Regulation of cell wall beta-glucan assembly: PTC1 negatively affects PBS2 action in a pathway that includes modulation of EXG1 transcription

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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 β1,6-glucan and caused killer resistance in wild type cells; while the exg1Δ mutant displayed modest increases in killer sensitivity and β1,6-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 β1,6-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: β1,3-glucan and β1,6-glucan. The β1,3-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 β1,6-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 β1,6-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 β1,6-glucan. KRE5 encodes a potential endoplasmic reticulum (ER) protein essential for β1,6-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 β1,6-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 β1,6-glucan is synthesized by a stepwise sequential process in the
secretory pathway (Brown et al. 1993; Roemer et al. 1993). A few genes implicated in β1,3-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 β1,3-glucan synthase activity and cell wall β1,3-glucan content, indicating that the KNR4 gene might play a functional role in β1,3-glucan synthesis. The Pkc1p-Mpk1p/Sltp 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 PKCl gene result in reductions in both β1,3-glucan and β1,6-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 β1,3 and β1,6-glucans (Nebreda et al. 1986; Vazquez de Aldana et al. 1991). EXG2 codes for a minor exo-β-glucanase homologous to Exgp (Nebreda et al. 1986; San Segundo et al. 1993). The BGL2 gene product is an endo-β1,3-glucanase specific for internal β1,3-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 β1,6-glucan. Indeed, genetic complementation analysis revealed that cwh48-1 was allelic to KRE6, which encodes a putative β1,6-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 β1,6-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 YEpl2-based yeast genomic DNA library. The centromeric vector pRS316 was used to subclone the CWH47 gene, and the 2μ-based plasmids YEp351 and YEpl2 were used for subcloning and overexpression of EXG1 and PBS2 genes. pBluescript II vectors were used for recombinant DNA constructions. Plasmid 2μ-EXG1 contains the EXG1 gene on a 3.6 kb HindIII fragment inserted in the HindIII site of YEp351. Plasmid 2μ-PBS2 contains the PBS2 gene on a 2.9 kb SpeI-SacI fragment cloned into the Xbal-SacI sites of YEp351.

Selection for killer resistance-inducing genes

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 transforming the wild-type strain SEY6210 with a YEpl2-based library, a total of 30000 transformants were washed from Petri dishes, and collected into 6 independent pools. About 10⁷ 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
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-32P]dCTP 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.), 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 Rothenstein (1983). A 727 bp SnaBI-BamH1 fragment was deleted from the PTC1/CWH47 gene and replaced with a 1.2 kb BamH1 fragment containing the URA3 gene, thereby deleting 86% of the coding region. After digestion by HindIII, the DNA fragment carrying the ptc1A deletion construct was gel-purified and used to transform a wild-type diploid strain (HAB251-15B) by selecting for uracil prototrophy. The same ptc1A deletion construct was used to transform the YPH499 and MAY1 strain, and the resulting transformants indicated that disruptions were at the PTC1/CWH47 locus (data not shown). Similarly, the exg1A deletion mutant was created by replacing an internal 138 bp EcoRI fragment of the coding region with a 1.8 kb EcoRI fragment containing the HIS3 gene.

RNA analysis

Total RNA was isolated from exponentially growing cells (at an OD600 ~ 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 XhoI-XbaI DNA fragment containing the EXG1 coding region was used to detect the EXG1 transcript, and a 0.6 kb BgII-XhoI 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 (Kaufner 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 ~ 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 β1,3-glucanase (zymolyase; ICN Pharmaceuticals, Irvine, Calif.) digestion and dialysis, the β1,6-glucan was collected and quantified as described by Boone et al. (1990). Total glucan (β1,3- plus β1,6-glucan) was determined as the hexose content before dialysis (Roemer and Bussey 1991), and the β1,3-glucan level was calculated by subtracting the β1,6-glucan content from the total glucan level. To determine the intracellular glycerol level, cells in log phase (OD600 ~ 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 *EXGI* 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 YEp24-based DNA library (see Materials and methods for details), we repeatedly isolated and overexpression (with 5 different members) contained a common 3.6 kb type level. Thus, disruption and overexpression of *pbs2A* (see Materials and methods for details), we repeatedly lead to elevated levels of the gene product (Rine 1991; maintained at high copy number, and this can often DNA library, as genes cloned in a 2μ plasmid are 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 Sphl-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 *EXGI*, the structural gene for an extracellular exo-β-glucanase (Vazquez de Aldana et al. 1991); and the 2.9 kb Sphl-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 *EXGI* 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 *exglA* and *pbs2A* mutants were hypersensitive to K1 killer toxin

As both *EXGI* and *PBS2* expressed from 2μ-based plasmids in a sensitive wild-type strain resulted in killer resistance, we examined the *exglA* and *pbs2A* 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 *EXGI* gene caused an approximately 2-fold reduction in cell survival after killer toxin treatment, while the *pbs2A* mutant displayed a cell survival approximately 20 times lower than the wild-type level. Thus, disruption and overexpression of *EXGI*, or *PBS2*, had opposing effects on killer toxin sensitivity.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Plasmid</th>
<th>Killer toxin survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEY6210</td>
<td>WT</td>
<td></td>
<td>1.60 ± 0.21</td>
</tr>
<tr>
<td>SEY6210</td>
<td>WT</td>
<td>2μ-EXGI</td>
<td>61.60 ± 9.40</td>
</tr>
<tr>
<td>SEY6210</td>
<td>WT</td>
<td>2μ-PBS2</td>
<td>54.20 ± 4.20</td>
</tr>
<tr>
<td>HAB852</td>
<td>ptc1Δ</td>
<td></td>
<td>40.04 ± 3.63</td>
</tr>
<tr>
<td>HAB853</td>
<td>exglA</td>
<td></td>
<td>0.82 ± 0.10</td>
</tr>
<tr>
<td>HAB852</td>
<td>exglA</td>
<td>2μ-PBS2</td>
<td>38.60 ± 0.60</td>
</tr>
<tr>
<td>HAB854</td>
<td>exglA ptc1Δ</td>
<td></td>
<td>25.10 ± 2.17</td>
</tr>
<tr>
<td>YPH499</td>
<td>WT</td>
<td></td>
<td>1.12 ± 0.26</td>
</tr>
<tr>
<td>MAY1</td>
<td>pbs2A</td>
<td></td>
<td>0.051 ± 0.009</td>
</tr>
</tbody>
</table>

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 ± 0.02:1.00) than the wild-type strain (0.94 ± 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 *trpl* near the centromere of Chromosome IV (18 out of 18 tetrads were parental ditype for *cwh47-1* and *trpl* 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 parental ditype for *cwh47-1* and *trpl* segregation. 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 glucan-related assembly, we examined the \( ptc1\Delta \) null mutant for glucan-related phenotypes. Unlike 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 \( ptc1\Delta \), \( pbs2\Delta \) single mutants and a \( ptc1\Delta \) \( pbs2\Delta \) double mutant (Table 3). While the \( ptc1\Delta \) single mutant was killer resistant, the \( ptc1\Delta \) \( pbs2\Delta \) double mutant became hypersensitive to killer toxin, a phenotype observed with the \( pbs2\Delta \) single mutant. These results demonstrate that the loss of function of the \( Pbs2p \) kinase is epistatic to the loss of function of the \( Ptclp/Cwh47p \) phosphatase.

\( Pbs2p \) is a member of the \( Pbs2p\)-Hoglp MAP kinase signal transduction cascade involved in osmoregulation, and activation of the \( Pbs2p\)-Hoglp casde by increased extracellular osmolarity results in intracellular glycerol accumulation (Brewster et al. 1993). We observed that, in standard YEPD medium, activation of the \( Pbs2p\)-Hoglp 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 casde. To independently check that disruption of the \( PTC1/CWH47 \) gene could alter the activity of the \( Pbs2p\)-Hoglp casde, we measured the intracellular glycerol levels of various strains grown in standard YEPD medium (Table 3). Under this low-osmolarity condition, the \( pbs2\Delta \) strain had a similar intracellular glycerol content to the wild type strain. However, the glycerol content in a \( ptc1\Delta \) null mutant increased about 50% over the wild-type level, indicating that the \( Pbs2p\)-Hoglp casde was activated by the \( PTC1/CWH47 \) disruption. The elevated glycerol content caused by the \( ptc1\Delta \) mutation could be suppressed by loss of function of \( PBS2 \) gene, since the \( pbs2\Delta \) \( ptc1\Delta \) double mutant displayed a wild-type glycerol level. This, again, demonstrated that \( PBS2 \) was epistatic to \( PTC1/CWH47 \).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Killer toxin survival (%)</th>
<th>Intracellular glycerol (nmole/10^7 cells)</th>
<th>Exo-β-glucanase^a (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YPH499</td>
<td>WT</td>
<td>1.12 ± 0.26</td>
<td>8.35 ± 0.40</td>
<td>0.420 ± 0.023</td>
</tr>
<tr>
<td>MAY1</td>
<td>( pbs2\Delta )</td>
<td>0.051 ± 0.009</td>
<td>9.74 ± 0.26</td>
<td>0.304 ± 0.017</td>
</tr>
<tr>
<td>YBJ1</td>
<td>( ptc1\Delta )</td>
<td>30.41 ± 4.78</td>
<td>12.83 ± 0.29</td>
<td>0.988 ± 0.037</td>
</tr>
<tr>
<td>YBJ2</td>
<td>( pbs2\Delta \ ptcl\Delta )</td>
<td>0.044 ± 0.009</td>
<td>8.72 ± 0.27</td>
<td>0.363 ± 0.019</td>
</tr>
</tbody>
</table>

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

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 β1,6-glucan (Bussey 1991). To determine if changes in \( EXG1 \), \( PBS2 \) or \( Ptc1p/Cwh47p \) activities also caused alterations in cell wall glucans, we examined the levels of β1,6-glucan and β1,3-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 β1,6-glucan levels. In contrast, the \( exgl\Delta \) and \( pbs2\Delta \) mutants displayed slight increases in the β1,6-glucan level. The levels of cell wall β1,3-glucan were unaffected by these manipulations, with the exception of a small elevation of the β1,3-glucan level in the \( pbs2\Delta \) mutant.

\( PBS2 \) overexpression and \( PTC1/CWH47 \) disruption both lead to \( EXG1 \) activation

The fact that overproducing \( PBS2 \) or deleting \( PTC1/CWH47 \) resulted in β1,6-glucan reductions indicated that the \( Pbs2p\)-Hoglp MAP kinase casde played a functional role in cell surface assembly, most likely through the regulation of genes directly involved in cell wall β1,6-glucan assembly. The \( EXG1 \) gene might be a downstream gene activated by the \( Pbs2p\)-Hoglp pathway, because \( EXG1 \) and \( PBS2 \) were both identified in the same screen, and because overproduction of the \( PBS2 \) gene or disruption of the
PTC1/CWH47 gene resulted in phenotypes very similar to those seen on EXG1 overexpression.

To explore this possibility, we tested if 2μ-PBS2 plasmids or a ptc1Δ mutation could lead to EXG1 activation by examining the EXG1 mRNA levels and the exo-β-glucanase activities. While both pbs2Δ and hog1Δ 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 2μ-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 Exglp 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 2µ-PBS2 plasmids. As shown in Table 2, killer toxin survival of the exglA ptc1Δ double mutant was lower than that of the ptc1Δ single mutant, though still significantly higher than the wild-type strain. Similarly, the exglA strain harboring 2µ-PBS2 plasmids also became less resistant to killer toxin than the 2µ-PBS2 containing wild type 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 2µ-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 β1,3-glucan assembly. The EXG1 and PBS2 were isolated as genes that, when overexpressed from a 2µ-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 2µ plasmid resulted in
killer resistance and a modest reduction in cell wall \( \beta 1,6 \)-glucan. In contrast, disruption of the \( \textit{EXG1} \) gene caused the mutant to become more sensitive to killer toxin, and led to a small increase in the \( \beta 1,6 \)-glucan level. These results demonstrate that changes in the \( \textit{Exglp} \) activity can lead to alterations in cell wall \( \beta 1,6 \)-glucan levels, thus providing experimental support for the expectation that the \( \textit{Exglp} \) \( \alpha \)-\( \beta \)-glucanase plays a functional role in cell wall glucan metabolism.

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

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

We showed that the \( \textit{Pbs2p} \) kinase and \( \textit{Ptc1p} \)/\( \textit{Cwh47p} \) phosphatase play opposing roles in maintaining killer toxin sensitivity and \( \beta 1,6 \)-glucan levels. Either \( \textit{PBS2} \) overexpression or \( \textit{PTC1}/\textit{CWH47} \) disruption result in killer resistance and \( \beta 1,6 \)-glucan reductions, while disruption of the \( \textit{PBS2} \) gene led to a killer-hypersensitive phenotype and a slight increase in the \( \beta 1,6 \)-glucan level. Although the reductions in \( \beta 1,6 \)-glucan level caused by \( \textit{PBS2} \) overexpression or \( \textit{PTC1}/\textit{CWH47} \) 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 \( \beta 1,6 \)-glucan level (e.g. an approximately 50% reduction in \( \textit{kre6} \), or in \( \textit{kre11} \)) can result in a very strong killer-resistant phenotype (Brown et al. 1993; Roemer and Bussey 1991); secondly, a comparable \( \beta 1,6 \)-glucan reduction (approximately 27%) caused by \( \textit{EXG1} \) overexpression results in a similar killer-resistant phenotype. Unlike killer resistance, the killer-hypersensitive phenotype is not well characterized. Although slight increases in both \( \beta 1,6 \)-glucan and \( \beta 1,3 \)-glucan levels were detected in the \( \textit{pbs2A} \) mutant, the molecular basis for killer hypersensitivity is still not clear. However, since reduced levels and/or altered structures of \( \beta 1,6 \)-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 \( \textit{PBS2} \) and \( \textit{PTC1}/\textit{CWH47} \), we examined genetic epistasis by comparing the phenotypes displayed by the single mutants and the double mutant. We found that the \( \textit{pbs2A} \textit{ptc1A} \) double mutant displayed the same phenotypes as the \( \textit{pbs2A} \) single mutant: while the \( \textit{ptc1A} \) single mutant showed killer resistance and elevated \( \textit{Exglp} \) glucanase activity, both the \( \textit{pbs2A} \textit{ptc1A} \) double mutant and the \( \textit{pbs2A} \) single mutant displayed hypersensitivity to killer toxin and reduced levels of \( \textit{Exglp} \) glucanase activity. These results demonstrate that the \( \textit{pbs2A} \) mutation is epistatic to the \( \textit{ptc1A} \) mutation. In addition, we showed that disruption of \( \textit{PTC1}/\textit{CWH47} \) resulted in activation of the \( \textit{Pbs2p-Hog1p} \) cascade, as indicated by an increase in the intracellular glycerol level. Together, these results provide genetic evidence suggesting that the \( \textit{Ptc1p} \) phosphatase negatively regulates the \( \textit{Pbs2p-Hog1p} \) MAP kinase cascade. Interestingly, the same relationship between \( \textit{Ptc1p} \) and \( \textit{Pbs2p-Hog1p} \) kinase cascade has recently been independently proposed (Maeda et al. 1994). In their study, these authors reported that the \( \textit{Pbs2p-Hog1p} \) MAP kinase cascade was regulated by a \( \textit{Snl1p-Ssk1p} \) two-component system, and that disruption of \( \textit{SLN1} \) resulted in constitutive activation of the MAP kinase cascade and lethality. Based on the observations that overexpression of the \( \textit{PTC1} \) gene, as well as the disruption of \( \textit{PBS2} \) or \( \textit{HOG1} \), could all suppress the lethal phenotype caused by a \( \textit{snl1A} \) null mutation, they proposed that \( \textit{Ptc1p} \) may inactivate the \( \textit{Pbs2p-Hog1p} \) MAP kinase cascade by dephosphorylating \( \textit{Pbs2p} \) and/or \( \textit{Hog1p} \).

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

See text for details.