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Rck1 and Rck2 MAPKAP kinases and the HOG pathway are required for oxidative stress resistance

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Summary

We demonstrate a role in oxidative and metal stress resistance for the MAPK-activated protein kinases Rck1 and Rck2 in *Saccharomyces cerevisiae*. We show that Hog1 is robustly phosphorylated in a Pbs2-dependent way during oxidative stress, and that Rck2 also is phosphorylated under these circumstances. Hog1 concentrates in the nucleus in oxidative stress. Hog1 localization is partially dependent on Rck2, as *rck2* cells have more nuclear Hog1 than wild-type cells. We find several proteins with a role in oxidative stress resistance using Rck1 or Rck2 as baits in a two-hybrid screen. We identify the transcription factor Yap2 as a putative target for Rck1, and the Zn^{2+} transporter Zrc1 as a target for Rck2. Yap2 is normally cytoplasmic, but rapidly migrates to the nucleus upon exposure to oxidative stress agents. In a fraction of untreated *pbs2* cells, Yap2 is nuclear. Zrc1 co-immunoprecipitates with Rck2, and *ZRC1* is genetically downstream of *RCK2*. These data connect activation of the Hog1 MAPK cascade with effectors having a role in oxidative stress resistance.

Introduction

Oxygen has the potential to cause a range of damage to the living cell through the action of intermediates with different reactivities and cellular distributions. As a result, oxidative damage occurs in all major classes of cellular molecules. Some compounds, like H_2O_2, are direct precursors of the highly reactive hydroxyl radical. Metal ions can contribute to oxidative stress by several mechanisms. Transition metal ions, like Fe^{2+} or Cu^{+}, catalyse the formation of hydroxyl radical through the Fenton reaction. Heavy metal poisoning, such as by Hg^{2+} or Cd^{2+}, can affect proteins with reactive sulphydryl groups, including proteins required for oxidative stress protection. Zinc is a cofactor of many enzymes. Zinc deficiency causes oxidative stress through malfunction of enzymes required for the oxidative stress response. Very high levels of zinc, on the other hand, increase H_2O_2 levels in mammalian cells (May and Contoreggi, 1982), and may lead to oxidative stress through inhibition of glutathione reductase and peroxidase by a mechanism similar to that of Cd^{2+} (Kim *et al*., 1999).

Dealing with oxidative stress requires co-ordination and regulation of the components of the various defence systems. For oxidative stress, several signalling pathways have been implicated to accomplish this. In mammals and fission yeast, stress-activated MAP kinase (MAPK) cascades have been shown to be important. The best-characterized stress-activated MAPK pathway is the HOG pathway of *Saccharomyces cerevisiae*. It comprises a three-layered MAPK cascade, represented by the MAPK Hog1, under control of the MAP kinase kinase (MAPKK) Pbs2 (Brewster *et al*., 1993). Upstream of Pbs2, the system bifurcates into two activating branches. One branch transduces the activating signal from the membrane protein Sln1 ultimately to the MAP kinase kinase kinases (MAPKKK) Ssk2 or Ssk22 (Posas and Saito, 1998). In the second branch, the transmembrane protein Sho1 transmits activation to the MAPKKK Ste11 (Posas *et al*., 1998). As a result of activation of the pathway by any of its upstream branches, Hog1 becomes tyrosine and threonine phosphorylated and migrates from the cytoplasm to the nucleus, where it affects the transcription factors Hot1 (Rep *et al*., 1999), Msn2/Msn4 (Rep *et al*., 2000), Sko1 (Proft *et al*., 2001) and Smp1 (de Nadal *et al*., 2003). The homologous pathway in *Schizosaccharomyces pombe*, the Sty1 MAPK cascade, has a similar structure, except that an upstream activator homologous to the Sho1 branch has not been identified. The Sty1 pathway is readily activated by several stress conditions, including osmotic shock, heat, DNA damage and oxidative stress (Millar *et al*., 1995; Shiozaki and Russell, 1995; Stettler *et al*., 1996; Toone *et al*., 1998). By contrast, the Hog1 pathway has been held to be specific for osmotic stress, and evidence for activation of the Hog1 pathway by oxidative stress has been lacking.

Some of the responses from a MAPK cascade are mediated directly from the MAPK to transcription factors.

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Other responses involve MAPK-activated protein kinases (MAPKAPK). In mammalian cells, MAPKAPK-2 phosphorylates an hnRNP, leading to enhanced translation of certain mRNAs (Rousseau *et al*., 2002). In budding yeast, the two MAPKAPKs, Rck1 and Rck2, were initially found as suppressors of fission yeast checkpoint mutations (Dahlkvist and Sunnerhagen, 1994; Dahlkvist *et al*., 1995). It was later established that Rck2 is phosphorylated by Hog1 under osmostress (Bilsland-Marchesan *et al*., 2000). Upon osmostress, there is a general down-regulation of translation, and this regulation is perturbed in *hog1* and *rck2* mutants (Teige *et al*., 2001; Uesono and Toh, 2002). Together, Rck1, Rck2, Mkp1 and Mkp2 (the two homologues from fission yeast) form a subfamily of protein kinases, which has the C-terminal MAPK-binding domain in common with mammalian MAPKAPKs, and in addition has a distinctive inserted sequence inside the catalytic domain, the 'glycine loop' (Asp and Sunnerhagen, 2003). Mkp1 and Mkp2 are under direct control of Sty1, the fission yeast Hog1 homologue (Sanchez-Piris *et al*., 2002; Smith *et al*., 2002; Asp and Sunnerhagen, 2003). Mutants lacking Mkp2 are slightly sensitive to arsenite (Sanchez-Piris *et al*., 2002).

The present work is an investigation of the activation of the *S. cerevisiae* Hog1 pathway by oxidative stress. We also characterize the interaction of Rck2 with Hog1 in this response. In addition, we identify two putative targets for Rck1 and Rck2. The first is the transcription factor Yap2. The second is the membrane protein Zrc1, a Zn^{2+} transporter. We thus define possible pathways for how Hog1 contributes to resistance to oxidative stress.

Results

Mutations in RCK1 *and* RCK2 *cause sensitivity to oxidative, but not to osmotic, stress*

It was previously shown that disruptions of the *RCK1* or *RCK2* genes do not cause osmosensitivity (Dahlkvist and Sunnerhagen, 1994; Bilsland-Marchesan *et al*., 2000), but that overexpression of the mutant *rck2-kd* allele, which is catalytically inactive, causes osmosensitivity in a wild-type background (Bilsland-Marchesan *et al*., 2000). In a search for phenotypes of the *rck1* and *rck2* null alleles, we found moderate sensitivities to oxidative stress agents, including metal ions. It is seen in Fig. 1A that the *rck2* mutation gives a pronounced growth defect after exposure to tert-butyl hydroperoxide (tBOOH), which is almost as severe as that of a *pbs2* mutant. The *rck1* mutation gives moderate sensitivity to tBOOH. For Cd^{2+}, a similar, but less pronounced, pattern is seen (Fig. 1B).

Other agents of oxidative stress were also examined in this group of mutants for sensitivities. Sensitivity of *hog1* mutants is also observed for paraquat and Cu^{2+} (Fig. 2 and data not shown) but neither for diamide (Fig. 2) nor for menadione (not shown). Out of the compounds shown in Fig. 2, the *rck2* mutation only conferred appreciable sensitivity to Cd^{2+}, thus not to NaCl. To examine whether the sensitivity of HOG pathway mutants (*hog1* and *pbs2*) would be additive with the *rck2* mutation, we included *hog1 rck2* and *pbs2 rck2* double mutants in the analysis. In all cases, the sensitivity of the double mutant was

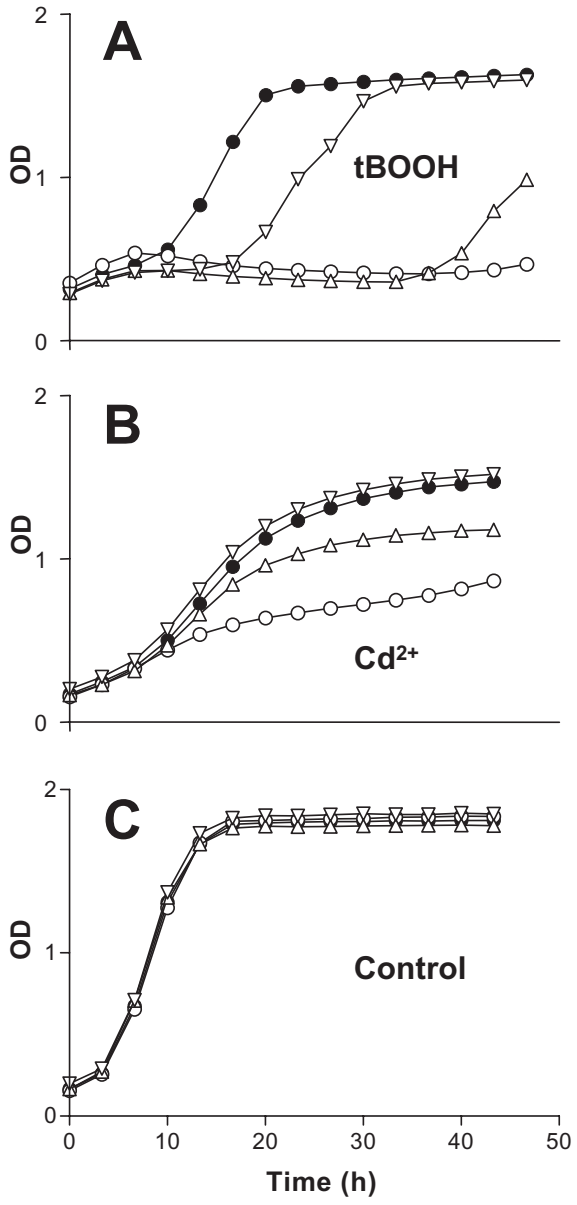

Fig. 1. Sensitivity of mutants to oxidative stress. The following isogenic strains were used: W303-1A (wild-type, ●), WDRCK1-L (*rck1*Δ, ▽); WDRCK2-T (*rck2*Δ, △), DMΔpbs2Wa (*pbs2*Δ, ○). Growth in YPAD at 30°C was recorded in a Bioscreen device as described in *Experimental procedures*. Data shown are the mean of three independent experiments; for clarity error bars are omitted. The media had the following additions: (A) 0.5 mM tertbutyl hydroperoxide (tBOOH); (B)15 μM Cd^{2+} (C) none (control).

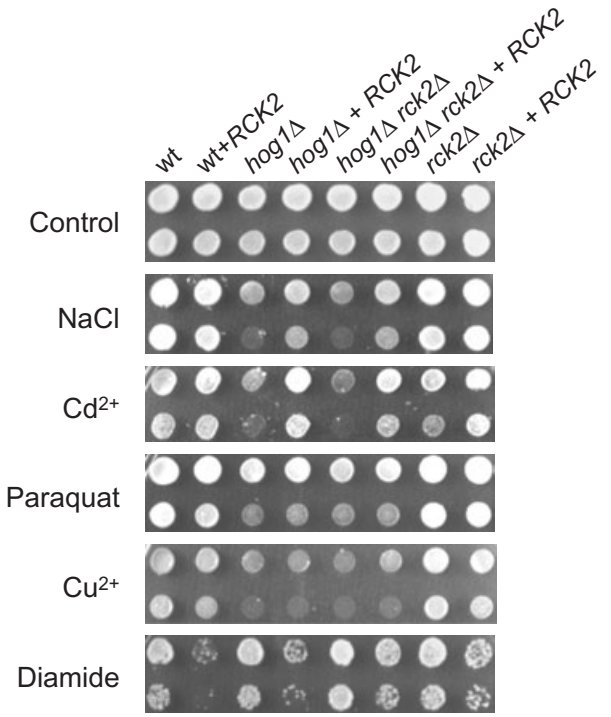

Fig. 2. Mutant sensitivity and suppression patterns for various oxidative stress agents. Stationary phase cultures were serially diluted 1:5 and spotted on plates.The following isogenic strains were used: W303-1A (wild-type), Δhog1Wa (*hog1*Δ), EBDH1,R2W (*hog1*Δ *rck2*Δ), WΔRCK2-T (*rck2*Δ). Strains were transformed with either empty high-copy vector pRS426 or pRSR2 (Bilsland-Marchesan *et al*., 2000; *RCK2* cloned into pRS426).The following concentrations were used: NaCl, 0.4 M; Cd^{2+}, 30 μM; paraquat, 0.25 mM; CuSO4, 1 mM; diamide, 0.8 mM.

indistinguishable from that of the more sensitive single mutant (*hog1* or *pbs2*; Fig. 2 and data not shown), indicating that *RCK2* acts in the same pathway as *HOG1* and *PBS2*.

Sensitivity to oxidative stress in HOG pathway mutants can be suppressed by overexpressing RCK2

It has previously been reported that *hog1* and *pbs2* mutants are sensitive to oxidative stress agents such as H_2O_2 (Singh, 2000). Because overexpression of *RCK2* efficiently suppresses the salt sensitivity of *hog1* mutants (Bilsland-Marchesan *et al*., 2000), and because *rck2* mutants now were shown to be sensitive to some oxidative stress agents, we wanted to examine the relationship between the HOG pathway and *RCK2* also with respect to oxidative stress. Recently, large-scale screens for protein–protein interactions have identified Rck1 as binding to Hog1, using both two-hybrid (Uetz *et al*., 2000) and protein complex purification methods (Ho *et al*., 2002). Likewise, in a directed two-hybrid test, we did find an interaction between Hog1 and the C-terminal portion of Rck1 (not shown). We therefore included *RCK1* in this analysis.

The effect on oxidative stress resistance of overexpression of *RCK1* or *RCK2* in a *hog* background was investigated. A distinct increase of growth is seen upon overexpression of *RCK2* in *hog1* or *pbs2* mutants exposed to tBOOH and Cd^{2+} (Figs 2 and 3A and B). Overexpres-

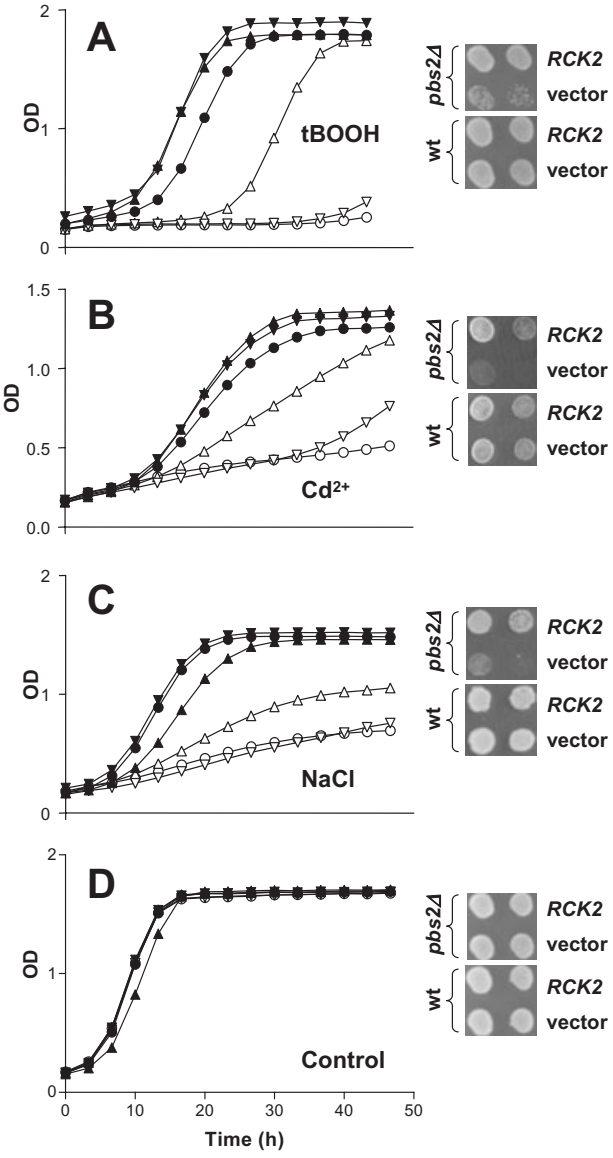

Fig. 3. Suppression of stress sensitivity in *pbs2*Δ cells by overexpression of *RCK1* or *RCK2*. Strain W303-1A (wild-type, filled symbols) and DMΔpbs2Wa (*pbs2*Δ, open symbols) strains were transformed with empty vector pRS426 (●/○), pRSR1 (*RCK1* cloned into pRS426, ▼/▽, or pRSR2 ▲/△). Left panels, stationary phase cultures in SC medium were diluted and either growth recorded in a Bioscreen device as in Fig. 1 (left) or were serially diluted 1 : 5 and spotted on plates (right).
A. tBOOH, 0.3 mM in liquid culture, 1 mM on plates.
B. Cd^{2+}, 30 μM in liquid culture, 60 μM on plates.
C. NaCl, 0.8 M in liquid culture, 0.4 M on plates.
D. Control (no stress agent).

sion of *RCK1* yields only a marginal improvement of resistance under these conditions. Overexpression of *RCK2* in a wild-type background also causes a small increase of growth rate and yield under exposure to tBOOH and Cd^{2+}. For *RCK1* overexpression, the effect under these conditions is more noticeable than in *hog* cells (Fig. 3A and B). It should be noted that the suppression by overexpression of *RCK1* or *RCK2* is at least as efficient as the suppression of osmosensitivity (Fig. 3C).

For other oxidative stress agents, distinct suppression patterns were observed upon overexpression of *RCK2*. While high-copy *RCK2* efficiently suppresses the sensitivity of a *hog1* or of a *pbs2* mutation towards NaCl, tBOOH, or Cd^{2+} (Figs 2 and 3), it has no effect on the sensitivity to Cu^{2+}, and instead causes hypersensitivity towards diamide, both in a wild-type and in a *hog* background. In this work, we focus on the group of oxidative stress agents where overexpression of *RCK2* increases resistance.

Pbs2-dependent phosphorylation of Hog1 during oxidative stress

Although HOG pathway mutants have been reported to be sensitive to oxidative stress, the reason for this was not clear. In previous papers (Schüller *et al*., 1994; Singh, 2000), the authors failed to observe Tyr phosphorylation of Hog1 after treatment with H_2O_2. Therefore, we wanted to ascertain whether the Hog1 MAPK was activated by phosphorylation after exposure to oxidative stress agents used in this work. It is seen in Fig. 4 that a distinct signal representing Hog1 doubly phosphorylated on Tyr and Thr appears after application of stress caused by H_2O_2, tBOOH, Cd^{2+} or Zn^{2+}. The authenticity of this signal is witnessed by the fact that it is absent in *hog1* mutants (Fig. 4, top, right). This Hog1 phosphorylation is dependent on an active HOG pathway, as shown by the absence of the corresponding signal in *pbs2* mutants (Fig. 4, middle). For H_2O_2, tBOOH and Cd^{2+}, the phosphorylation peaks around 60 min post exposure, and then declines to baseline levels within 2–4 h. We compared the Hog1 dual phosphorylation after osmotic stress (0.4 M NaCl) to that found after oxidative stress, and judge that the peak signal intensity obtained from any of the oxidative stress agents is at least as high as that seen after osmostress (Fig. 4, bottom, right).

We also tested other oxidative stress agents for the ability to activate Hog1. Neither paraquat, Cu^{2+}, nor diamide, all at 1 mM, caused detectable Hog1 phosphorylation within this time range (data not shown).

Rck2 is phosphorylated upon oxidative stress

Hog1 phosphorylates Rck2 upon osmotic shock (Bilsland-Marchesan *et al*., 2000), and we wanted to establish whether the same signalling pathway operates during oxidative stress. Using antibodies against Rck2, we examined the time-course of phosphorylation after tBOOH treatment. Figure 5A shows that Rck2 is phosphorylated

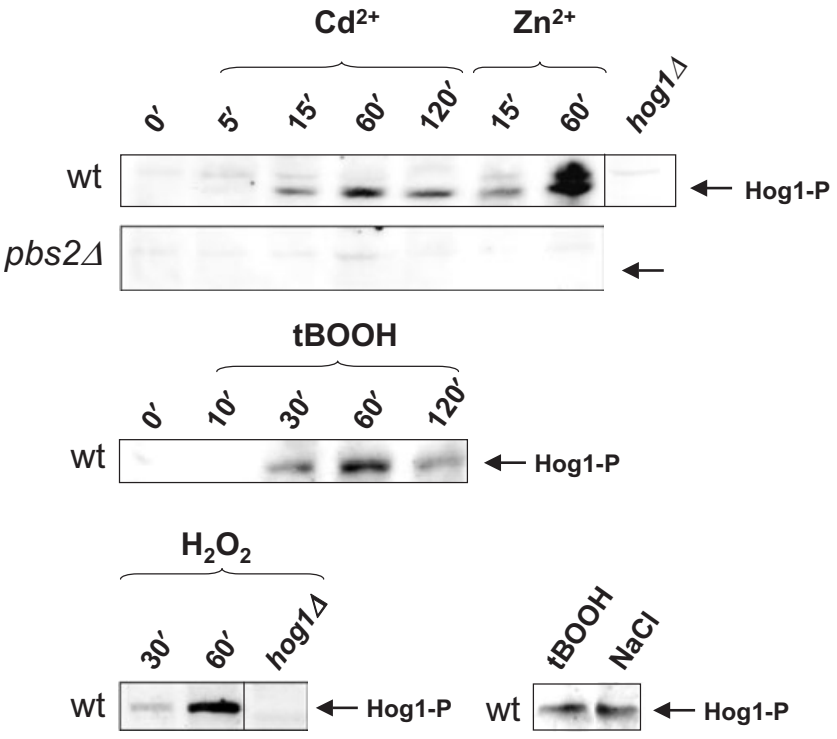

Fig. 4. Tyrosine phosphorylation of Hog1 during oxidative stress. W303-1A (wild-type), DMΔpbs2Wa (*pbs2*Δ) or Δhog1Wa (*hog1*Δ) cells grown in YPAD were exposed to different stress agents for the indicated times. The following concentrations were used: Cd^{2+}, 30 μM; $ZnCl_2$, 10 mM; tBOOH, 3 mM; H_2O_2, 1 mM. The *hog1*Δ negative control (top, rightmost lane) was treated with Cd^{2+} for 30 min. In the bottom right panel, tBOOH treatment was for 60 min, and NaCl treatment for 10 min. Protein extracts prepared under denaturing conditions were electrophoresed on 10% polyacrylamide gels, electroblotted to filters and probed with α-phospho-Tyr-Thr-p38 antibodies, recognizing doubly phosphorylated Hog1 species. Arrows indicate the position of the tyrosine/threonine phosphorylated Hog1 species.

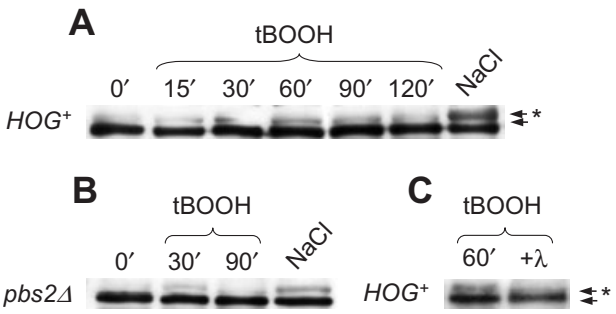

Fig. 5. Phosphorylation of Rck2 during oxidative stress.
A. Cells of strain WΔRCK1-L (*rck1*Δ to obviate cross-reaction of the α-Rck2 antibodies with an Rck1-dependent band migrating close to Rck2) were exposed to 3 mM tBOOH for the indicated times or to 0.4 M NaCl for 10 min.
B. Strain EBΔR1,p2Wa (*rck1*Δ *pbs2*Δ) was treated in an identical manner.
Protein samples were electrophoresed on 8% polyacrylamide gels and analysed by Western blot using α-Rck2 antibodies. Upper band, marked with an asterisk, represents the phosphorylated species.
C. λ phosphatase treatment of a protein sample from WΔRCK1-L cells exposed to tBOOH for 60 min.

with kinetics similar to Hog1 phosphorylation, peaking around 60 min after addition of this stress agent. However, the level of phosphorylation is clearly lower than that seen after hyperosmotic shock (Fig. 5; Teige *et al*., 2001). Also, the phosphorylated Rck2 species seen after oxidative stress migrate closer to the main Rck2 band than those caused by hyperosmosis. To examine if this phosphorylation is dependent on the HOG pathway, we did a corresponding analysis in *pbs2* cells. As seen in Fig. 5B, increased Rck2 phosphorylation can be detected after tBOOH treatment also in such cells, although with less intensity than in the wild-type case. For hyperosmotic stress in *pbs2* cells, Rck2 phosphorylation appears to occur with lower number of phosphates than in the wild-type case.

Localization of Hog1 is affected by oxidative stress and by the presence of Rck2

During osmostress, Hog1 is rapidly, within 5 min, phosphorylated and concentrated in the nucleus (Ferrigno *et al*., 1998; Reiser *et al*., 1999). Because we observe similar phosphorylation levels of Hog1 during oxidative stress, we wanted to see whether it behaves the same also with respect to subcellular localization, using a centromeric Hog1-GFP construct. In agreement with earlier reports, a certain signal from Hog1 was also seen in the nuclei of untreated cells (Fig. 6A). In contrast to the distinct nuclear concentration of Hog1 after osmostress, we observed a less pronounced enrichment of Hog1 in the nucleus after oxidative stress in wild-type cells (Fig. 6B). In untreated *rck2*Δ mutants, the fraction of nuclear Hog1 is greater than in the wild-type (Fig. 6C). This difference is further accentuated after oxidative stress treatment (Fig. 6D).

Rck1 and Rck2 interact in the two-hybrid system with proteins involved in oxidative stress resistance

In order to obtain a wider picture of the cellular role of Rck1 and Rck2, and to identify putative targets for these kinases, we performed genome-wide two-hybrid screens, using two novel Gal4-activation domain (AD) fusion libraries of genomic *S. cerevisiae* DNA. Several screens were performed, using clones expressing full-length or partial Rck1 or Rck2 protein fusions as bait. A common property of many preys identified in these screens is their involvement in various aspects of resistance to oxidative stress and/or transmembrane transport, as summarized in Table 1. Some of the preys were retested in the two-hybrid system for reactivity towards Rck1 or Rck2. In each case, they were found to react with both kinases, with the exception of Yap2, which gave a positive signal only with Rck1.

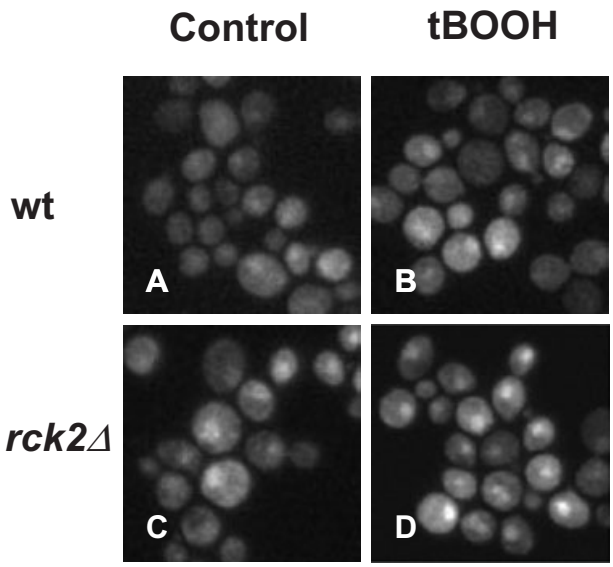

Fig. 6. Subcellular localization of Hog1 after oxidative stress. Hog1 was visualized using the centromeric plasmid pVR65-WT (Reiser *et al*., 1999) expressing Hog1-eGFP from the *HOG1* promoter, at 63× magnification.
A. Wild-type W303-1A cells.
B. W303-1A, 30 min after application of 1.5 mM tBOOH.
C. WΔRCK2-T (*rck2*Δ) cells.
D. WΔRCK2-T (*rck2*Δ), 30 min after application of 1.5 mM tBOOH.
The accumulation of Hog1-GFP in the nucleus in each case was quantified from random photographic fields using ADOBE PHOTOSHOP software. For each cell, the average light intensity in the area manually defined as the nucleus (N) was computed and compared to the average light intensity in the remainder of the cell area (cytoplasm, C). The following values for the relative nuclear enrichment (N–C)/C were obtained (mean and 95% confidence interval): wild-type, 0.44 ± 0.05; wild-type with tBOOH, 0.71 ± 0.07; *rck2*Δ, 0.57 ± 0.04; *rck2*Δ with tBOOH, 1.01 ± 0.08.

Table 1. Prey clones obtained in Rck1 and Rck2 two-hybrid screens.

Gene	Description	Interacting bait product
Yap2	Transcription factor required for Cd^{2+} and oxidative stress resistance	Rck1
Zrc1	Zn^{2+} transporter, vacuolar membrane	Rck1, Rck2
Rod1	ODNB and organic molecules resistance, plasma membrane/cytoplasm	Rck2
Yfr043c	Putative membrane protein, homology to *E. coli* Fe^{2+} transporter	Rck2
Pdr12	Multidrug resistance, ABC transporter	Rck1
Aqr1	Multidrug resistance, major facilitator superfamily	Rck1
Kre11	ER to Golgi transport, TRAPP complex	Rck1, Rck2

The transcription factor Yap2/Cad1 was originally found by virtue of resistance to Cd^{2+} upon overexpression (Wu *et al*., 1993). Overexpression of *YAP2* also results in increased resistance to Zn^{2+}, iron chelators and 1,10-phenantroline (Bossier *et al*., 1993; Wu *et al*., 1993; Lesuisse and Labbe, 1995), whereas the *yap2* mutation yields only weak sensitivity to such compounds except in combination with *yap1* (Wu *et al*., 1993; Stephen *et al*., 1995; Fernandes *et al*., 1997), indicating partially overlapping roles in the response to oxidative stress.

ZRC1/*OSR1* was originally identified as a gene-conferring resistance to Zn^{2+} and Cd^{2+} when overexpressed (Kamizono *et al*., 1989). It is a vacuolar membrane transporter pumping Zn^{2+} from the cytoplasm into the lumen of the vacuole (MacDiarmid *et al*., 2000; Miyabe *et al*., 2000). The *ZRC1* gene is under control of the Zap1 transcription factor, and is induced under conditions of zinc deficiency (Lyons *et al*., 2000).

Other proteins identified in this screen represent membrane transporters of metal ions and organic compounds, and a protein required for ER to Golgi protein translocation (Table 1). The phenotypes associated with the cognate genes indicate a role in oxidative stress resistance. Thus, *rod1* mutants are sensitive to *o*-dinitrobenzene, a glutathione *S*-transferase inhibitor (Andoh *et al*., 2002), and to a variety of other compounds. Rod1 was previously found to complex with Rck1 in a large-scale screen of physical protein interactions (Ho *et al*., 2002). The ABC transporter Pdr12 is required for resistance to citric acid, a compound recently demonstrated to activate Hog1 (Lawrence *et al*., 2004). Aqr1 belongs to the major facilitator superfamily, and contributes to resistance to quinidine and crystal violet (Tenreiro *et al*., 2002). Kre11 is a member of the TRAPPII complex, required for ER to Golgi protein transport (Sacher *et al*., 2000). In the current work, we focus on two of these putative interactants, Yap2 and Zrc1, and their interaction with Rck1 and Rck2 respectively.

Interactions between Rck1 and Yap2

We examined whether a physical interaction between Yap2 and Rck1 indicated by the two-hybrid system could be verified by an independent method. In Fig. 7A, it is seen that HA-Rck1 is coprecipitated with GFP-Yap2. We further investigated possible genetic interactions between *YAP2* and *RCK1*. We found that the sensitivity of *rck1* mutants, but not of *rck2* mutants, to tBOOH can be fully suppressed by overexpression of *YAP2* (Fig. 7B).

Yap2 translocates to the nucleus on exposure to oxidative stress agents

The subcellular localization of Yap2 in oxidative stress has not been previously reported. We investigated the localization of Yap2 by fluorescence microscopy of a Yap2-Gfp fusion expressed from an episomal 2μ-based construct. In untreated cells, Yap2 is exclusively cytoplasmic (Fig. 8A). Soon after addition of Cd^{2+}, Yap2 translocates to the nucleus in wild-type cells. Already at 2 min post exposure, a minor fraction of wild-type cells have accumulated Yap2 in the nucleus, and at 15 min, over 75% of cells carry Yap2 in the nucleus (Fig. 8B). With other oxidative stress agents, Yap2 goes to the nucleus with similar kinetics and extent. Thus, nuclear Yap2 is seen in the majority of cells around 15 min after addition of tBOOH, Zn^{2+} or diethyl maleate (Fig. 8C and D, and data not shown). At longer times (2–3 h after initial exposure), Yap2 re-enters the cytoplasm, even if the exposure to the oxidative stress agent persists (data not shown).

We noted that in a population of *pbs2* cells, a significant fraction (about 10%) carry Yap2 in the nucleus also in the absence of added oxidative stress agents, something that was never observed in wild-type cells (cf. Fig. 8A and E). Yap2 does enter the nucleus upon tBOOH treatment in *pbs2* mutants to the same degree as in wild-type cells (Fig. 8F).

Genetic and physical interactions between Rck2 and the metal ion transporter Zrc1

We examined the *in vivo* interaction between Rck2 and Zrc1 using coimmunoprecipitation. In Fig. 9A, a band representing Zrc1-myc immunoprecipitated with α-Rck2 antibodies is indeed clearly visible. In Fig. 9B–D, we examined the effect on resistance to high Zn^{2+} by different genetic changes affecting *ZRC1* and *RCK2*. In wild-type cells, overexpression of *RCK2* gives a distinct increase in growth rate (Fig. 9B and C). A *zrc1*Δ mutation gives very marked sensitivity to high Zn^{2+} (Fig. 9C and D). This sen-

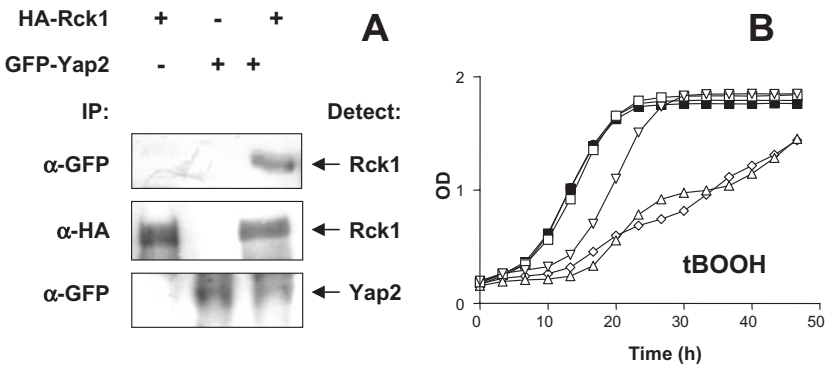

Fig. 7. Interactions between Rck1 and Yap2.
A. Coimmunoprecipitation of Rck1 and Yap2. Cells expressing HA-tagged Rck1 (EBgHAR1Wa, lane 1), GFP-tagged Yap2 (EBgGY2Wa, lane 2) or both (EBgHAR1gGY2Wa, lane 3) were grown in YP-galactose to induce expression of the tagged proteins. Lysates were immunoprecipitated with α-GFP (top) for test of a binding between Rck1 and Yap2, with α-HA (middle) for verification of expression of HA-Rck1 or with α-GFP (bottom) for verification of expression of GFP-Yap2. Detection on filters was with α-HA or α-GFP as indicated.
B. Suppression of sensitivity to tBOOH in *rck1* mutants by overexpression of *YAP2*. Growth in SC medium in the presence of 0.3 mM tBOOH was recorded as in Fig. 1. The following combinations of strains and plasmids were used: W303-1A (wild-type), empty pRS426 vector (●); W303-1A, pRSY2 (*YAP2* expressed from its own promoter in high-copy) (■); WDRCK1-L (*rck1*Δ), pRS426 (▽); *rck1*Δ, pRSY2 (□); WDRCK2-T (*rck2*Δ), pRS426 (△); rck2D, pRSY2 (◇).

sitivity, however, is unaffected by overexpression of *RCK2* (Fig. 9C and D). Overexpression of *ZRC1*, on the other hand, improves Zn^{2+} resistance considerably, both in a wild-type and in an *rck2* background (Fig. 9B–D). This places *ZRC1* genetically downstream of *RCK2*.

Discussion

It has long been held that the *S. cerevisiae* HOG pathway would be activated in response to osmotic stress only, and not to, for example oxidative or metal stress. This would be in contrast to the situation in fission yeast, mammals and plants, where the homologous stress-activated MAPKs and their upstream components respond to a wide variety of environmental stress agents. Even in the more closely related yeast *Candida albicans*, the Hog1 homologue is activated upon oxidative stress (Alonso-Monge *et al.*, 2003). In *S. cerevisiae*, activation of the

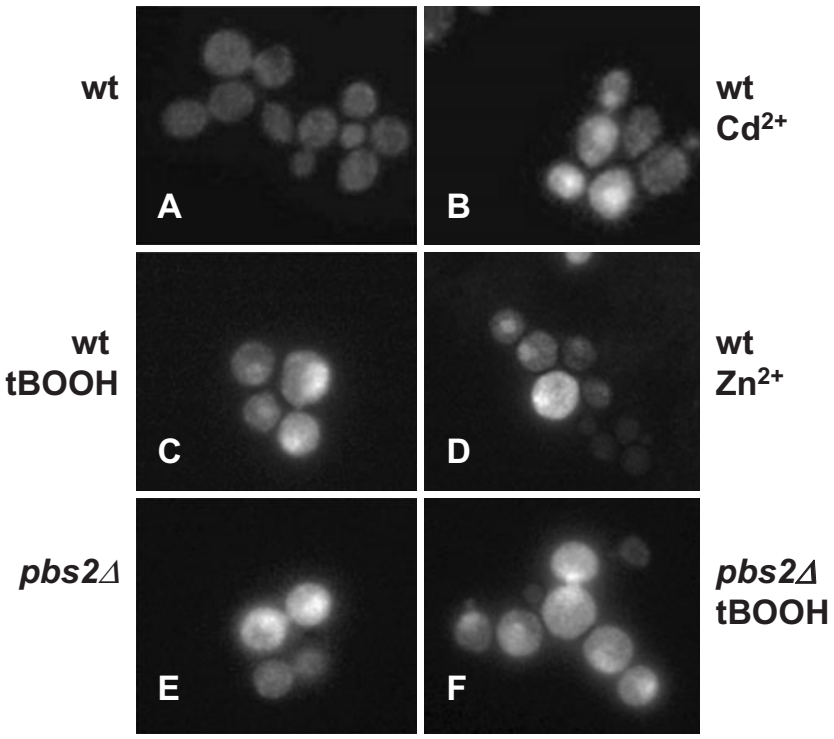

Fig. 8. Subcellular localization of Yap2. Yap2 was visualized using the multicopy plasmid pRSY2G, expressing Yap2-eGFP from the *YAP2* promoter. Images were taken at 15 min after addition of the respective stress agent except in D, which was taken at 30 min.
A. Wild-type W303-1A cells, no stress agent.
B. W303-1A, Cd^{2+} (30 μM).
C. W303-1A, tBOOH (1.5 mM).
D. W303-1A, Zn^{2+} (10 mM).
E. DMΔpbs2Wa (*pbs2*Δ), no stress agent.
F. *pbs2*Δ, tBOOH (1.5 mM).

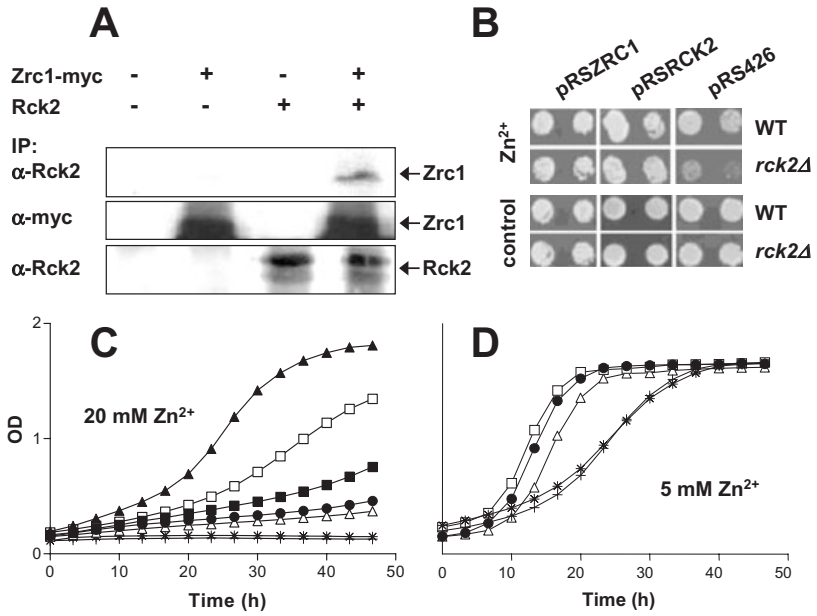

Fig. 9. Interactions between Rck2 and Zrc1.
A. Coimmunoprecipitation of Rck2 and Zrc1. W303-1A (wild-type) or WΔRCK2-T (*rck2*Δ) cells were transformed with empty vector pRS426, or with pRSZrc1-myc (expressing Zrc1 C-terminally tagged with myc). Lysates were immunoprecipitated with α-Rck2 (top) for test of a binding between Rck2 and Zrc1, with α-myc (middle) for verification of expression of Zrc1-myc, or with α-Rck2 for verification of expression of Rck2 (bottom).
B. Suppression of sensitivity to high Zn^{2+} in *rck2* mutants by overexpression of *ZRC1* as seen in plate assays. Procedure as in Fig. 2, right panels. Combinations of the following strains and plasmids were used as indicated: W303-1A (wild-type), WΔRCK2-T (*rck2*Δ); pRSZrc1 (high-copy *ZRC1*), pRSRCK2 (high-copy *RCK2*), empty vector pRS426.
C. Growth assays in liquid showing suppression of sensitivity to high Zn^{2+} in *rck2* mutants by overexpression of *ZRC1*, and lack of suppression in *zrc1* mutants by overexpression of *RCK2*. Cells were grown in SC containing 20 mM Zn^{2+}. Procedure as in Fig. 1. The following combinations of strains and plasmids were used: BY4741 (wild-type), empty pRS426 vector (●); wild-type, pRSRCK2 (▲); wild-type, pRSZrc1-myc (■); BYrck2 (*rck2*Δ), pRS426 (△); *rck2*Δ, pRSZrc1-myc (□); BYzrc1 (*zrc1*Δ), pRS426 (*); *zrc1*Δ, pRSRCK2 (+).
D. As in C but 5 mM Zn^{2+}.

parallel Pkc-dependent MAPKs pathway by oxidative stress has been demonstrated (Alic *et al*., 2003).

There is published evidence that *S. cerevisiae* HOG pathway-deficient mutants are sensitive to oxidative stress. Thus, Singh (2000) showed that *hog1* and *sln1 ssk1* mutants are sensitive to H_2O_2. However, no tyrosine phosphorylation of Hog1 on oxidative stress was observed in that work. Likewise, no Hog1 phosphorylation was seen at 10 min after addition of 0.4 mM H_2O_2 (Schüller *et al*., 1994). We show here that Hog1 is phosphorylated to high levels upon oxidative and metal stress, as seen with Cd^{2+}, Zn^{2+}, H_2O_2 and tBOOH. Likely explanations for the discrepancy between our results and those in earlier work are dosage and timing. Singh (2000) used 10 mM H_2O_2, which entails high cell lethality. We show in this article that Hog1 is tyrosine phosphorylated upon oxidative and metal stress, and that this phosphorylation is dependent on *PBS2*. The peak of Hog1 phosphorylation occurs relatively late, at 45–60 min after application of oxidative stress, significantly later than the time point examined by Schüller *et al*. (1994). This slower kinetics contrasts with the rapid activation of Hog1 by hyperosmotic stress, and with activation of the homologue in *S. pombe*, Sty1, upon oxidative stress. This opens the possibility that the activation mechanism could be different in *S. cerevisiae*. Pertinent to this issue, the *S. cerevisiae* membrane-bound receptor Sln1 lacks PAS domains, which are believed to sense oxidative stress, whereas such domains are present in the *S. pombe* homologues, Mak1-3. Recently, it has been shown that the HOG pathway is activated upon heat or citric acid stress (Winkler *et al*., 2002; Lawrence *et al*., 2004). For heat stress, this activation requires the Sho1, but not the Sln1, activation branch of the pathway (Winkler *et al*., 2002). A role for Hog1 in survival in stationary phase, mediated by the transcription factor Smp1 has recently been demonstrated (de Nadal *et al*., 2003). Clearly, the response of the HOG pathway is not limited to hyperosmotic stress. Particularly relevant to our present work, Hog1 has been demonstrated to become Tyr phosphorylated upon heavy metal ion stress (M. Tamás, pers. comm.).

In line with our previous finding that Rck2 becomes phosphorylated upon hyperosmotic shock (Bilsland-Marchesan *et al*., 2000), we now see that Rck2 is phosphorylated also during oxidative stress. However, the degree of Rck2 phosphorylation is markedly lower than

under hyperosmotic shock. This is somewhat surprising, given that the level of Hog1 phosphorylation is similar under the two conditions, and given that Rck2 appears to be more significantly implicated in the defence against oxidative than hyperosmotic stress (see below). In fission yeast, oxidative stress induces robust phosphorylation of the Rck2 homologue Cmk2/Mkp2 (Sanchez-Piris *et al.*, 2002). Possibly, this reflects a more vigorous role of the Sty1 pathway in *S. pombe* than of the homologous Hog1 pathway in protection against oxidative stress. Phosphorylation of Rck2 in oxidative stress is only partially HOG-dependent, indicating the possibility that Rck2 could also be phosphorylated by another kinase under these conditions. Another observation pointing in the same direction is that we do observe oxidative stress-induced phosphorylation of a mutated version of Rck2 (Teige *et al.*, 2001), where both Hog1-dependent phosphorylation sites have been changed to non-phosphorylatable residues (data not shown). That the interaction between Hog1 and Rck2 in oxidative stress could be functionally quite complex is further indicated by the distinct patterns of sensitivity that are seen for different oxidative stress agents upon deletion of *HOG1* and/or overexpression of *RCK2.* Thus, diamide and Cu^{2+} deviate drastically in this respect from the group of tBOOH, Cd^{2+} and Zn^{2+}. The former compounds are also those where *hog* mutants are sensitive, but Hog1 phosphorylation has not been detected. Together, these observations are consistent with Hog1 and Rck2 each contributing to oxidative stress resistance independently of each other under some circumstances.

Hog1 becomes phosphorylated after oxidative stress to a level comparable to that seen after hyperosmotic stress. Still, Hog1 concentration in the nucleus is less pronounced after oxidative than hyperosmotic stress. The mechanistic reason for this difference is not clear, but one obvious difference between activation of Hog1 through oxidative versus hyperosmotic stress is the slower kinetics. During oxidative stress, phosphorylation peaks at 45 min (Fig. 4; this article), compared to at 5 min after osmotic shock (Ferrigno *et al.*, 1998). Even though phosphorylation of Hog1 by Pbs2 upon osmotic stress does cause a translocation of Hog1 from the cytoplasm to the nucleus (Ferrigno *et al.*, 1998), the localization of Hog1 is not a simple function of its phosphorylation status. Relocalization of Hog1 to the cytoplasm does not require the tyrosine phosphatases Ptp2 and Ptp3. Furthermore, Ptp2 acts as a nuclear anchor for Hog1, and Ptp3 as a cytoplasmic anchor (Mattison and Ota, 2000). We have found Hog1 to accumulate more in the nucleus in *rck2*Δ than in wild-type cells. This suggests a role for Rck2 as a cytoplasmic anchor for Hog1, and would be consistent with the reported cytoplasmic localization of Rck2 (Melcher and Thorner, 1996). Potentially, the difference in Hog1 localization after hyperosmotic versus oxidative stress is related to the difference in Rck2 phosphorylation level under the same two conditions. The association between Hog1 and Rck2 is constitutive and relatively tight (Bilsland-Marchesan *et al.*, 2000), as well as the association between the homologues Sty1 and Mkp1 in *S. pombe* (Asp and Sunnerhagen, 2003). In mammalian cells, the cytoplasmic localization of the MAPK p38 is regulated by its binding to MAPKAPK-2 (Ben-Levy *et al.*, 1998). The physiological relevance of the attenuated nuclear concentration during oxidative, compared to osmotic, stress could be that cytoplasmic targets of Hog1 are more significant under those conditions.

The physiological role of Rck2 was initially defined in the context of hyperosmotic stress (Bilsland-Marchesan *et al.*, 2000; Teige *et al.*, 2001). Here, we present evidence that the major role of Rck2 may be in dealing with oxidative, rather than osmotic, stress. First, *rck2* null mutants are sensitive to several agents of oxidative stress (this paper), but not noticeably sensitive to osmotic stress (Dahlkvist and Sunnerhagen, 1994; Bilsland-Marchesan *et al.*, 2000). Second, overexpression of *RCK2* gives increased resistance to several agents of oxidative stress even in a wild-type background, but has no effect on osmoresistance in wild-type cells. Third, our two-hybrid screen using Rck2 as the bait identified proteins involved in resistance to oxidative and metal stress, but not to osmotic stress. A similar argument based on interacting proteins found in the two-hybrid system can be made about Rck1.

We find that the transcription factor Yap2, similar to Yap1, is cytoplasmic in unstressed cells, but migrates to the nucleus after exposure to oxidative stress. The localization of Yap1 is governed by a direct cysteine oxidation mechanism, which masks a nuclear export signal (Delaunay *et al.*, 2000). The kinetics of nuclear translocation of Yap2 under oxidative stress is much faster than activation of Hog1, and Yap2 goes to the nucleus also in *pbs2* mutants. Therefore, the Hog1 pathway is not required for nuclear import of Yap2. Our finding that Yap2 is nuclear in a fraction of *pbs2* cells even under normal growth conditions could indicate that such mutant cells are unable to deal with low levels of oxidative stress, and could accumulate oxidized species inducing Yap2 to translocate to the nucleus. Alternatively, *PBS2*-dependent phosphorylation may be required to keep Yap2 cytoplasmic. Our present data cannot distinguish between these alternatives. Yap2 binds to Rck1, which is cytoplasmic under the conditions we have studied (data not shown). The role of Rck1 might be in regulating nuclear re-export of Yap2, because activation of Rck1 by the Hog pathway would be too slow for it to have a role in its initial nuclear import.

Rck2 binds to Zrc1, a membrane-bound Zn^{2+} transporter. Zrc1 is believed to protect cells from excess Zn^{2+} when cells are suddenly shifted from zinc-deficient to zinc-replete conditions (MacDiarmid *et al.*, 2003). Rck2 is

important for survival under high Zn^{2+}, as *rck2* mutants are quite sensitive, and overexpression of *RCK2* yields hyper-resistance in a wild-type background. However, in a *zrc1* background, overexpression of *RCK2* has no effect (Fig. 9C and D). This indicates that Zrc1 is the major effector of the zinc resistance caused by Rck2. Further experiments will establish how the interaction with Rck2 enhances the capacity of Zrc1 to manage homeostasis under high zinc conditions.

In this work, we provide a road map for signal transduction from the Hog1 MAPK cascade, through the MAPKAP kinases Rck1 and Rck2, to proteins acting at different levels of cellular homeostasis. The summed weight of evidence, including sensitivity of mutants, indicates that this branch of the pathway is particularly important for handling of oxidative stress. Our results demonstrate that the Hog1 MAPK cascade is activated during oxidative stress, and that MAPKAP kinases affect the response at multiple levels: subcellular localization of the MAPK, interactions with transcription factor and membrane-bound transporters. The physiological significance of many of the individual protein–protein contacts remains to be worked out, however. The establishment of a role for the Hog1 pathway in metal and oxidative stress resistance will provide additional tools for the study of mechanisms of oxidative stress resistance, using the efficient genetics in budding yeast, which will be applicable in other organisms.

Experimental procedures

Strains and culture conditions

All *S. cerevisiae* strains used are shown in Table 2. For non-selective conditions, the medium was YPAD (1% yeast extract, 2% peptone, 2% glucose, 100 mg l^{-1} of adenine). For maintenance of plasmids, SD medium was used (0.67% yeast nitrogen base, 2% glucose, plus supplements as required; 40 mg l^{-1} each of uracil, histidine and tryptophan; 100 mg l^{-1} of adenine, 150 mg l^{-1} of leucine). In experiments where GFP fusions expressed from episomal constructs were to be visualized, cells were grown overnight in SD, and then transferred to YPD 2 h before microscopy.

Plasmid construction

pRSR1 was constructed by subcloning a 3.6 kb *Bst*YI fragment containing the *RCK1* gene into the *Bam*HI site of pRS426. A high-copy plasmid expressing *YAP2* from its own promoter was made by polymerase chain reaction (PCR) amplification from genomic DNA of strain W303-1A, and insertion between the *Bam*HI and *Xho*I sites of pRS426, to make pRSY2. From pRSY2, an analogous construct expressing a Yap2-eGFP fusion was made by inserting an eGFP-HIS3MX6 cassette, obtained by PCR amplification from pFA6a-GFP(S65T)-HIS3MX6 (Longtine *et al*., 1998) with primers EBY2fusF2 and EBY2fusR1, using homologous recombination *in vivo* (Muhlrad *et al*., 1992), to make pRSY2G. A high-copy plasmid expressing Zrc1 C-terminally tagged with GFPuv (Stratagene) was made by PCR amplification from genomic DNA using SSZRC1fus-F and SSZRC1fus-R. The PCR product was cotransformed into yeast with pRS426Gfp-UV digested with *Hin*dIII, to yield pRSZrc1GFP-UV. To construct a plasmid expressing Zrc1 C-terminally tagged with myc, a PCR product was made using primers SSZrc1-Myc-F and EBGfp-to-MycR, and template pFA6a-13myc-HIS3MX6 (Longtine *et al*., 1998). This was cotransformed into yeast with pRSZrc1GFP-UV digested with *Sma* I, finally to yield PRSZrc1-myc. For recovery of plasmids from yeast for transformation of *Eschichia coli*, DNA was purified on Qiagen columns using a modification of the protocol for plasmid preparation from *E. coli* supplied by the

Table 2. *S. cerevisiae* strains used.

Name	Genotype	Source or reference
UMY1705	*MATa ura3-52 his3-Δ200 ade2-101 lys2-801 trp1-901 tyr1-501 gal4-Δ452*	A. Byström (= JB974 from M. Johnston)
PJ69-4A	*MATa ade2 trp1-109 leu2-3112 ura3-52 his3-200 gal4Δ gal80Δ GAL2:ADE2 lys2::GAL1:HIS3 met2::GAL7:lacZ*	James *et al*. (1996)
PJ69-4α	*MATα ade2 trp1-109 leu2-3112 ura3-52 his3-200 gal4Δ gal80Δ GAL2:ADE2 lys2::GAL1:HIS3 met2::GAL7:lacZ*	James *et al*. (1996)
W303-1A	*MATa ade2-1 leu2-3112 ura3-1 his3-11 trp1-1a can100*	H. Ronne
WΔRCK1-L	*MATa rck1::LEU2 ade2-1 leu2-3112 ura3-1 his3-11 trp1-1a can100*	Dahlkvist and Sunnerhagen (1994)
WΔRCK2-T	*MATa rck2::TRP1 ade2-1 leu2-3112 ura3-1 his3-11 trp1-1a can100*	Dahlkvist and Sunnerhagen (1994)
WΔRCK1,2-LT	*MATa rck1::LEU2 rck2::TRP1 ade2-1 leu2-3112 ura3-1 his3-11 trp1-1a can100*	Dahlkvist and Sunnerhagen (1994)
Δhog1Wa	*MATa hog1::TRP1 ade2-1 leu2-3112 ura3-1 his3-11 trp1-1a can100*	S. Hohmann
DMΔpbs2Wa	*MATa pbs2::kanMX6 ade2-1 leu2-3112 ura3-1 his3-11 trp1-1a can100*	Bilsland-Marchesan *et al*. (2000)
EBΔR1,P2Wa	*MATa rck1::LEU2, pbs2::kanMX4, ade2-1, leu2-3,112, ura3-1, his3-11 trp1-1a can100*	This work
EBDH1,R2W	*MATa rck2:: kanMX4, hog1::LEU2 ade2-1 leu2-3112 ura3-1 his3-11 trp1-1a can100*	This work
EBR2-HAWa	*MATa RCK2:HA$_{(3)}$:HIS3MX6 ade2-1 leu2-3112 ura3-1 his3-11 trp1-1a can100*	This work
EBgGY2Wa	*MATa HIS3MX6:GAL1:eGFP:YAP2 ade2-1 leu2-3112 ura3-1 his3-11 trp1-1a can100*	This work
EBgHAR1-gGY2Wa	*MATa kanMX6:GAL1:HA$_{(3)}$:RCK1 HIS3MX6:GAL1:eGFP:YAP2 ade2-1 leu2-3112 ura3-1 his3-11 trp1-1a can100*	This work
EBgHAR1Wa	*MATa kanMX6:GAL1:HA$_{(3)}$:RCK1 ade2-1 leu2-3112 ura3-1 his3-11 trp1-1a can100*	This work
BY4741	*MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*	Brachmann *et al*. (1998)
BYzrc1	*MATa zrc1::kanMX6 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*	EUROSCARF
BYrck2	*MATa rck2::HIS3MX6 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*	EUROSCARF

manufacturer. Yap2- or Zrc1-expressing plasmids were verified by restriction digestion and by the ability to confer hyperresistance to Cd^{2+}, or to complement the Zn^{2+}-sensitivity of *zrc1* mutants respectively.

Assays of sensitivity to oxidative stress agents

Cells were grown to stationary phase in liquid YPAD, or in SC plus supplements if selection for plasmids was necessary. Serial dilutions were made in YPAD in 96-well microtitre plates, cells were replica plated onto agar medium containing the various compounds to be tested, and grown at the indicated temperatures. Alternatively, cells were taken from pregrowth to at least 36 h of stationary phase. Samples were diluted 70-fold, and growth was continued at 30°C in 400 µl microchambers containing medium with the compounds to be tested, with shaking in a Labsystems Bioscreen C Microbiology Workstation. Cell density was measured as turbidity using a wide-band visible light filter.

Construction of high-complexity genomic Gal4-AD fusion libraries from S. cerevisiae

Genomic DNA was prepared from strain UMY1705. This strain is *his3* and *ade2* deficient, to avoid unwanted complementation of the corresponding markers in the recipient strain. It also has the entire *GAL4* coding sequence removed, to eliminate the possibility of activation of marker genes by *GAL4*-containing clones in the library. The DNA was sheared by sonication to average sizes 2 or 4 kb, pooled into two separate size fractions, and ligated to 5′-phosphorylated *Xho*l adapters. The DNA was then size fractionated on a sucrose gradient. λACT2 (Durfee *et al*., 1993) was digested with *Xho*l, and the ends were thereafter partially filled using Klenow enzyme and dTTP. The two pools of adapter-ligated and size-fractionated genomic DNA were then separately ligated to the λACT2 vector, and the mixtures were packaged into λ particles *in vitro* using Stratagene GigaPack™ packaging extract and used to infect *E. coli* LE392. By PCR analysis of individual plaques from the resulting libraries, it was estimated that the 'short insert' library has an insert size range of 0.3–3 kb (average size 0.8 kb), and a complexity of 1.8×10^6 independent recombinants; 45% of the clones carry an insert. The 'long insert' library has an insert size range of 2–6 kb (average size 3 kb), and a complexity of 1.4×10^6 independent recombinants; 40% of the clones carry an insert. For conversion into plasmid (pACT2) form, samples of the λ phage library stocks were used to infect the *E. coli* λ lysogenic strain BNN322 (Elledge *et al*., 1991). The resulting ampicillin-resistant colonies ($>10^7$) were pooled and used to prepare standard plasmid lysates. These plasmid libraries were then transformed into *S. cerevisiae* strain PJ69-4A in large batches, and transformants were pooled and frozen at −70°C. By plating aliquots of the transformants on SC lacking leucine, it was estimated that $>10^7$ yeast transformants were obtained for each library.

Two-hybrid screening

Two-hybrid bait clones were constructed using gap repair *in vivo* (Muhlrad *et al*., 1992). Full-length or partial coding sequences from *RCK1* or *RCK2* were PCR amplified from *S. cerevisiae* FY1679 genomic DNA with Roche Expand High Fidelity™ system. Hybrid primers carried about 20 nucleotides of sequence homology to the bait gene and 30 nucleotides of homology to either side of the cloning site in the Gal4 DNA-binding domain (Gal4-DB) vector pGBT9 (Bartel *et al*., 1993). These PCR products were then cotransformed in 100-fold molar excess with pGBT9 restricted with *Bam* HI and *Eco*R1 into *S. cerevisiae* PJ69-4α. Randomly sampled plasmids recovered into *E. coli* from yeast tryptophan prototrophs obtained in this manner were checked by restriction and were found to contain an insert of the correct size in >90% of all cases. Some of these plasmids were retransformed into yeast, and the size of the expressed fusion protein was checked by Western blot analysis, using α-Gal4 antibodies. It was found that all the examined plasmids expressed hybrid proteins of the expected size (not shown). This indicates that the majority of bait plasmids constructed by gap repair were recombinant and encoded full-length protein.

About 100 colonies of bait transformants were pooled, grown in liquid selective medium and used for mass mating on filters (Bendixen *et al*., 1994) with an aliquot of PJ69-4a transformed with one of the *S. cerevisiae* genomic DNA Gal4-AD fusion libraries.

Diploid cells with a functional two-hybrid interaction were selected on medium lacking tryptophan, leucine and histidine, and containing 3 mM 3-aminotriazole (3-AT) and 2 mg l^{-1} of adenine. The identity and reading frame of genes in prey plasmids was verified by partial sequencing.

Positive prey clones obtained in the library screens were retested by reconstructing clones encoding full-length protein by PCR and inserting into the AD vector pACT-2 by gap repair, as described above for bait plasmids. In all cases, the full-length prey clones reacted as strongly with the respective bait clone in the two-hybrid system as the initial library clone encoding a partial protein fused with Gal4-AD (not shown).

Fluorescence microscopy

Cells were imaged after fixation for 20 min in 80% methanol at −20°C. DNA was visualized by the addition of 4,6-diamino-2-phenylindole (DAPI) to a concentration of 20 ng ml^{-1} after fixation. Intracellular fluorescence was visualized in a Leica DM RXA microscope at 100 or 63× magnification with a Fluotar lens. The following filters were used: all variants of GFP, Leica 513852, DAPI, Leica 513824. Photographs were taken with a Hamamatsu ORCA-cooled CCD camera.

Protein preparation and Western blot

Cells were grown to mid-log phase, centrifuged and washed once in buffer A: 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 0.2% Triton X-100, complemented with Complete^R protease inhibitor mix (Roche). Cells were then lysed in the same buffer using a FastPrep 120 apparatus at speed 4 for 15 s.

Twenty micrograms of total protein per lane was loaded on SDS-polyacrylamide gels, electrophoresed and blotted onto PROTRAN nitrocellulose filter (Schleicher and Schuell) in a semidry blotting device (Sigma Aldrich). Primary antibodies were: polyclonal α-native Rck2 (Ramne *et al*., 2000),

monoclonal α-phospho-Tyr180-phospho-Thr182-p38 (New England Biolabs), monoclonal α-HA, monoclonal α-GFP (Sigma) or monoclonal α-c-Myc (Santa Cruz Biotechnology). Detection was with the ECL Western Blotting Analysis System (Amersham Pharmacia), using as secondary antibodies HRP-linked α-mouse monoclonal IgG (Amersham Pharmacia) or α-rabbit Ig-POD F_{ab} fragment (Roche). For phosphatase treatment, extracts were prepared in phosphatase buffer (50 mM Tris/HCl, pH 8.0; 2 mM DTT; 0.1% Triton X-100; 1 mM PMSF) and incubated with 400 U of λ phosphatase (New England Biolabs) for 15 min at 30°C.

Coimmunoprecipitation

Protein extracts (0.5 mg) were first precleared by the addition of Pansorbin (formalin-fixed *S. aureus* cells), incubated for 2 h at 4°C and centrifuged. For precipitation, 4 μg of the precipitating antibody was added to the supernatant. After 4 h of head-over-head incubation at 4°C, Pansorbin was added, and head-over-head incubation was continued overnight at 4°C. Precipitates were collected by high-speed centrifugation, washed in buffer A containing 0.25% NP-40 (final NaCl concentration 50 mM for the Rck1–Yap2 interaction, and 150 mM for the Rck2–Zrc1 interaction) and finally resolved in SDS–PAGE.

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Note added in proof

Independently of our research, Tyr phosphorylation of Hog1 upon H_2O_2 treatment has been reported recently Haghnazari, E., and Heyer, W.D. (2004) The Hog1 MAP kinase pathway and the Mec1 DNA damage checkpoint pathway independently control the cellular responses to hydrogen peroxide. *DNA Repair* **3:** 769–776.

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