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ORIGINAL PAPER

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Regulation of cell wall β -glucan assembly: *PTC1* Negatively affects *PBS2* Action in a pathway that includes modulation of *EXG1* transcription

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Abstract Analysis of genes involved in yeast cell wall β -glucan assembly has led to the isolation of *EXG1*, *PBS2* and *PTC1*. *EXG1* and *PBS2* were isolated as genes that, when expressed from multicopy plasmids, led to a dominant killer toxin-resistant phenotype. The *PTC1* gene was cloned by functional complementation of the calcofluor white-hypersensitive mutant *cwh47-1*. *PTC1/CWH47* is the structural gene for a type 2C serine/threonine phosphatase, *EXG1* codes for an exo- β -glucanase, and *PBS2* encodes a MAP kinase kinase in the Pbs2p-Hog1p signal transduction pathway. Overexpression of *EXG1* on a 2μ plasmid led to reduction in a cell wall β 1,6-glucan and caused killer resistance in wild type cells; while the *exg1 Δ* mutant displayed modest increases in killer sensitivity and β 1,6-glucan levels. Disruption of *PTC1/CWH47* and overexpression of *PBS2* gave rise to similar β -glucan related phenotypes, with higher levels of *EXG1* transcription, increased exo- β -glucanase activity, reduced β 1,6-glucan levels, and resistance to killer toxin. Genetic analysis revealed that loss of function of the *PBS2* gene was epistatic to *PTC1/CWH47* disruption, indicating a functional role for the Ptc1p/Cwh47p phosphatase in the Pbs2p-Hog1p signal transduction pathway. These results suggest that Ptc1p/Cwh47p and Pbs2p play opposing regulatory roles in cell wall glucan assembly, and that this is effected in part by modulating Exg1p activity.

Key word Cell wall · Killer toxin resistance
glucanase · Protein kinase · Protein phosphatase

Introduction

β -Glucans, homopolymers of glucose, are the main structural components responsible for the shape and rigidity of the yeast cell wall (Fleet and Phaff 1981). Based upon their chemical linkage characteristics, β -glucans can be subdivided into two distinct polymer types: β 1,3-glucan and β 1,6-glucan. The β 1,3-glucan is the more abundant component, containing approximately 1500 glucose residues per molecule. It consists of a predominantly 1,3-linked linear molecule with approximately 3% 1,6-linkages at branchpoints (Manners et al. 1973a). The β 1,6-glucan is a smaller and highly branched molecule comprised largely of 1,6-linked residues with a small proportion of 1,3-linked residues. The average size of β 1,6-glucan is approximately 140–200 residues per molecule (Boone et al. 1990; Manners et al. 1973b).

Studies on K1 killer toxin-resistant mutants have led to the identification and molecular characterization of several *KRE* (killer resistant) genes required for the synthesis of β 1,6-glucan. *KRE5* encodes a potential endoplasmic reticulum (ER) protein essential for β 1,6-glucan synthesis (Meaden et al. 1990). *KRE6* and *SKN1* are a pair of functional homologs coding for type II integral membrane proteins located in the Golgi apparatus (Roemer and Bussey 1991; Roemer et al. 1993, 1994). *KRE11* codes for a 63 kDa cytoplasmic protein (Brown et al. 1993). *KRE1* encodes a serine/threonine-rich protein, which is probably anchored to the plasma membrane by its hydrophobic C-terminal tail (Boone et al. 1990). *KRE9* is the structural gene for a small, serine/threonine-rich secretory O-glycoprotein (Brown and Bussey 1993). Null mutations in these genes disrupt the normal synthesis of β 1,6-glucan in various ways leading to reduced levels of the polymer and a killer-resistant phenotype. Based on the molecular and genetic analyses of these *kre* null mutants, it has been suggested that β 1,6-glucan is synthesized by a stepwise sequential process in the

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secretory pathway (Brown et al. 1993; Roemer et al. 1993). A few genes implicated in β 1,3-glucan synthesis have also been described. Recently, DiDomenico's group cloned the *KNR4* gene by functional complementation of a K9 killer toxin resistant mutant (Hong et al. 1994). The *KNR4* gene encodes a highly charged, acidic protein. Disruption of the gene led to reductions in both β 1,3-glucan synthase activity and cell wall β 1,3-glucan content, indicating that the *KNR4* gene might play a functional role in β 1,3-glucan synthesis. The Pkc1p-Mpk1p/Slt2p MAP kinase cascade has also been suggested to be involved in cell wall glucan assembly, because mutants in this kinase cascade display cell lysis phenotypes (Errede and Levin 1993; Torres et al. 1991), and because mutations in the *PKC1* gene result in reductions in both β 1,3-glucan and β 1,6-glucan levels in the cell wall (Roemer et al. 1994; Shimizu et al. 1994).

Saccharomyces cerevisiae produces several endo- and exo- β -glucanases during vegetative growth, and it has been hypothesized that these enzymes are involved in various morphogenetic events, such as cell expansion, budding, conjugation and sporulation, that require controlled and localized hydrolysis of cell wall β -glucans (Abd-el-al and Phaff 1968; Farkas et al. 1973; Fleet and Phaff 1981; Hien and Fleet 1983; Sanchez et al. 1982). To date, four glucanase genes, *EXG1*, *EXG2*, *BGL2* and *SPR1/SSG1*, have been cloned in *S. cerevisiae*. *EXG1* codes for an abundant, apparently non-specific, exo- β -glucanase that is active in vitro on both β 1,3 and β 1,6-glucans (Nebreda et al. 1986; Vazquez de Aldana et al. 1991). *EXG2* codes for a minor exo- β -glucanase homologous to Exg1p (Nebreda et al. 1986; San Segundo et al. 1993). The *BGL2* gene product is an endo- β 1,3-glucanase specific for internal β 1,3-linkages (Klebl and Tanner 1989; Mrsa et al. 1993). *SPR1/SSG1* encodes a sporulation-specific exo- β -glucanase sharing extensive sequence homology with *EXG1* (Muthukumar et al. 1993; San Segundo et al. 1993). Possibly because of the redundant nature of these and other yeast glucanases, deletion analyses of glucanase genes have failed to give rise to obvious cell wall defects. Therefore, the physiological roles of glucanases in cell wall β -glucan metabolism remain unclear.

To search for new genes involved in cell wall assembly, Ram et al. (1994) carried out a genetic screen for mutants hypersensitive to calcofluor white, a drug which interferes with the extracellular assembly of the cell wall components. A total of 53 calcofluor white hypersensitive complementation groups were isolated, which included both low-mannan and low-glucan mutants. Results from secondary screenings revealed that three of the low-glucan mutants (*cwh41-1*, *cwh47-1* and *cwh48-1*) were also more resistant to K1 killer toxin than the wild-type strain, suggesting that these mutants had defects in the synthesis of β 1,6-glucan. Indeed, genetic complementation analysis revealed that *cwh48-1* was allelic to *KRE6*, which encodes a putative β 1,6-

glucan synthase component (Ram et al. 1994; Roemer and Bussey 1991). Since *cwh41-1* and *cwh47-1* were not complemented by any of the known *kre* mutants, they represent new genes possibly involved in cell wall β 1,6-glucan assembly. In this study, we report the cloning of the *CWH47* gene and the characterization of its functional role in glucan metabolism. In addition, we report the isolation of genes that, when overproduced, lead to a dominant killer-resistant phenotype.

Materials and methods

Yeast and bacterial strains and growth media

The *Saccharomyces cerevisiae* strains used are listed in Table 1. Growth conditions and media for yeast were as described (Bussey et al. 1982). Standard procedures were used for genetic crosses, sporulation of diploids and dissection of tetrads (Sherman et al. 1982). Yeast transformations were made by the lithium acetate method of Ito et al. (1983). Killer survival assays for killer toxin sensitivity were performed as described previously (Hutchins and Bussey 1983). *Escherichia coli* strain XL1-Blue was used for the propagation of all plasmids. LB and 2YT media were used for bacterial culture (Sambrook et al. 1989).

Plasmids

A pRS316-based yeast genomic DNA library (provided by Dr. C. Boone, Simon Fraser University, BC, Canada) was used to clone the *CWH47* gene. The *EXG1* and *PBS2* genes were isolated from a YE24-based yeast genomic DNA library. The centromeric vector pRS316 was used to subclone the *CWH47* gene, and the 2 μ -based plasmids YE351 and YE24 were used for subcloning and overexpression of *EXG1* and *PBS2* genes. pBluescript II vectors were used for recombinant DNA constructions. Plasmid 2 μ -EXG1 contains the *EXG1* gene on a 3.6 kb *HindIII* fragment inserted in the *HindIII* site of YE351. Plasmid 2 μ -PBS2 contains the *PBS2* gene on a 2.9 kb *SpeI-SacI* fragment cloned into the *XbaI-SacI* sites of YE351.

Selection for killer resistance-inducing genes

The selection was performed by using a 2 μ plasmid-based yeast genomic DNA library, as genes cloned in a 2 μ plasmid are maintained at high copy number, and this can often lead to elevated levels of the gene product (Rine 1991; Rine et al. 1983). After transforming the wild-type strain *SEY6210* with a YE24-based library, a total of 30000 transformants were washed from Petri dishes, and collected into 6 independent pools. About 10^7 cells from each pool were treated with killer toxin, plated out and incubated at 18°C for 4 to 7 days (Brown et al. 1993). After incubation, growing colonies of different sizes were picked and quantitatively checked for killer resistance by measuring cell survival after killer toxin treatment for 3 h. Plasmids from the killer resistant transformants were extracted, amplified in *E. coli*, and re-transformed into *SEY6210*. Plasmids that conferred killer resistance on re-transformation were analyzed further by restriction mapping, subcloning and DNA sequencing.

DNA purification and recombinant DNA techniques.

Yeast DNA was isolated by the procedure of Hoffman and Winston (1987). Plasmid DNA was prepared from *E. coli* as described by

Table 1 Yeast strains used in this study

Strain	Genotype	Source
AR27	<i>MATα ura3-52</i>	Ram et al. (1994)
AR49	<i>MATα lys2</i>	Ram et al. (1994)
ARC14	<i>MATα cwh47-1 ura3-52 lys2</i>	Ram et al. (1994)
SEY6210	<i>MATα leu2-3,112 ura3-52 his3-Δ200 lys2-801 trp1-Δ901 suc2-Δ9</i>	S.D. Emr
HAB251-15B	SEY6210 autodiploid	Roemer and Bussey (1991)
HAB635	<i>MATα kre1Δ::HIS3</i> in SEY6210	Boone et al. (1990)
TR92	<i>MATα kre6Δ::HIS3</i> in SEY6210	Roemer and Bussey (1991)
HAB806	<i>MATα kre11Δ::URA3</i> in SEY6210	Brown et al. (1993)
HAB851	<i>MATα/MATα ptc1Δ::URA3/PTC1</i> in HAB251-15B	This study
HAB852	<i>MATα ptc1Δ::URA3</i> in SEY6210	This study
HAB853	<i>MATα exg1Δ::HIS3</i> in SEY6210	This study
HAB854	<i>MATα exg1Δ::HIS3 ptc1Δ::URA3</i> in SEY6210	This study
YPH499	<i>MATα leu2 ura3 his3 lys2 trp1 ade2</i>	M.C. Gustin
JBV10	<i>MATα hog1-Δ1::TRP3</i> in YPH499	M.C. Gustin
MAY1	<i>MATα pbs2-Δ1::LEU2</i> in YPH499	M.C. Gustin
YBJ1	<i>MATα ptc1Δ::URA3</i> in YPH499	This study
YBJ2	<i>MATα ptc1Δ::URA3 pbs2Δ1::LEU2</i> in YPH499	This study

Sambrook et al. (1989). DNA sequence was determined by the dideoxy-chain termination method of Sanger et al. (1977) using the Sequenase kit (U.S. Biochemicals, Cleveland, Ohio) with [α -³⁵S]dATP as a substrate (Amersham Canada, Oakville, Ont.). Restriction endonucleases, Klenow and T4 DNA polymerases, shrimp alkaline phosphatase and T4 DNA ligase were purchased from Bethesda Research Laboratories Inc. (Gaithersburg, Md.), Pharmacia LKB Biotechnology (Piscataway, N.J.), Boehringer Mannheim Biochemicals (Indianapolis, Ind.), Stratagene Cloning Systems (La Jolla, Calif.), or U.S. Biochemicals (Cleveland, Ohio) and were used according to the instructions of the manufacturers. Radioactive probes for DNA-DNA and RNA-DNA hybridizations were labelled with [α -³²P]dCTP (Amersham Canada) using the Oligolabelling kit from Pharmacia.

Gene disruption

Deletion disruption mutants were constructed by the method of Rothstein (1983). A 727 bp *Sna*BI-*Bam*HI fragment was deleted from the *PTC1/CWH47* gene and replaced with a 1.2 kb *Bam*HI fragment containing the *URA3* gene, thereby deleting 86% of the coding region. After digestion by *Hind*III, the DNA fragment carrying the *ptc1 Δ* deletion construct was gel-purified and used to transform a wild-type diploid strain (HAB251-15B) by selecting for uracil prototrophy. The same *ptc1 Δ* deletion construct was also used to transform the YPH499 and MAY1 (*pbs2 Δ*) haploid strains directly to create the isogenic *ptc1 Δ* single and *pbs2 Δ ptc1 Δ* double mutants. Southern blot analysis (Southern 1975) of genomic DNA from the resulting transformants indicated that disruptions were at the *PTC1/CWH47* locus (data not shown). Similarly, the *exg1 Δ* deletion mutant was created by replacing an internal 138 bp *Eco*RI fragment of the coding region with a 1.8 kb *Eco*RI fragment containing the *HIS3* gene.

RNA analysis

Total RNA was isolated from exponentially growing cells (at an OD₆₀₀ of 0.2–0.3) in YNB medium by a glass-bead lysis method (Rose et al. 1990). After fractionation on a 1% agarose gel in 20 mM MOPS (morpholinepropanesulfonic acid), 5 mM sodium acetate,

1 mM EDTA, 0.66 M formaldehyde, the RNA samples were transferred onto a Nytran filter and hybridized with [α -³²P]dCTP-labelled probes (Fourney et al. 1988). A 1.1 kb *Xho*I-*Xba*I DNA fragment containing the *EXG1* coding region was used to detect the *EXG1* transcript, and a 0.6 kb *Bgl*II-*Xho*I DNA fragment from the *L29* ribosomal protein gene (provided by Dr. P. Belhumeur, Université de Montréal, PQ, Canada) was used as an internal control for the amount of mRNA loaded in each lane (Kaufman et al. 1983). Autoradiograms were scanned with a LKB Ultrascan XL Laser Densitometer.

Cell extract preparation and exo- β -glucanase assay

Cell extracts were prepared from cultures in exponential growth phase (OD₆₀₀ $\approx$ 0.4), and used to determine the cell-associated exo- β -glucanase activity as described by Santos et al. (1979). Briefly, samples were incubated with *p*-nitrophenyl- β -D-glucoside (*p*-NPG, 0.25% final concentration) in 1 ml of 50 mM sodium acetate buffer (pH 5.2) at 37°C for 1 h and the reaction stopped by putting the tubes in boiling water for 3 min. After removing the protein precipitates by centrifugation, the concentration of released *p*-nitrophenol was measured by adding 0.9 ml of 4% Na₂CO₃ to 0.1 ml of the supernatant and reading the optical density at 415 nm. Protein concentration was determined using the BioRad Protein Assay with bovine serum albumin as a standard.

Analyses of cell wall glucans and intracellular glycerol

Alkali-insoluble glucans were isolated from stationary-phase yeast cells or isolated cell walls. After β 1,3-glucanase (zymolyase; ICN Pharmaceuticals, Irvine, Calif.) digestion and dialysis, the β 1,6-glucan was collected and quantified as described by Boone et al. (1990). Total glucan (β 1,3- plus β 1,6-glucan) was determined as the hexose content before dialysis (Roemer and Bussey 1991), and the β 1,3-glucan level was calculated by subtracting the β 1,6-glucan content from the total glucan level. To determine the intracellular glycerol level, cells in log phase (OD₆₀₀ $\approx$ 0.4) were collected and the glycerol levels were measured enzymatically as previously described (Blomberg and Adler 1989; Blomberg et al. 1988).

Results

Isolation of *EXG1* and *PBS2* as killer resistance-inducing genes

To identify additional genes involved in the regulation and synthesis of cell wall glucan, we carried out a selection for genes that, when overproduced, could cause killer resistance in a wild-type strain. The selection was performed by using a 2μ plasmid-based yeast genomic DNA library, as genes cloned in a 2μ plasmid are maintained at high copy number, and this can often lead to elevated levels of the gene product (Rine 1991; Rine et al. 1983). After screening approximately 30,000 colonies transformed with a YE24-based DNA library (see Materials and methods for details), we repeatedly recovered two different classes of plasmids from the killer resistant transformants. One class of plasmids (with 5 different members) contained a common 3.6 kb *HindIII* genomic DNA fragment sufficient to confer killer-resistance; while the other class (with 2 distinct isolates) shared a common 2.9 kb *SpeI-SacI* DNA fragment capable of causing killer resistance. Further restriction enzyme mapping and DNA sequence analyses showed that the 3.6 kb *HindIII* fragment contained *EXG1*, the structural gene for an extracellular exo- β -glucanase (Vazquez de Aldana et al. 1991); and the 2.9 kb *SpeI-SacI* fragment carried the *PBS2* gene, which codes for a protein kinase related to the MAP (mitogen-activated protein) kinase kinase family (Boguslawski and Polazzi 1987; Brewster et al. 1993).

Overproducing *EXG1* on a 2μ -based plasmid caused wild-type cells to become resistant to killer toxin with an approximately 40-fold increase in cell survival after toxin treatment. Similarly, a 2μ -*PBS2* plasmid led to an approximately 30-fold increase in survival of killer toxin treated cells (Table 2).

The *exg1Δ* and *pbs2Δ* mutants were hypersensitive to K1 killer toxin

As both *EXG1* and *PBS2* expressed from 2μ -based plasmids in a sensitive wild-type strain resulted in killer resistance, we examined the *exg1Δ* and *pbs2Δ* mutants to see if loss of function of the genes could also cause alterations in killer sensitivity. As shown in Table 2, both mutants were more sensitive to the toxin than the isogenic wild-type strain. Disruption of the *EXG1* gene caused an approximately 2-fold reduction in cell survival after killer toxin treatment, while the *pbs2Δ* mutant displayed a cell survival approximately 20 times lower than the wild-type level. Thus, disruption and overexpression of *EXG1*, or *PBS2*, had opposing effects on killer toxin sensitivity.

Table 2 Effects on K1 killer toxin survival

Strain	Genotype	Plasmid	Killer toxin survival (%)
SEY6210	WT	—	1.60 ± 0.21
SEY6210	WT	2μ -EXG1	61.60 ± 9.40
SEY6210	WT	2μ -PBS2	54.20 ± 4.20
HAB852	<i>ptc1Δ</i>	—	40.04 ± 3.63
HAB853	<i>exg1Δ</i>	—	0.82 ± 0.10
HAB852	<i>exg1Δ</i>	2μ -PBS2	38.60 ± 0.60
HAB854	<i>exg1Δ ptc1Δ</i>	—	25.10 ± 2.17
YPH499	WT	—	1.12 ± 0.26
MAY1	<i>pbs2Δ</i>	—	0.051 ± 0.009

Cloning of the *CWH47* gene

The *cwh47-1* mutant was isolated as a calcofluor white-hypersensitive mutant. Initial studies indicated that the *cwh47-1* allele displayed a higher cell wall mannose:glucose ratio ($1.39 \pm 0.02:1.00$) than the wild-type strain ($0.94 \pm 0.04:1.00$) and was resistant to K1 killer toxin; suggesting that the mutant had defects in β -glucan assembly (Ram et al. 1994). To further characterize the gene identified by the mutant, we cloned the *CWH47* gene by functional complementation of the calcofluor white-hypersensitive phenotype of *cwh47-1*. Five overlapping genomic fragments complementing the *cwh47-1* mutation were isolated from a yeast genomic DNA library. Restriction mapping and subcloning analyses localized the complementing activity to a 2.6 kb *HindIII* fragment. Nucleotide sequences derived from this DNA fragment were identical to the sequence of *PTC1*, a homolog of the mammalian protein serine/threonine phosphatase 2C (Maeda et al. 1993), and the restriction map of this 2.6 kb *HindIII* fragment also matched that of *PTC1*. We genetically mapped the *cwh47-1* locus to the same region as *ptc1*, which is tightly linked to *trp1* near the centromere of Chromosome IV (18 out of 18 tetrads were parental ditype for *cwh47-1* and *trp1* segregation). In addition, the *ptc1Δ* null mutant resembled the *cwh47-1* allele in displaying a calcofluor white hypersensitive phenotype (data not shown). To determine directly if *cwh47-1* was allelic to *ptc1*, we crossed the *ptc1Δ::URA3* mutant with the original *cwh47-1* allele and analyzed meiotic tetrads from the resulting diploid strain. Of the 10 tetrads tested, all 4 spores from each tetrad were calcofluor white-hypersensitive, with the *URA3* marker segregating 2:2. In addition, the diploid strain displayed hypersensitivity to calcofluor white. These results demonstrate that the *ptc1Δ* mutation is tightly linked to the *cwh47-1* locus, and fails to complement the calcofluor white hypersensitive phenotype caused by the *cwh47-1* mutation. These data indicate that *CWH47* is *PTC1*, and suggest a functional role for the *PTC1* phosphatase in yeast cell wall assembly. We will refer to this gene as *PTC1/CWH47* for the remainder of this paper.

Disruption of *PTC1/CWH47* results in killer toxin resistance

To test if the *PTC1/CWH47* gene is involved in cell wall assembly, we examined the *ptc1Δ* null mutant for glucan-related phenotypes. Like the original *cwh47-1* allele, the null mutant was more resistant to K1 killer toxin than the wild-type strain, with an approximately 25-fold increase in cell survival after toxin treatment (Table 2).

Epistasis analysis of the *Ptc1p/Cwh47p* phosphatase with the *Pbs2p* kinase

The observation that the *Ptc1p/Cwh47p* phosphatase and the *Pbs2p* kinase had opposing effects on killer sensitivity raised the possibility that they functionally interacted with each other. To examine this conjecture genetically, we made an epistasis analysis between the *PBS2* and *PTC1/CWH47* genes by comparing the killer phenotypes of *ptc1Δ*, *pbs2Δ* single mutants and a *ptc1Δ pbs2Δ* double mutant (Table 3). While the *ptc1Δ* single mutant was killer resistant, the *ptc1Δ pbs2Δ* double mutant became hypersensitive to killer toxin, a phenotype observed with the *pbs2Δ* single mutant. These results demonstrate that the loss of function of the *Pbs2p* kinase is epistatic to the loss of function of the *Ptc1p/Cwh47p* phosphatase.

Pbs2p is a member of the *Pbs2p-Hog1p* MAP kinase signal transduction cascade involved in osmoregulation, and activation of the *Pbs2p-Hog1p* cascade by increased extracellular osmolarity results in intracellular glycerol accumulation (Brewster et al. 1993). We observed that, in standard YEPD medium, activation of the *Pbs2p-Hog1p* cascade by *PBS2* overexpression led to an approximately 40% increase in glycerol content (from 8.35 ± 0.40 to 11.86 ± 0.35 nmole per 10^7 cells). Thus the intracellular glycerol level can be used as an indicator for the activity of the cascade. To independently check that disruption of the *PTC1/CWH47* gene could alter the activity of the *Pbs2p-Hog1p* kinase cascade, we measured the intracellular glycerol levels of various strains grown in standard YEPD medium (Table 3). Under this low-osmolarity condition, the *pbs2Δ* strain had a similar

intracellular glycerol content to the wild type strain. However, the glycerol content in a *ptc1Δ* null mutant increased about 50% over the wild-type level, indicating that the *Pbs2p-Hog1p* cascade was activated by the *PTC1/CWH47* disruption. The elevated glycerol content caused by the *ptc1Δ* mutation could be suppressed by loss of function of *PBS2* gene, since the *pbs2Δ ptc1Δ* double mutant displayed a wild-type glycerol level. This, again, demonstrated that *PBS2* was epistatic to *PTC1/CWH47*.

Effects of *EXG1*, *PBS2* and *PTC1/CWH47* genes on cell wall β -glucan assembly

K1 killer toxin uses β 1,6-glucan as a component of its cell wall receptor, and changes in killer sensitivity often reflect alterations in the structure and/or level of cell wall β 1,6-glucan (Bussey 1991). To determine if changes in *Exg1p*, *Pbs2p* or *Ptc1p/Cwh47p* activities also caused alterations in cell wall glucans, we examined the levels of β 1,6-glucan and β 1,3-glucan isolated from these various strains (Fig. 1). Consistent with the killer resistant phenotype, *EXG1* overproduction, *PBS2* overexpression and *PTC1/CWH47* disruption all led to modest but reproducible reductions in cell wall β 1,6-glucan levels. In contrast, the *exg1Δ* and *pbs2Δ* mutants displayed slight increases in the β 1,6-glucan level. The levels of cell wall β 1,3-glucan were unaffected by these manipulations, with the exception of a small elevation of the β 1,3-glucan level in the *pbs2Δ* mutant.

PBS2 overexpression and *PTC1/CWH47* disruption both lead to *EXG1* activation

The fact that overproducing *PBS2* or deleting *PTC1/CWH47* resulted in β 1,6-glucan reductions indicated that the *Pbs2p-Hog1p* MAP kinase cascade played a functional role in cell surface assembly, most likely through the regulation of genes directly involved in cell wall β 1,6-glucan assembly. The *EXG1* gene might be a downstream gene activated by the *Pbs2p-Hog1p* pathway, because *EXG1* and *PBS2* were both identified in the same screen, and because overproduction of the *PBS2* gene or disruption of the

Table 3 Epistasis analysis between *pbs2Δ* and *ptc1Δ*

Strain	Genotype	Killer toxin survival (%)	Intracellular glycerol (nmole/ 10^7 cells)	Exo- β -glucanase ^a (U/mg protein)
YPH499	<i>WT</i>	1.12 ± 0.26	8.35 ± 0.40	0.420 ± 0.023
MAY1	<i>pbs2Δ</i>	0.051 ± 0.009	9.74 ± 0.26	0.304 ± 0.017
YBJ1	<i>ptc1Δ</i>	30.41 ± 4.78	12.83 ± 0.29	0.988 ± 0.037
YBJ2	<i>pbs2Δ ptc1Δ</i>	0.044 ± 0.009	8.72 ± 0.27	0.363 ± 0.019

^a One unit of activity is defined as the amount of enzyme that release 1 μ mole of *p*-nitrophenol per h at 37° C

Fig. 1A, B Cell wall β -glucan levels. Alkali-insoluble glucans were isolated from stationary-phase cells and quantified as previously described (Boone et al. 1990; Roemer and Bussey 1991). The *pbs2 Δ* and *hog1 Δ* (see Fig. 2) mutants were kindly provided by Dr. M. Gustin. Since they were constructed in the *YPH499* strain background, the glucan levels of the *pbs2 Δ* mutant were only compared with those of *YPH499*. Likewise, the glucan levels of the other mutant strains were compared only with their isogenic wild type strain *SEY6210*. **A**, β 1,6-glucan levels. **B**, β 1,3-glucan levels. The data represent the average results of at least three independent experiments. The error bars represent standard deviations. See Materials and methods for details.

PTC1/CWH47 gene resulted in phenotypes very similar to those seen on *EXG1* overexpression.

To explore this possibility, we tested if *2 μ -PBS2* plasmids or a *ptc1 Δ* mutation could lead to *EXG1* activation by examining the *EXG1* mRNA levels and the exo- β -glucanase activities. While both *pbs2 Δ* and *hog1 Δ* null mutants contained less *EXG1* transcript than the wild-type strain (Fig. 2, compare lane 1 with lanes 2 and 3), we observed elevated levels of the *EXG1* mRNA in wild-type strains harboring the *2 μ -PBS2* plasmid (Fig. 2, compare lanes 1 and 4, 6 and 7), as well

as in the *ptc1Δ* mutant (Fig. 2, compare lanes 6 and 8). But, *PBS2* overproduction in the *hog1Δ* mutant strain did not increase the *EXG1* mRNA level (Fig. 2, compare lanes 3 and 5), indicating that the observed *EXG1* activation was dependent on an intact Pbs2p-Hog1p kinase cascade. In parallel with the alterations in *EXG1* mRNA levels, *pbs2Δ* and *hog1Δ* displayed lower levels of exo- β -glucanase activity than the wild type cells (Fig. 3, compare lanes 1–3); and *PBS2* overexpression or *PTC1/CWH47* disruption resulted in increased levels of exo- β -glucanase activity (Fig. 3, compare lanes 1 and 4, 6–8). Together, these results indicate that the Pbs2p-Hog1p MAP kinase cascade plays a role in *EXG1* regulation.

As another test for genetic epistasis between *pbs2Δ* and *ptc1Δ* mutations, we examined the exo- β -glucanase activities in isogenic *pbs2Δ*, *ptc1Δ* and *pbs2Δ ptc1Δ* strains (Table 3). While the *ptc1Δ* single mutant showed an approximately 2-fold increase in exo- β -glucanase activity, the *pbs2Δ ptc1Δ* double mutant displayed an approximately 15% reduction in exo- β -glucanase activity similar to the activity observed in the *pbs2Δ* single mutant. These results provided further evidence indicating that the loss of function of the Pbs2p kinase is epistatic to the loss of function of the Ptc1p/Cwh47p phosphatase.

A null mutation in *EXG1* partially alleviates the killer-resistant phenotype caused by *PBS2* overexpression and *PTC1/CWH47* disruption

To further test if Exg1p was a downstream target regulated by both Pbs2p and Ptc1p/Cwh47p, we examined the effect of *EXG1* disruption on the killer-resistant phenotype caused by *ptc1Δ* null mutation and 2 μ -*PBS2* plasmids. As shown in Table 2, killer toxin survival of the *exg1Δ ptc1Δ* double mutant was lower than that of the *ptc1Δ* single mutant, though still significantly higher than the wild-type strain. Similarly, the *exg1Δ* strain harboring 2 μ -*PBS2* plasmids also became less resistant to killer toxin than the 2 μ -*PBS2* containing wild type

Fig. 3 Exo- β -glucanase activity. Cell extracts prepared from exponentially growing cultures were used to measure the exo- β -glucanase activities as described in Materials and methods. As controls for the exo- β -glucanase assay, exo- β -glucanase activities in the *EXG1* disruption mutants and an *EXG1* overproducing strain were measured (lanes 9–11). The data shown represent the average results of at least three independent experiments. The error bars represent standard deviations

strain. These results demonstrate that the loss of function of *EXG1* partially suppresses the killer resistant phenotype caused by *PTC1/CWH47* disruption and *PBS2* overexpression, and suggest that *EXG1* is a downstream target of Ptc1p/Cwh47p and Pbs2p. However, other targets must also contribute to the observed killer resistant phenotypes caused by *ptc1Δ* and 2 μ -*PBS2* plasmids, since these strains still retained considerable resistance to killer toxin in the absence of the *EXG1* gene.

Discussion

In this study we searched for additional genes involved in yeast cell wall β 1,6-glucan assembly. The *EXG1* and *PBS2* were isolated as genes that, when overexpressed from a 2 μ -based plasmid, resulted in killer resistance. The *PTC1/CWH47* gene was cloned by functional complementation of a novel killer-resistant mutant *cwh47-1* isolated from a screen for calcofluor white hypersensitive mutants (Ram et al. 1994).

EXG1 codes for an abundant exo- β -glucanase produced during vegetative growth, and has long been expected to have a function in cell wall β -glucan assembly (Nebreda et al. 1986). In this study, we showed that overexpressing the *EXG1* from a 2 μ plasmid resulted in

Fig. 2A, B *EXG1* transcript levels. **A** Northern blot analysis of *EXG1* mRNA. Total RNA samples isolated from exponentially growing cultures were analyzed by Northern blotting as described in Materials and methods. The blot was hybridized with ³²P-labelled probes specific for *EXG1* mRNA (upper panel) and the L29 ribosomal protein transcript (lower panel), which serves as a control for sample loading. The *EXG1* transcript of the *hog1Δ* mutant displayed a slightly higher mobility than that in the other strains. This is probably a gel running artifact, because the L29 transcript in this lane showed a similarly higher mobility. **B** Histogram of the *EXG1* transcript levels. The relative *EXG1* transcript levels were quantified by densitometric scanning of the autoradiograms and expressed as the ratios of *EXG1* to L29 signals. The data shown in the histogram represent the average results obtained by scanning several different autoradiograms derived from the same blot. The error bars represent standard deviations

killer resistance and a modest reduction in cell wall β 1,6-glucan. In contrast, disruption of the *EXG1* gene caused the mutant to become more sensitive to killer toxin, and led to a small increase in the β 1,6-glucan level. These results demonstrate that changes in the Exg1p activity can lead to alterations in cell wall β 1,6-glucan levels, thus providing experimental support for the expectation that the Exg1p exo- β -glucanase plays a functional role in cell wall glucan metabolism.

Although Exg1p is active in vitro on both laminarin and pustulan (linear β 1,3-glucan and β 1,6-glucan model substrates, respectively) (Nebreda et al. 1986), we found that overproducing or deleting the *EXG1* gene led to detectable in vivo alterations only in the β 1,6-glucan level. We suggest two possible explanations for this observation that are not mutually exclusive. Exg1p is an exo- β -glucanase, and since exo- β -glucanases hydrolyze glucan polymers from the non-reducing end, Exg1p would preferentially break down the smaller and highly branched β 1,6-glucan over the long linear β 1,3-glucan. Alternatively, one could hypothesize that Exg1p functions specifically as a β 1,6-glucanase on the in vivo cell wall glucans.

PBS2 is a component of the Pbs2p-Hog1p MAP kinase cascade involved in osmoregulation (Brewster et al. 1993), and *PTC1* encodes a protein phosphatase and was isolated as a synthetic lethal mutation in association with a lesion in the protein tyrosine phosphatase gene *PTP2* (Maeda et al. 1993). Both *PBS2* and *PTC1* are regulatory genes not previously implicated in cell wall assembly. In this study, we provide experimental evidence indicating that the Pbs2p kinase and Ptc1p phosphatase are both involved in the regulation of cell wall β 1,6-glucan assembly.

We showed that the Pbs2p kinase and Ptc1p/Cwh47p phosphatase play opposing roles in maintaining killer toxin sensitivity and β 1,6-glucan levels. Either *PBS2* overexpression or *PTC1/CWH47* disruption result in killer resistance and β 1,6-glucan reductions, while disruption of the *PBS2* gene led to a killer-hypersensitive phenotype and a slight increase in the β 1,6-glucan level. Although the reductions in β 1,6-glucan level caused by *PBS2* overexpression or *PTC1/CWH47* disruption were small (approximately 15% or 25% reductions, respectively), they could very likely be the basis for the observed killer-resistant phenotypes for two reasons. Firstly, it has been well documented that a modest reduction in the β 1,6-glucan level (e.g. an approximately 50% reduction in *kre6 Δ* , or in *kre11 Δ*) can result in a very strong killer-resistant phenotype (Brown et al. 1993; Roemer and Bussey 1991); secondly, a comparable β 1,6-glucan reduction (approximately 27%) caused by *EXG1* overexpression results in a similar killer-resistant phenotype. Unlike killer resistance, the killer-hypersensitive phenotype is not well characterized. Although slight increases in both β 1,6-glucan and β 1,3-glucan levels were detected in the *pbs2 Δ* mutant, the molecular basis for killer

hypersensitivity is still not clear. However, since reduced levels and/or altered structures of β 1,6-glucan can lead to killer resistance, one can also imagine that some changes in cell wall structure that retain a functional toxin receptor may lead to increased killer sensitivity.

Genetic epistasis analysis is a powerful tool to study interactions between genes within a regulatory hierarchy, even without a detailed knowledge of the gene products (Avery and Wasserman 1992). To analyze possible interactions between *PBS2* and *PTC1/CWH47*, we examined genetic epistasis by comparing the phenotypes displayed by the single mutants and the double mutant. We found that the *pbs2 Δ ptc1 Δ* double mutant displayed the same phenotypes as the *pbs2 Δ* single mutant: while the *ptc1 Δ* single mutant showed killer resistance and elevated Exg1p glucanase activity, both the *pbs2 Δ ptc1 Δ* double mutant and the *pbs2 Δ* single mutant displayed hypersensitivity to killer toxin and reduced levels of Exg1p glucanase activity. These results demonstrate that the *pbs2 Δ* mutation is epistatic to the *ptc1 Δ* mutation. In addition, we showed that disruption of *PTC1/CWH47* resulted in activation of the Pbs2p-Hog1p cascade, as indicated by an increase in the intracellular glycerol level. Together, these results provide genetic evidence suggesting that the Ptc1p phosphatase negatively regulates the Pbs2p-Hog1p MAP kinase cascade. Interestingly, the same relationship between Ptc1p and Pbs2p-Hog1p kinase cascade has recently been independently proposed (Maeda et al. 1994). In their study, these authors reported that the Pbs2p-Hog1p MAP kinase cascade was regulated by a Sln1p-Ssk1p two-component system, and that disruption of *SLN1* resulted in constitutive activation of the MAP kinase cascade and lethality. Based on the observations that overexpression of the *PTC1* gene, as well as the disruption of *PBS2* or *HOG1* could all suppress the lethal phenotype caused by a *sln1 Δ* null mutation, they proposed that Ptc1p may inactivate the Pbs2p-Hog1p MAP kinase cascade by dephosphorylating Pbs2p and/or Hog1p.

Recently, another yeast MAP kinase cascade, the Pkc1p-Mpk1p/Slt2p cascade, has been implicated in the regulation of the Bgl2p glucanase activity. Shimizu et al. (1994) reported the characterization of *hpo2*, which was a novel allele of the *PKC1* gene. They observed a 2- to 3-fold increase in the level of the Bgl2p endo- β -glucanase in the *hpo2/pkc1* mutant, and demonstrated that disruption of the *BGL2* gene partially alleviated the hypo-osmolarity-sensitive growth defect of the *hpo2* mutant. Based on these phenotypes, Shimizu et al. (1994) suggested that the Pkc1p kinase cascade negatively regulates the production of the Bgl2p endo- β -glucanase. In this study, we provide evidence suggesting that the Pbs2p-Hog1p MAP kinase cascade plays a role in Exg1p glucanase regulation. We show that disruption of the *PBS2* or *HOG1* gene slightly decreased the *EXG1* mRNA level and

Fig. 4 A working model of interactions among the Ptc1p/Cwh47p phosphatase, Pbs2p-Hog1p kinase cascade, Exg1p glucanase, and β 1,6-glucan. The Pbs2p-Hog1p kinase cascade is shown in the box. $\rightarrow$ indicates positive activation, and $\vdash$ indicates negative regulation. See text for details

exo- β -glucanase activity, while *PBS2* overproduction or *PTC1/CWH47* disruption caused an approximately 2-fold activation in *EXG1* transcription and a 2 to 3-fold increase in exo- β -glucanase activity. In addition, we demonstrate that disruption of the *EXG1* gene partially suppressed the killer resistant phenotype caused by *PBS2* overproduction or *PTC1* disruption, indicating the observed cell wall defects could be attributed partially to the elevated Exg1p exo- β -glucanase activity. The fact that the *exg1Δ* mutation did not completely suppress the killer-resistant phenotype strongly suggests that the Pbs2p-Hog1p MAP kinase cascade also regulates other genes involved in the cell wall assembly, possibly other glucanases or genes required for glucan biosynthesis.

As a summary of our results, we present a working model relating functions of the Pbs2p-Hog1p pathway, the Ptc1p/Cwh47p phosphatase and the Exg1p glucanase in cell wall β 1,6-glucan metabolism (Fig. 4). This model is based largely on analyses of cell wall related phenotypes. Although some of these phenotypes were relatively modest, the alterations in *EXG1* gene transcription, exo- β -glucanase activity, sensitivity to K1 killer toxin, cell wall polymer levels, as well as studies on various genetic interactions, were all consistent with the proposed model. However, we cannot yet formally distinguish if *EXG1* expression is directly regulated by a transcription factor component of the Pbs2p-Hog1p MAP kinase cascade, or is controlled indirectly by some other downstream target of the cascade. In addition, we stress that it is very likely that the Pbs2p-Hog1p cascade also regulates other genes involved in cell wall glucan assembly, such as other

glucanases or genes required for glucan biosynthesis. Further investigations to identify the putative transcription factor involved in *EXG1* regulation, and to search for additional downstream targets of the Pbs2p-Hog1p cascade should provide more insights into the regulation of cell wall assembly.

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