Impaired PRPP-synthesizing capacity compromises cell integrity signalling in *Saccharomyces cerevisiae*

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In *Saccharomyces cerevisiae*, *PRS* genes comprise a family of five paralogous genes. Previously, it has been shown that in the cell the gene products are organized into two interacting complexes, one of which is a heterodimer and the other a heterotrimer. Here, it has been demonstrated that in addition to supplying the cell with the key metabolic intermediate PRPP [5-phospho-D-ribosyl-1-(z)-pyrophosphate], the gene products contribute to the maintenance of cell integrity. Specifically, the phosphorylation of Rim1, one of the end points of the cell integrity signalling pathway, is significantly impaired following deletion of any one of the *PRS* genes, in particular *PRS1* and *PRS3*. This is reflected in changes in the expression of the alternative 1,3-β-glucan synthase catalytic subunit, Fks2, as measured by its promoter activity.

Yeast two-hybrid analysis has shown that Prs1, specifically the non-homologous region, NHR1-1 and Prs3, and to a lesser extent Prs2 and Prs4, interact with the MAPK (mitogen-activated protein kinase) of the cell integrity pathway, Slt2. When *PRS1* is lacking, the basal level of phosphorylation of Slt2 is increased. Furthermore, *prs1A* and *prs3A* strains have an increased chitin content under normal growth conditions. z-Factor sensitivity and Calcofluor White resistance associated with the lack of Prs1 and Prs3 corroborate the involvement of these two gene products in cell integrity signalling. It is postulated that Prs polypeptides play a significant role in the remodelling of the cell wall and may have a direct involvement in cell integrity signalling.

**INTRODUCTION**

PRPP [5-phospho-D-ribosyl-1-(z)-pyrophosphate] is an essential biosynthetic precursor for the synthesis of purine, pyrimidine nucleotides and the pyridine nucleotides NAD+ and NADP+ (Becker, 2001). We have shown that the *Saccharomyces cerevisiae* genome contains five unlinked paralogous genes, each capable of encoding PRPP synthetase (Prs: EC 2.7.6.1; ATP : D-ribose-5-phosphate diphosphotransferase). A systematic phenotypic analysis has been carried out with our collection of strains representing all possible combinations of deletions of the five *PRS* genes. The results obtained define three phenotypes: (i) a synthetic lethal phenotype when *PRS1* or *PRS3* was deleted from a *prs5A* strain – simultaneous deletion of *PRS2* and *PRS4* in combination with loss of *PRS1* or *PRS3* also results in inviability; (ii) a second phenotype that is encountered in strains containing deletions of *PRS1* and *PRS3* together or in combination with lack of *PRS2* or *PRS4* manifests itself as a reduction in growth rate, enzyme activity and nucleotide content; and (iii) deletion of *PRS2*, *PRS4* or *PRS5* or combinations thereof has reduced enzyme activity, but are unimpaired in growth and nucleotide profiles (Carter et al., 1994; Hernando et al., 1998, 1999). Three viable triple deletion combinations, *prs2Δ prs4Δ prs5Δ*, *prs1Δ prs3Δ prs4Δ* and *prs1Δ prs2Δ prs3Δ*, define three minimal subunits, Prs1/Prs3, Prs2/Prs5, Prs4/Prs5, respectively An extensive two-hybrid (Y2H) analysis suggested the existence in the wild-type of two interacting functional entities which may have compensatory function since in the absence of one entity or one of its components the yeast cell can still survive (Hernando et al., 1998, 1999). In accordance with our data, interactions between Prs polypeptides have been identified in large-scale Y2H and high-throughput MS analyses (Bader et al., 2003; Ito et al., 2001; Uetz et al., 2000) (http://www.bind.ca/ or http://mips.gsf.de/proj/yeast/tabs/interaction/).

Since none of the *PRS* deletant strains had an obvious phenotype with the exception of slow growth in *prs1Δ* and *prs3Δ* strains, it was significant that a *prs3* mutation was uncovered in a colony-sectoring screen analysis for mutations which are co-lethal with *whi2*. Mutation of *WHI2* results in caffeine sensitivity (Binley et al., 1999) and it is...
now known that Whi2 is a binding partner for Psr1 phosphatase and both proteins are necessary for a full activation of the general stress response (Kaida et al., 2002). We have shown that caffeine sensitivity is characteristic of all PRS deletant strains with those lacking PRS1 and/or PRS3 being the most sensitive. A possible link between Psr1 and the maintenance of cell integrity is provided by the observation that in prs1Δ and prs3A strains the caffeine-induced release of alkaline phosphatase can be counteracted by stabilizing the medium with 1 M sorbitol; this is a strong indication that the maintenance of cell integrity is compromised in these strains. Furthermore, electron microscopy indicated that in PRS deletant strains there is a higher incidence of plasma membrane invaginations and accumulation of cytoplasmic vesicles than in the wild-type (Schneider et al., 2000).

Cell integrity is dependent upon a functional Pck1-Slt2 signalling cascade (Gustin et al., 1998; Hohmann, 2002; Klis et al., 2002). The pathway is a MAPK (mitogen-activated protein kinase) cascade consisting of MAPKK kinase Bck1, the redundant MAPK kinases Mkk1 and Mkk2, and the MAP kinase Slt2 (Heinisch et al., 1999). Inputs into the pathway are mediated via sensors located in the plasma membrane, Mid2 and members of the Wsc family (Popolo et al., 2001; Rajavel et al., 1999; Verna et al., 1997). There are two known outputs: the transcription factors SBF, which interfere with cell wall synthesis (de Nobel et al., 2000). Solid media contained 2 % agar. Yeast transformations were carried out using the high efficiency yeast transformation method (Gietz et al., 1992), the 'lazy bones' method (Woods & Gietz, 2001) or the 'Plate' method (Eibl, 1992). *Escherichia coli* DH5α was used for plasmid propagation. *E. coli* cells were cultured in LB-medium and transformed by standard methods (Ausubel et al., 1995).

**METHODS**

**Strains and growth conditions.** *S. cerevisiae* strains used in this study are listed in Table 1. Yeast cultures were grown in YEPD (1 % yeast extract, 2 % Bacto peptone, 2 % glucose) or in synthetic complete (SC) medium (0-17 % yeast nitrogen base without amino acids and NH₄Cl, 0-5 % (NH₄)₂SO₄, 2 % glucose) supplemented with the appropriate amino acids or nucleobases (Kaiser et al., 1994). Solid media contained 2 % agar. Yeast transformations were carried out under the high efficiency yeast transformation method (Gietz et al., 1992), the 'lazy bones' method (Woods & Gietz, 2001) or the 'Plate' method (Eibl, 1992). *Escherichia coli* DH5α was used for plasmid propagation. *E. coli* cells were cultured in LB-medium and transformed by standard methods (Ausubel et al., 1995).

**Plasmids.** Plasmid YEplac195-PKCI (URA3/2 μ) harbours a genomic fragment containing PKCI cloned into the HindIII and EcoR1 restriction sites of YEplac195 (Gietz & Sugino, 1988) and was kindly provided by Dr M. J. R. Stark (University of Dundee). The pHPS100 series of plasmids was kindly provided by Dr J. J. Heinisch (University of Osnapbrück, Germany). This is a CENARS vector containing both a lacZ-RML1 fusion and a lacZ reporter gene preceded by five lexA binding sites. This lexA-RML1 fusion activates a β-galactosidase reporter construct after phosphorylation of the Rlm1 moiety by Slt2. pHPS100-T, pHPS100-U and pHPS100-L carry the selection markers TRP1, URA3 and LEU2, respectively (Kirchrath et al., 2000). FKS2(-706/-1)-lacZ containing the 2 μ origin of replication was generously provided by Dr P. de Groot (University of Amsterdam, The Netherlands) and is described by de Nobel et al. (2000) and Zhao et al. (1998). pGAD424-Slt2 (originally created by Dr Maria Molina, Universidad Complutense, Madrid, Spain) was kindly provided by Dr Hélène Martin-Yken (INSA, Toulouse, France) after obtaining permission from Dr Molina. To obtain Slt2 in pGBT9 the 1-5 kb BamHI/BglII fragment from pGAD424-Slt2 containing the ORF of Slt2 was cloned into BamHI-restricted pGBT9 and checked by restriction analysis.

**Phenotypic growth assays.** These were carried out under appropriate conditions by spotting 3–5 μl serial tenfold dilutions of a culture of OD₆₀₀ = 1.0 onto solid medium. Growth was recorded after 48–72 h. CFW was added to YEPD plates at the concentrations indicated. Incubation was carried out at either 28 or 37 ℃ for 2–3 days.
Table 1. Yeast strains used in this study

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<th>Strain</th>
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<td>Pj69-4A</td>
<td>MATα trpl-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GALI&lt;sub&gt;UAS&lt;/sub&gt;-GALI&lt;sub&gt;TATA&lt;/sub&gt;-HIS3</td>
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α-Factor sensitivity. When testing α-factor sensitivity appropriately supplemented liquid SC medium was buffered by the addition of succinic acid to a final concentration of 85 mM and NaOH to a final concentration of 19 mM (Ketela et al., 1999). Cultures of MATα strains containing either control plasmids or plasmids bearing genes of interest were diluted to 2 × 10<sup>6</sup> cells ml<sup>-1</sup> in buffered SC medium. Aliquots (50 μl) of each strain were removed and spread on YEPD or selective medium. α-Factor (Sigma) was then added to the cultures at a final concentration of 1 or 2 μM. The cultures were incubated further at 30°C over a period of 6–7 h. Aliquots (50 μl) were removed at intervals of 100 min and plated out on appropriate media. The plates were incubated for 48 h at 30°C and the number of colonies formed on each plate was determined. α-Factor sensitivity was measured by the difference between the number of colonies on pre- and post-α-factor plates for each strain.

Measurement of Fks2 promoter activity. Individual transformants containing the FKS2(−706/−1)-lacZ reporter plasmid were grown in duplicate in 10 ml selective medium to a density of 1 × 10<sup>8</sup> cells ml<sup>-1</sup>. One set of duplicates was grown at 37°C for a further 4 h and the second set was grown at 28°C for the same length of time. Cells were then harvested by centrifugation in 15 ml Falcon tubes (6000 r.p.m., 4°C) and washed once in 1 ml sterile ice-cold H<sub>2</sub>O, and, if not to be used immediately, the pellet was stored at −80°C until required. For cell disruption 300 μl Z-buffer (60 mM Na<sub>2</sub>HPO<sub>4</sub>, 12 H<sub>2</sub>O, 40 mM NaH<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 10 mM KCl, 1 mM MgSO<sub>4</sub>·7H<sub>2</sub>O) was added to the cells followed by 0.5 g acid-washed glass beads (425–600 μm diam.) and the whole was vortexed at maximum speed for 1 min followed by 1 min incubation on ice. This step was repeated three times before centrifugation as described above (Guarente, 1983). The supernatant was transferred to a fresh tube and protein concentration was determined according to the method of Bradford (1976) The β-galactosidase assay was performed using O-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate. Depending on the protein concentration, 20–200 μl of crude extract in a final volume of 1 ml Z-buffer was combined with 200 μl ONPG (4 mg ml<sup>−1</sup>). After a pale yellow colour developed, 0.5 ml 1 M Na<sub>2</sub>CO<sub>3</sub> was added to stop the reaction. The specific activity of β-galactosidase [U (mg protein<sup>−1</sup>)] was calculated as described by Miller (1972).

Yeast two-hybrid analysis (Y2H). The Gal4pDBD-Prs1-5 and Gal4pAd-Prs1-5 fusion plasmids (Hernando et al., 1999), and pGBT9-Snf1 and pGAD424-Snf4 (Roder et al., 1999) have been described previously. The truncated versions of Prs1, pGAD424-Prs1(ANHR1-1) and pGBT9-(ANHR1-1), were created by PCR to remove NHR1-1 which extends from nt 604 to 925 in the ORF of Prs1. For the other two constructs Prs1 in pGAD424 was bisected using the BamHI restriction site located at nt 746–751 of the PRS1 ORF; the two halves of the ORF were cloned into pGAD424 as an EcoRI/BamHI fragment to give pGAD424-Prs1(Δ746–1281) and as a BamHI/BglII fragment to give pGAD424-Prs1(Δ1–745) (A. Carter, unpublished data). The host strain Pj69-4A (James et al., 1996) was transformed with the two plasmids whose interaction was to be tested. At least three independent transformants were inoculated singly into 10 ml SC minus leucine and tryptophan and incubated overnight at 30°C. Crude extracts were prepared and assayed for β-galactosidase activity as described above with ONPG as substrate. The transformants were also tested for their ability to grow on selective media lacking adenine or histidine plus 150 mM 3-aminotriazole.

Rlm1 transcriptional activation. Transformants containing one of the pHPS100 series of plasmids were grown in selective medium at 28°C to OD<sub>600</sub> = 0.5, harvested, rinsed once with an equal
volume of Z-buffer (see above) and centrifuged, and the pellet was taken up in 1/5 volume Z-buffer. The cells were broken by two cycles of freezing and thawing and β-galactosidase activity was assayed by using 20 μl crude extract with the chemiluminescent substrate Galacto-Star, according to the manufacturer’s instructions (Applied Biosystems). β-Galactosidase activity was expressed as relative light units (RLU) (μg protein)−1 using a tube luminometer, Lumar LB 9507 (Berthold Technologies). Protein concentration was determined according to the method of Bradford (1976). When measuring the influence of temperature or α-factor on Rlm1 activation, the culture was divided into two aliquots, one served as the control and the other was incubated at 39 °C or treated with α-factor at a concentration of 2 μM for 4 h before determining β-galactosidase activity.

**Measurement of chitin content.** This was performed according to the protocol kindly provided by Dr Laura Popolo (University of Milan, Italy). A 50 ml culture was grown overnight at a density of 1·0 × 10^7 cells ml^−1 and harvested by centrifugation (6000 r.p.m., 4 °C). The cells were washed twice with 1·5 ml H_2O, the wet weight determined and the cells resuspended at a concentration of 1 g wet weight (ml H_2O)^−1; 1 ml 6% KOH was added per 0·1 g cells and the cell suspension was incubated at 80 °C for 90 min. After cooling to room temperature, 0·1 ml glacial acetic acid was added and centrifuged at 13000 r.p.m. for 10 min. The pellet was washed twice in 50 mM sodium phosphate buffer, pH 6·3. At this stage the pellet could be stored at −20 °C. Chitinase (Sigma C-1650; 40 μl; 17 mg per 400 μl 50 mM sodium phosphate buffer, pH 6·3) was added to the pellet and the volume brought up to 600 μl with sodium phosphate buffer, pH 6·3. A control was set up containing 40 μl chitinase and 540 μl 50 mM sodium phosphate buffer, pH 6·3. The suspensions were incubated at 37 °C for 2 h. After vortexing thoroughly, a 400 μl sample was transferred to a fresh 1·5 ml Eppendorf tube to which 25 μl β-glucuronidase (Sigma G0876) was added. The tubes were incubated at 37 °C for 1 h, boiled for 1 min and centrifuged at 12000 r.p.m. for 5 min. A sample of supernatant (50 μl) was transferred to a glass tube and the final volume was brought up to 250 μl with H_2O. K_2HPO_4·H_2O (250 μl 0·27 M, pH 9·0) was added to the tube and the whole was boiled for 8 min. The sample was cooled to room temperature and 3 ml 1× Reissig solution (10× stock: 10 g dimethylaminobenzaldehyde, 12·5 ml 10 M HCl, 87·5 ml glacial acetic acid) was added. After vortexing the tubes were incubated at 37 °C for 40 min and the absorbance was measured at 585 nm. A standard curve was prepared using appropriately diluted N-acetylgallosamine (10 mg ml^−1 stock). For chitin measurements following exposure to CFW, cells were cultured in the presence of 100 μg CFW ml^−1 at 28 °C for 4 h prior to harvesting.

**Western blotting.** Yeast cells were grown overnight at 28 °C to mid-exponential phase and diluted to OD_{600}=0·3 (9 × 10^7 cells ml^−1) in a volume of 200 ml YEPD. The cultures were allowed to grow for one generation before removing a 10 ml (1 × 10^8·2 × 10^6 cells) aliquot which was processed as described by Reinoso-Martin et al. (2003) and Schuetzer-Muehlbauer et al. (2003). Four further 10 ml aliquots were dispensed into individual pre-warmed universals and incubated at 37 °C. At hourly intervals thereafter the contents of a single universal were processed as indicated above. Each individual pellet was resuspended in 100 μl loading buffer (40 mM Tris/HCl, pH 6·8, 8·0 M urea, 5% SDS, 0·1 mM EDTA, 10 mg bromophenol blue) which was supplemented with 1% β-mercaptoethanol and 10% 1 M Tris-base. For the sample taken at time zero a volume of the suspension, equivalent to approximately 4 × 10^6 cells, was loaded onto the gel. To compensate for cell growth during the 4 h incubation period, the remaining samples were diluted with an appropriate amount of loading buffer to a concentration which corresponded to 4 × 10^6 cells. Cell extracts were separated in SDS-8% polyacrylamide gels and transferred to Hybond-P PVDF membrane (0·2 μm; Amersham Bioscience) using the Mini Trans-Blot Electrophoretic Transfer system (Bio-Rad) according to the manufacturer’s instructions. Phosphorylated Slt2 was detected using anti-phospho-p44/p42 MAPK (Thr202/Tyr204) as the primary antibody (1:2000 dilution; New England Biolabs) (Martin et al., 2000) and detected by goat anti-rabbit antibody (1:5000 dilution; Sigma) with the ECL+ detection system from Amersham Bioscience. Slt2 was detected by anti-GST-Slt2 antibody (1:1000 dilution) (Martin et al., 1993) generously provided by Drs Maria Molina and Humberto Martin (Universidad Complutense de Madrid, Spain) and detected as described above.

**RESULTS**

**The requirement for Prs in the yeast cell response to stress**

VariousprsΔstrains were examined for their growth characteristics following exposure to elevated temperature (Kamada et al., 1995) and CFW (Ketela et al., 1999). Fig. 1 shows that at 37 °C prs1Δ and prs3Δ are impaired in their growth and the double deletant prs1Δ prs3Δ is inviable. The temperature-sensitive phenotype of prs1Δ prs3Δ can be reversed by the addition of 1 M sorbitol to the medium (data not shown). No thermosensitive phenotype was observed in a prs4Δ or prs5A strain or in the triple deletant strain prs2Δ prs4Δ prs5Δ.

The ability of the PRS deletants to grow in the presence of CFW is also shown in Fig. 1; prs1Δ, prs3Δ and prs1Δ prs3Δ prsΔ strains have acquired resistance to CFW, whereas the prs1Δ prs3Δ and the prs1Δ prs2Δ prs3Δ strains are, in contrast, sensitive to this compound. Deletion of PRS2, PRS4 or PRS5 singly or in combination confers a slight, albeit variable, degree of resistance to CFW.

It is known that exposure to α-factor induces morphological changes in the cell wall which activate the cell integrity pathway (Buehler & Errede, 1997). Treatment with 1 μM α-factor over a period of 6 h revealed that in comparison to the wild-type, strains lacking PRS1 displayed a 46% reduction and strains lacking PRS3 a 73% reduction, in viability. Simultaneous deletion of PRS1 and PRS3 has an additive effect since viability decreased more rapidly within 2 h and reached a similar level to the prs3Δ strain, 77%, after 6 h (Fig. 2a). Surprisingly, the α-factor sensitivity of prs1Δ prs3Δ can be overcome by the additional deletion of either PRS2 or PRS4; these triple deletants retained their wild-type response even when the α-factor concentration was increased to 2 μM. Deletion of PRS2 PRS4 PRS5 had no effect on the response of a MATα cell to α-factor (Fig. 2b).

**Overexpression of PKC1 partially counteracts PRS deletant-induced cell integrity pathway malfunction**

The slightly reduced growth of prs1Δ and prs3Δ observed at 37 °C (Fig. 1) and the α-factor sensitivity of prs1Δ and prs3Δ strains (Fig. 2a) are alleviated by overexpression of Pkc1, a well-documented upstream regulator of the cell integrity pathway (Heinisch et al., 1999). The α-factor sensitivities
of prs1Δ, prs3Δ strains (Fig. 2c) and the wild-type (data not shown) each containing Pkc1 are in agreement with each other when exposed to α-factor at a concentration of 2 μM. However, overexpression of Pkc1 could correct neither the thermosensitivity nor the α-factor sensitivity of the double deletant prs1Δ prs3Δ (data not shown). With respect to growth on CFW at a concentration of 300 μg ml⁻¹, overexpression of Pkc1 in a prs1Δ prs3Δ strain has a growth-promoting effect which is, however, not sufficient to confer CFW resistance. Conversely, overexpression of Pkc1 reduces growth in the wild-type and has a sensitizing effect on the growth of prs1Δ and prs3Δ strains under the above conditions (data not shown).

Sensitivity to α-factor is also encountered in strains carrying a deletion of MID2, a gene which encodes a putative cell surface sensor known to activate the cell integrity pathway via Pkc1 (Ketela et al., 1999; Philip & Levin, 2001; Rajavel et al., 1999). Deletion of PRS1 and/or PRS3 in a strain lacking MID2 does not exacerbate the inherent α-factor sensitivity of a mid2Δ strain. The independent nature of the apparently similar prs1Δ- and/or prs3Δ- and mid2Δ-induced phenotypes is underlined by the observation that overexpression of either PRS1 or PRS3 does not correct the mid2Δ-associated α-factor-sensitive phenotype (data not shown).

Chitin levels in PRS deletant strains

CFW resistance goes hand in hand with altered chitin synthesis (Roncero & Duran, 1985; Roncero et al., 1988).

Therefore, we decided to measure the chitin content of certain PRS deletant strains in the presence or absence of CFW. As is shown in Fig. 3, strains deleted for PRS2, PRS4 or PRS5, singly or combined, respond in the same way as the wild-type, i.e. they increase their chitin content at least fourfold upon exposure to CFW. On the other hand, deletion of PRS1 or PRS3 causes a threefold increase in chitin content and impairs the ability of the cell to increase its chitin content upon exposure to CFW. For the double deletant prs1Δ prs3Δ there is a 50 % increase in chitin content at 28 °C, whereas in the presence of CFW the chitin content is virtually doubled.

Rlm1 activation in prsΔ strains

The observations described above suggest an involvement of Prs1 and Prs3 polypeptides with the cell integrity pathway. Therefore, it seemed a logical step to examine one of the end points of the cell integrity pathway, the transcription factor Rlm1 (Heinisch et al., 1999), in strains lacking one or other of the Prs polypeptides. To this end, a plasmid carrying a lexA-Rlm1 fusion, which when phosphorylated activates a β-galactosidase reporter on the same plasmid (Kirchrath et al., 2000), thus providing a measure of Rlm1 activation under the cell integrity pathway, was introduced into various prsΔ strains. The loss of any of the Prs polypeptides had a dramatic effect on Rlm1 activation under normal growth conditions: deletion of PRS5 reduced Rlm1 activation by 30 %; however, lack of PRS2 or PRS4 alone or in combination with PRS5 reduced Rlm1 transcriptional...
activation further by 80% (Fig. 4a). The most pronounced effect on Rlm1 activation is observed when PRS1 and/or PRS3 is removed; in a prs1Δ prs3Δ strain Rlm1 activation is virtually abolished (Fig. 4b). However, when the growth temperature is increased to 39 °C, strains which lack PRS2 or PRS4 or PRS2 PRS4 PRS5 are capable of increasing Rlm1 activation. Interestingly, the loss of PRS5 or the loss of PRS1 and/or PRS3 alone results in Rlm1 activation remaining virtually unaltered at the higher temperature.
considerable loss of Rlm1 activation, does not alter the α-factor-induced reduction in Rlm1 response. However, deletion of PRS3 results in a twofold increase, whereas a prs1Δ prs3Δ strain is severely impaired in its Rlm1 response following exposure to α-factor. The same holds true for the triple deletant strains prs1Δ prs2Δ prs3Δ and prs1Δ prs3Δ prs4Δ (Fig. 4b).

Next, we examined the effect of overexpression of Pkc1 on the impaired Rlm1 response of strains lacking PRS1 and/or PRS3 under normal growth conditions. Introduction of Pkc1 in a prs1Δ strain carrying the Rlm1 reporter plasmid had a positive effect on the transcriptional activation of Rlm1, increasing it by approximately twofold at 28°C, 39°C and following exposure to α-factor (Fig. 5a). When PRS3 was lacking, overexpression of Pkc1 increased Rlm1 activation threefold at 28 and 39°C, but only 1.7-fold after exposure to α-factor (Fig. 5b). Overexpression of Pkc1 was also able to correct the defective activation of Rlm1 in a prs1Δ prs3Δ strain at 28°C by effecting a sixfold increase; when α-factor was tested in the same strain the response was improved almost fourfold to just slightly above the Rlm1 activation observed in the prs1Δ prs3Δ strain grown at 28°C. However, Pkc1 overexpression does not improve Rlm1 activation sufficiently to allow the temperature-sensitive phenotype of the prs1Δ prs3Δ strain to be overcome (Fig. 5c).

**FKS2 activity in PRS deletant strains**

Introduction of the FKS2-lacZ reporter which contains the FKS2 promoter from −706 to −1 linked to the β-galactosidase reporter module into the wild-type and PRS deletant strains allowed us to determine the effect of the loss of Prs polypeptides on the expression of the FKS2 gene, which encodes an alternative catalytic subunit of 1,3-β-glucan synthase (Inoue et al., 1995) and is expressed under conditions of cellular stress and Rlm1 activation. As shown in Fig. 6, in the wild-type FKS2 promoter activity is almost three times higher following incubation at 37°C than at 28°C. Deletion of either PRS2 or PRS4 has no effect on this response. Deletion of PRS1, however, although it reduces FKS2 promoter activity at 28°C, apparently does not affect the heat-inducibility. Deletion of PRS3 reduces the basal FKS2 promoter activity slightly, but completely abolishes the heat-inducibility. In a prs5Δ strain the basal activity is hardly altered, but there is a significant reduction in β-galactosidase activity at 37°C. A strain lacking PRS1 and PRS3 exhibited a reduced basal FKS2 promoter activity which was further reduced.
following growth at 37°C. This is in accordance with the Rlm1 activation in the same strain under temperature stress (Fig. 4b).

**Interactions of Prs polypeptides with components of the cell integrity pathway**

To determine whether or not the effects of the Prs-dependent cell integrity-related phenotypes are caused by direct interaction between Prs polypeptides and elements of the cell wall integrity pathway, we undertook a Y2H analysis. The yeast reporter strain PJ69-4A was transformed with appropriate plasmids to give pairwise combinations of PRS1–PRS5 with SLT2 fused to either the activation or the binding domain of GAL4. The transformants were tested for β-galactosidase activity and their ability to grow on selective media for histidine and adenine prototrophy. In addition to several negative controls, two positive controls were used: one measuring an interaction between Prs4 and Prs5 and the other, which is Prs-independent, measuring the interaction between Snf1 and Snf4. The results are summarized in Table 2. It can be seen that each of the Prs polypeptides is capable of interacting with Slt2 in either orientation with the strongest interaction occurring with Prs3. All pairwise combinations grow in the absence of histidine, and adenine prototrophy is encountered in all combinations with the exception of Slt2 and Prs5 in either orientation, although the interaction of Prs5 and Slt2 supports a β-galactosidase activity above that of the background.

As a way of confirming these data we used three truncations of Prs1 to investigate the interaction between Prs1 and Slt2. As is obvious from Table 2, deletion of NHR1-1 (Carter et al., 1994) completely abolishes the Prs1/Slt2 interaction. Interaction of Prs1 with Prs2 or Prs3 is not destroyed by deletion of NHR1-1 (Hernando et al., 1999). On the other hand, when the PRS1 ORF was split into approximately equal parts there was no loss of interaction between Prs1 and Slt2. In contrast, neither Prs2 nor Prs3 was capable of interacting with either Prs1(Δ1-745) or Prs1(Δ746-1281) (data not shown).

**Table 2. Y2H interactions of Prs with MAPKs of the cell integrity pathway**

PJ69-4A transformants were tested for β-galactosidase activity and growth on adenine-free (ADE auxotrophy) and histidine-free plus 150 mM 3-aminotriazole (HIS auxotrophy) SC media. pGBT9-Snf1/pGAD424-Snf4 and pGBT9-Prs5/pGAD424-Prs4 were used as positive controls and the combinations pGBT9/pGAD424, pGBT9-Slt2/pGAD424 and pGBT9/pGAD424-Slt2 served as negative controls. Values are means ± SD (n = 6 or n = 3) for Prs1/Slt2 and Prs1-truncated/Slt2 combinations, respectively.

<table>
<thead>
<tr>
<th>Plasmid combinations</th>
<th>Relative β-galactosidase activity (U mg⁻¹ ± SD)</th>
<th>ADE auxotrophy</th>
<th>HIS auxotrophy</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGBT9/pGAD424</td>
<td>3.2 ± 0.1</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>pGBT9-Slt2/pGAD424</td>
<td>1.6 ± 0.3</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>pGBT9-Slt2/pGAD424-Prs1</td>
<td>19.3 ± 2.3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pGBT9-Slt2/pGAD424-Prs2</td>
<td>13.9 ± 1.1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pGBT9-Slt2/pGAD424-Prs3</td>
<td>22.9 ± 0.1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pGBT9-Slt2/pGAD424-Prs4</td>
<td>17.2 ± 1.0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pGBT9-Slt2/pGAD424-Prs5</td>
<td>6.1 ± 0.7</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>pGBT9/pGAD424-Prs1(ΔNHR1-1)</td>
<td>2.9 ± 0.79</td>
<td>−</td>
<td>−</td>
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<tr>
<td>pGBT9-Slt2/pGAD424-Prs1(ΔNHR1-1)</td>
<td>2.8 ± 0.076</td>
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<td>−</td>
</tr>
<tr>
<td>pGBT9/pGAD424-Prs1(Δ746-1281)</td>
<td>2.9 ± 0.38</td>
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<td>−</td>
</tr>
<tr>
<td>pGBT9-Slt2/pGAD424-Prs1(Δ746-1281)</td>
<td>13.1 ± 1.56</td>
<td>+</td>
<td>+</td>
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<tr>
<td>pGBT9/pGAD424-Prs1(Δ1-745)</td>
<td>3.1 ± 0.43</td>
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<td>−</td>
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<tr>
<td>pGBT9-Slt2/pGAD424-Prs1(Δ1-745)</td>
<td>12.4 ± 0.88</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pGBT9/pGAD424-Slt2</td>
<td>2.6 ± 1.0</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>pGBT9-Prs1/pGAD424-Slt2</td>
<td>13.3 ± 0.1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pGBT9-Prs2/pGAD424-Slt2</td>
<td>19.1 ± 0.3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pGBT9-Prs3/pGAD424-Slt2</td>
<td>32.5 ± 1.0</td>
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<td>+</td>
</tr>
<tr>
<td>pGBT9-Prs4/pGAD424-Slt2</td>
<td>10.7 ± 0.2</td>
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<td>pGBT9-Prs5/pGAD424-Slt2</td>
<td>5.1 ± 0.6</td>
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<td>−</td>
</tr>
<tr>
<td>pGBT9-Prs1(ΔNHR1-1)/pGAD424</td>
<td>2.7 ± 0.05</td>
<td>−</td>
<td>−</td>
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<tr>
<td>pGBT9-Prs1(ΔNHR1-1)/pGAD424-Prs2</td>
<td>3.3 ± 0.78</td>
<td>−</td>
<td>−</td>
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<tr>
<td>pGBT9-Snf1/pGAD424-Snf4</td>
<td>11.1 ± 0.08</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pGBT9-Prs5/pGAD424-Prs4</td>
<td>12.8 ± 0.5</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>
metabolic disturbances in any organism have far-reaching and unexpected consequences. In this report we have demonstrated that altering the capacity of the yeast cell for synthesizing PRPP, a key biosynthetic intermediate, through the deletion of individual PRS genes. Crude extracts of PRS deletant strains, cultivated as described in Methods, were separated on 8% SDS-PAGE and transferred to a PVDF membrane followed by probing with anti-phospho-p44/42 MAPK (Thr202/Tyr204) antibodies. In the wild-type strain there was a faint signal corresponding to Slt2-P at time zero which increases in intensity with respect to time of incubation at 37 °C. In contrast in the prs1Δ strain there was a strong signal at time zero which was maintained over a period of 4 h incubation at 37 °C. A strain lacking PRS3 was also phosphorylated at time zero and this signal increased steadily over a period of 4 h. For control purposes the same membranes were stripped and reprobed with anti-GST-Slt2 antibody (Fig. 7).

**DISCUSSION**

Metabolic disturbances in any organism have far-reaching and unexpected consequences. In this report we have demonstrated that altering the capacity of the yeast cell for synthesizing PRPP, a key biosynthetic intermediate linking carbon and nitrogen metabolism (Khorana et al., 1958), causes the cell to display cell wall damage-related phenotypes and interferes with signalling in the cell integrity pathway. Stress conditions, such as elevated temperature and mating pheromone treatment, trigger the Slt2 MAPK pathway which is essential for maintenance of the cell wall (Heinisch et al., 1999). Our finding that 1 M sorbitol prevented prs1Δ and prs3Δ strains from releasing alkaline phosphatase and reversed their caffeine sensitivity (Schneiter et al., 2000) suggested that the PRS gene products may in some way be connected with the maintenance of cell integrity. To extend these results we performed a number of experiments aimed at investigating the influence of the five gene products responsible for the production of PRPP on the cell integrity pathway. The data presented in Fig. 1 show that strains lacking PRS1 or PRS3 are resistant to CFW. These two genes encode one of the Prs complexes postulated on the basis of the Y2H analysis and is also one of the three minimal functional units identified by genetic analysis (Hernando et al., 1999). The existence of the Prs1/Prs3 complex has also been confirmed immunologically. Using our collection of PRS deletant strains in which PRS1 has been replaced by a functional GFP-PRS1 fusion (Schneiter et al., 2000), we were able to demonstrate that in a strain carrying a deletion of PRS3 the GFP signal is lost, whereas in all other strains bearing the GFP-PRS1 fusion the GFP signal is clearly visible (M. Oufir, unpublished data). Therefore, we postulate that the phenotypes of temperature-sensitivity, CFW resistance (Fig. 1) and caffeine sensitivity (Schneiter et al., 2000) are associated with loss of the Prs1/Prs3 complex. Such phenotypes have not been observed in strains lacking PRS2, PRS4 or PRS5 whose products form the second complex defined by Y2H and the other two minimal functional units, Prs2/Prs5 and Prs4/Prs5 (Hernando et al., 1999). The triple deletant YN96-72 (prs2A prs4A prs5A) which relies on the Prs1/Prs3 minimal functional unit for provision of PRP has a phenotype which with respect to temperature sensitivity is indistinguishable from the wild-type. The other two triple deletant strains YN95-36 (prs1Δ prs2A prs3Δ) and YN95-25 (prs1Δ prs3A prs4Δ) are both temperature-sensitive and the latter is also CFW-resistant (Fig. 1), suggesting that there is an inherent difference in the minimal functional units Prs2/Prs5 and Prs4/Prs5. However, with regard to z-factor sensitivity all three triple deletants behave as wild-type (Fig. 2b). It would appear that the z-factor sensitivity observed in prs1Δ and prs3A strains is a result of the dysfunction of the cell integrity pathway, since overexpression of PKCl in these strains corrects the phenotype (Fig. 2c).

We also examined the possibility of the involvement of Mid2 which is a putative cell wall stress sensor and upstream activator of the cell integrity pathway and whose deletion invokes phenotypes similar to those of prs1Δ and/or prs3A strains, e.g. z-factor and caffeine sensitivity, and CFW resistance (Ketela et al., 1999; Rajavel et al., 1999). The viability of a mid2Δ prs1Δ prs3Δ strain suggests that the

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**Fig. 7. Slt2 phosphorylation in crude extracts of wild-type (WT) YN94-1, prs1Δ (YN96-50) and prs3Δ (YN96-52) strains.** The strains were grown in YEPD to mid-exponential phase at 28 °C (time point zero) and then heat-shocked at 37 °C for the times indicated (h). The level of phospho-Slt2 was detected immunologically and the filter was stripped and reprobed with GST-Slt2 antibodies as described in Methods.
impact of the metabolic disturbance caused by altering the PRPP-synthesizing capacity of the cell impinges on the cell integrity pathway via Wsc sensors (Zu et al., 2001) or a cell internal signalling pathway parallel to the MID2-dependent signal transduction pathway.

It has been shown that when global transcript profiles of yeast exposed to CFW and Zymolyase are analysed with the REduce and Quontology algorithms there is an upregulation of Rlm1-regulated genes (Boorsma et al., 2004). The transcription factor Rlm1, one of the end points of the cell integrity pathway, is directly phosphorylated by Slt2 (Dodou & Treisman, 1997; Kirchrath et al., 2000; Watanabe et al., 1995, 1997). Induction of FKS2 expression is regarded as a measure of cell wall damage (de Nobel et al., 2000) and is regulated by both the cell wall integrity pathway and independently thereof (Jung & Levin, 1999). We have demonstrated that activation of Rlm1, as measured with the pHSP-100 series of plasmids, is reduced in each of the five prsΔ strains, with the possible exception of prsΔA, in comparison to the wild-type. However, prs2Δ, prs4Δ and prs2A prs4Δ prs5A strains are able to increase Rlm1 phosphorylation at 39 °C (Fig. 4a). This heat-induced response of Rlm1 may be sufficient to mediate the wild-type response of the FKS2 promoter observed in prs2Δ and prs4Δ strains (Fig. 6). Rlm1 activation and FKS2 promoter activity are also correlated in a prs5Δ strain since a temperature-dependent response was not observed for either reporter plasmid. In prs1Δ and prs3Δ strains Rlm1 activation is reduced by 90% and is no longer responsive to increased temperature; this is reflected in the decreased levels of FKS2 promoter activity measured in the same strains (Figs 4 and 6).

The chitin measurements further emphasize the involvement of the Prs1/Prs3 complex in cell wall construction. First, the elevated chitin content in prs1Δ, prs3A and prs1Δ prs3Δ strains points to a response on the part of the cell to a disturbance in the cell wall synthesis machinery. Second, in these strains the CFW-induced increase in chitin synthesis observed in the wild-type and strains compromised for, or lacking, the Prs2/Prs4/Prs5 complex is impaired (Fig. 3). This chitin-associated strengthening of the cell wall would suggest that the Prs1/Prs3 complex plays some role in the compensatory mechanism induced by perturbations of the yeast cell wall. This role is likely to be subtle since in none of the recent genome-wide analyses of the response to cell wall mutations has any of the PRS genes been identified. However, clustering of the data obtained following treatment with cell-wall-damaging agents (Garcia et al., 2004) or from strains carrying mutations affecting different aspects of cell wall construction (Lagorce et al., 2003) has indicated an enrichment of gene categories whose products are involved in metabolism and energy generation, and PRS genes can be included in these categories since their products are responsible for the synthesis of PRPP which is essential for nucleotide production. For instance UTP is required in the penultimate step of chitin synthesis to produce UDP- N-acetylglucosamine (Orlean, 1997; Roncero, 2002; Ruiz-Herrera et al., 2002). We have shown previously that the UTP + UDP + UMP content of strains lacking PRS1 and/or PRS3 is reduced by 75 and 80 %, respectively, in a prs1Δ prs2Δ prs3Δ strain. A prs1Δ prs3Δ prs4Δ strain contains only about 10 % of the UTP + UDP + UMP level of the wild-type, whereas in the triple deletant prs2Δ prs4Δ prs5Δ the level is indistinguishable from the wild-type. The levels of adenine and guanine tri-, di- and mono-nucleotides are also reduced in line with the levels of UTP + UDP + UMP (Hernando et al., 1998, 1999). These reduced nucleotide levels may be the primary signal which causes the cell wall defects in prsΔ strains. However, this does not appear to be the whole story.

Removal of NHR1-1, which is a characteristic of the PRS1 ORF and is also found in the Prs-associated proteins of rat and man (Becker, 2001; Hernando et al., 1999), completely abolishes the Prs1/Slt2 interaction (Table 2). Other work has shown that deletion of NHR1-1 does not impair the interaction of Prs1 with the other Prs polypeptides (A. Carter, unpublished data). In contrast, there is still a Prs1/Slt2 interaction when Slt2 is co-transformed independently with the two halves of Prs1. It remains for these Prs1/Slt2 interactions to be validated by co-immunoprecipitation.

In the wild-type, incubation at 37 °C leads to an increase in the signal for phosphorylated Slt2 from that obtained at 28 °C in agreement with published data (Lagorce et al., 2003; Martin et al., 2000; Rodriguez-Pachon et al., 2002). Western blot analysis shows that, in contrast to the wild-type, in a prs1Δ strain the signal corresponding to phosphorylated Slt2 is stronger at 28 °C and is maintained over a period of 4 h during incubation at 37 °C (Fig. 7). Interestingly, incubation at 37 °C for a further 2 h resulted in a drastic reduction in the strength of the signal (data not shown). The phosphorylated form of Slt2 in a prs3A strain shows the same profile in a Western blot as that of the wild-type, albeit with a stronger signal at time zero. However, this signal is not lost upon further incubation at 37 °C (data not shown).
The loss of the phospho-Slt2 signal at elevated temperature in the absence of PRS1 together with the Y2H interaction of Slt2 with both Prs1 and Prs3 would suggest that the Prs1/Prs3 complex may have a supporting role in the functioning of the cell integrity pathway. Intriguing possibilities are that it may provide a dedicated source of PRPP for ATP production for Slt2 phosphorylation or that the complex functions as a scaffold or regulates a phosphatase, such as Msg5 (Collister et al., 2002; Flandez et al., 2004; Hahn & Thiele, 2002; Huang & Symington, 1995; Martin et al., 2000) known to be essential for maintaining a low level of signalling through the cell integrity pathway.

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REFERENCES


