (2×10^7) were incubated with 50 μg mL^{-1} calcofluor white and visualized by fluorescent and brightfield microscopy. Fluorescent images of wild-type (A) and *vrg4-2* cells (C) are compared with brightfield images of wild type (B) and *vrg4-2* cells (D). All cells were viewed with a 60X oil immersion lens

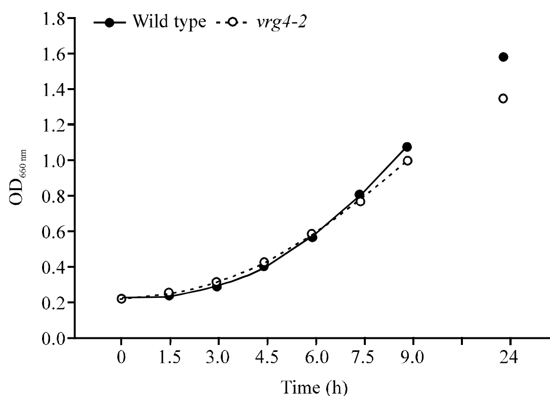

Fig. 4: Growth curve of *vrg4-2* cells. The growth curve of *vrg4-2* cells and wild-type cells in YPAD medium at 30°C

a sex pheromone produced by the opposite type (Terrance and Lipke, 1987). The resultant sex-specific adhesion increases the efficiency of mating. A fusion protein of α-agglutinin green fluorescence protein was constructed and its incorporation into the cell walls of the mutant and wild type cells was measured (Gonzalez and Lipke, 2003). There was no apparent difference in the amount of fluorescence incorporated into the mutant versus the wild type cells. Therefore, *VRG4* is not required for anchoring mannoproteins onto the cell wall.

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