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Rck1 up-regulates Hog1 activity by down-regulating Slt2 activity in *Saccharomyces cerevisiae*

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ABSTRACT

We previously reported that the over-expression of KDX1 up-regulates RCK1 gene expression. To further understand the function of Rck1, microarray analysis was performed using a RCK1 over-expressing strain. Based on microarray and Northern blot analyses, we determined that the expression of KDX1 was down-regulated when RCK1 was over-expressed. Furthermore, we determined that phosphorylated forms of Slt2 and Mkk2 were down-regulated by the over-expression of RCK1. Ptp2, a phosphatase that is regulated by the Slt2 MAP kinase pathway, was down-regulated by the over-expression of RCK1. Ptp2 is a negative regulator of Hog1; thus, the phosphorylated form of Hog1 was up-regulated by RCK1 over-expression. A point mutation of lysine 152 to arginine resulted in a failure to up-regulate Hog1 and the subsequent down-regulation of CTT1, which is a Hog1 pathway target gene. Furthermore, using microarray and Northern blot analyses, we determined that genes that are regulated by Msn2/Msn4 were up-regulated by Rck1 and that this was the result of Hog1 activation by RCK1 over-expression. Together, our results suggest that Rck1 inhibits Slt2 MAP kinase pathway activity and then Ptp2, which subsequently activates Hog1.

1. Introduction

The cell wall integrity (CWI) pathway plays an important role in maintaining the cell structure and protecting *Saccharomyces cerevisiae* from external stress [1–3]. Virtually all microbes are exposed to various forms of stress and protect themselves by regulating the CWI pathway [1–4]. Most cell wall stresses are recognized by a sensor system located on the plasma membrane that transfers signals to intracellular MAP kinase proteins [5–7].

In *S. cerevisiae*, the CWI pathway is involved in the responses to high temperature, osmotic stress, actin depolymerization, mating pheromones, and cell wall damaging agents [4–8]. The sensor proteins Mid2, Wsc1-4, and Mtl1 are located on the plasma membrane and recognize stress signals. These proteins transfer signals to the MAPKKK Bck1, the MAPKKs Mkk1 and Mkk2, and then to the MAPK Slt2 by activating Pkc1. Activated Slt2 triggers Rlm1 and the transcription factor SBF, finally activating the gene expression involved in cell wall biogenesis and the stress response [9–14].

Hog1 also plays an important role in response to osmotic stress and regulates the gene expression involved in the stress response pathway [15–17]. Furthermore, Hog1 is involved in the responses to low temperature, oxidative stress, and heavy metal stress [18–20]. In the fission yeast *Schizosaccharomyces pombe*, the Hog1 homolog Sty1 is involved in the stress response pathway in a manner similar to that in *S. cerevisiae* [21]. The Hog1 pathway is generally activated by Pbs2 via phosphorylation, and activated Hog1 migrates into the nucleus where it activates various transcriptional activators such as Hot1, Msn2/4, Sko1, and Smp1. These transcriptional activators regulate the expression of genes involved in various stress responses [22–27].

Recently, the interaction between Rck2 and Hog1 has been reported to be required for the response to osmotic and oxidative stresses, and Hog1 has been shown to interact with the C-terminal region of Rck2, resulting in the phosphorylation of Rck2 at Ser519 [28–30]. Rck1 is a Rck2 homolog in *S. cerevisiae* [31]. Rck1 gene expression is regulated by cell wall stress, and Kdx1 up-regulates *RCK1* expression [32]; however, the function of Rck2 has not been identified.

Recently, our group has found that Rck1 is involved in Hog1 phosphorylation in response to cell wall stress and that it regulates MAP kinase pathway components. In this study, we demonstrate the molecular mechanism of Rck1's role in the cell wall stress response.

2. Materials and methods

2.1. Strains, media, and growth conditions

The yeast strains used in this study included *S. cerevisiae* BY4741 (*MATa, his3Δ1, leu2Δ0, met15Δ0, and ura3Δ0*) and its

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derivatives. Tag strains in the BY4741 background (WT *PKC1*-3HA, WT *BCK1*-3HA, WT *MKK1*-3HA, WT *MKK2*-3HA, Δ*mkk1* Δ*rck1 MKK2*-3HA, Δ*mkk2* Δ*rck1 MKK1*-3HA, WT *PTP2*-3HA, Δ*rck1 PTP2*-3HA, and WT *RCK1*-3HA) were obtained using a PCR-mediated gene modification method. Yeast strains were grown in 1% yeast extract, 2% peptone, 2% glucose (YPD) or a synthetically defined (SD) medium (6.7 g/L yeast nitrogen base) supplemented with the necessary auxotrophic supplements. The yeast cells were grown overnight at 30 °C in YPD. The culture was diluted to an OD of 0.2 and grown at 30 °C for 2 h 30 min. The cultures were divided into two parts; one part was allowed to continue growing under the same conditions, whereas the other was supplemented with 5 units/ml of zymolyase 100T (Seikagaku Corp) for various times. At the indicated times, cells were collected for RNA extraction and protein extraction.

2.2. Plasmids

The plasmids pRS426-KDX1, pRS425-RCK1, and pRS415-RCK1-3HA were constructed as follows: A multicopy plasmid containing *KDX1* from YEp13-KDX1 was constructed using PCR-based gene amplification. *KDX1* was subcloned from a library of YEp13 plasmids as a 3.8-kb (XhoI/SpeI) DNA fragment and introduced into the *XhoI* and *SpeI* sites of the pRS426 plasmid. To construct the plasmid pRS425-RCK1, a 4.1-kb (XhoI/BamHI) DNA fragment containing *RCK1* was prepared using PCR and 5′-CTCGAGGAACTATAGCTAAATAACCCA-3′ and 5′-GGATCCCGAGCCACAATATGCGGCAACG-3′ as the forward and reverse primers, respectively. The nucleotide sequences of the constructs were confirmed by sequencing. The amplified fragments were then subcloned into pRS425. To construct the pRS415-RCK1-3HA plasmid, a 4.1-kb (XhoI/BamHI) DNA fragment containing *RCK1* tagged with a triple hemagglutinin (HA) tag was prepared using PCR and 5′-CTCGAGGAACTATAGCTAAATAACCCA-3′ and 5′-GGATCCCGAGCCACAATATGCGGCAACG-3′ as the forward and reverse primers, respectively.

2.3. Site-directed mutagenesis of RCK1

To obtain the pRS415-*RCK1*(K152R) – HA plasmid, a 4.1-kb (XhoI/BamHI) fragment from genomic DNA was cloned into the pRS415 vector. The forward and reverse primers used were 5′-CTCGAGGAACTATAGCTAAATAACCCA-3′ and 5′-GGATCCCGAGCCACAATATGCGGCAACG-3′, respectively. To obtain the Rck1-K152R mutant, the wild-type *RCK1* gene was mutated using the QuikChange site-directed mutagenesis kit (Stratagene) and the following primers: 5′-AACACGGATGACCAAGCTCCTGTTGCCATCAGAGCAATCATAAA-3′ and 3′-GATGGCAACAGGAGCTTGGTCATCCGTGTTGATGC-5′.

2.4. Cell viability assays

Yeast cells were grown overnight at 30 °C in SD medium lacking leucine. The cells were washed in distilled water and resuspended in 1 M sorbitol and 0.01 M EDTA to a density of 1×10^8 cells/ml. Zymolyase was added to a final concentration of 0.8 u/ml, and the cells were incubated at 30 °C for 1 h. The cells were then collected by centrifugation and washed twice with resuspension buffer. The cells (1×10^6 cells/ml) were plated on a fresh SD plate lacking leucine and incubated overnight at 30 °C for 2–3 days.

2.5. Microarray experiment

Microarray analysis was performed using two independent RNA samples from the Δ*rck1* and *RCK1* overexpressing strains. Yeast cultures were grown to mid-log phase in SD-leu medium and then subjected to cell wall stress (5 U/ml zymolyase, 2 h). Total RNA was extracted using TRIzol reagent (Life Technologies) and performed cDNA microarray. The microarray data were analyzed using clustering program MeV4.7.1 (eBiogen Co., Seoul, Korea) and was deposited on http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE49340.

2.6. Northern blot analysis

Yeast cells were grown to mid-log phase and then subjected to cell wall stress (5 U/ml zymolyase) for the indicated times. Total RNA was extracted using the TRIzol reagent (Life Technologies). Equal amounts (6 μg) of total RNA were separated using 1% formaldehyde agarose gel electrophoresis and transferred to nylon membranes where they were hybridized with a ^{32}P-labeled probe at 65 °C in Rapid-hybridization buffer (Amersham). The hybridized membranes were washed twice with 2X SSC containing 0.1% SDS at

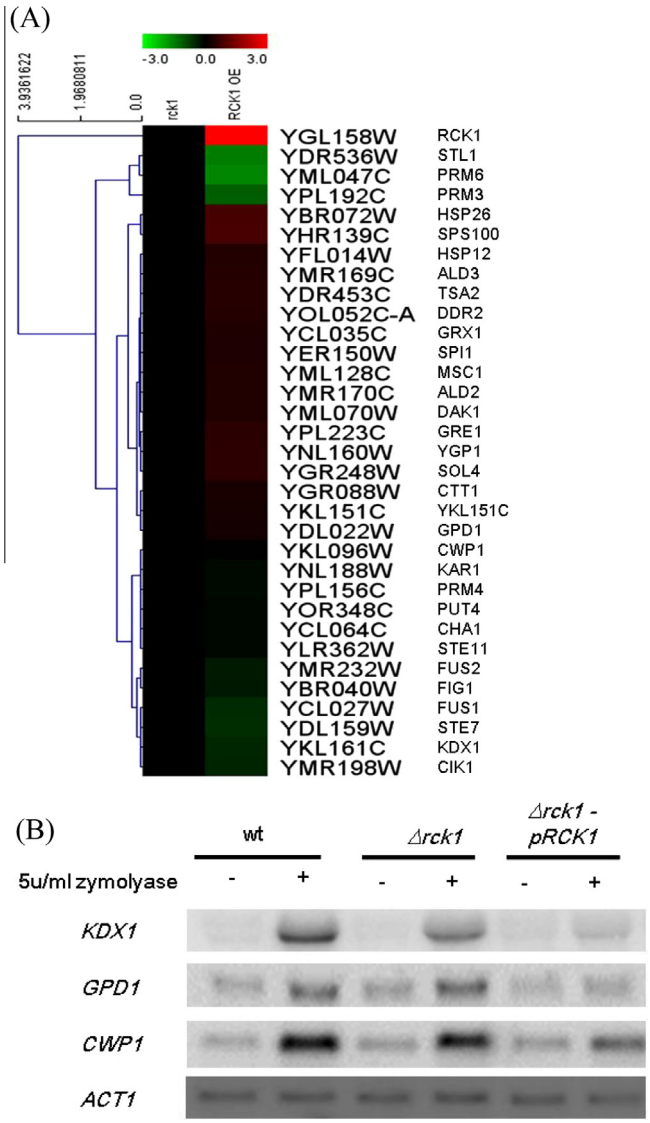

Fig. 1. Rck1 down-regulates *KDX1* gene expression. (A) Microarray analysis was performed using wild type and *RCK1*-over-expressing cells, and the genes that are up- or down-regulated by Rck1 are shown. *HSP26* and *GRX1* were up-regulated several fold, and *GPD1*, *CWP1*, and *KDX1* were down-regulated. (B) The microarray data were confirmed by Northern blot analysis. Total RNAs were extracted from the indicated cells, resolved using agarose gels, and analyzed using Northern blots.

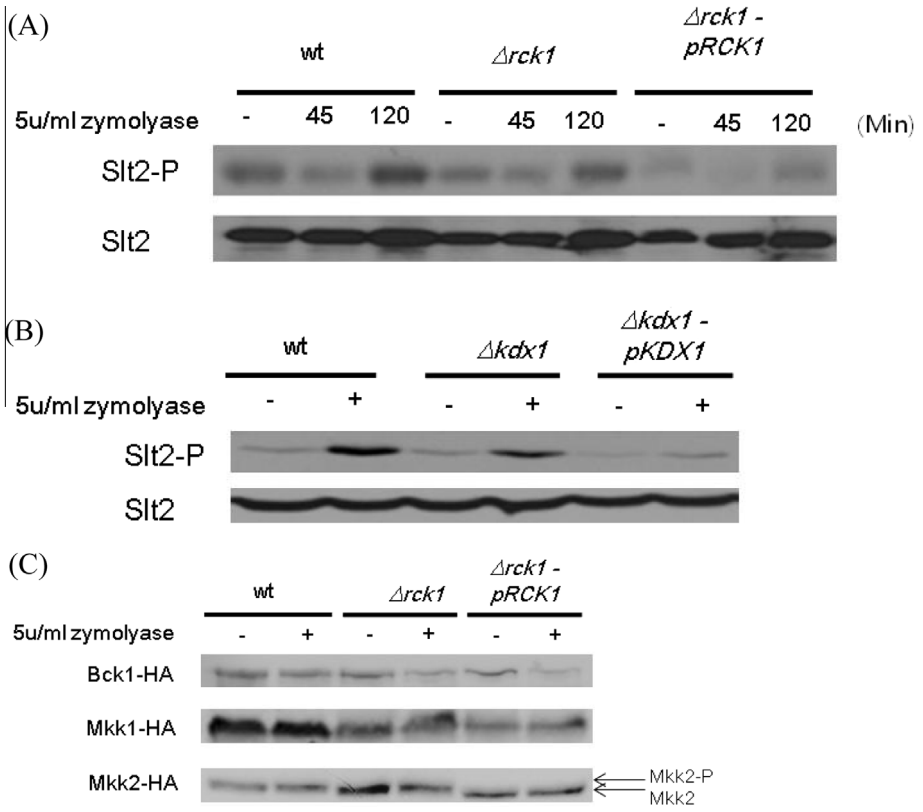

Fig. 2. MAP kinase proteins are down-regulated by *RCK1* over-expression. (A) To identify the effects of Rck1 on Slt2, the Slt2-P expression level was measured. Total proteins were extracted from the indicated cells, resolved using PAGE, and Western blotted using an antibody that specifically detects Slt2-P. Total Slt2 was used as a loading control. (B) To confirm the effects of Kdx1 on Slt2 activity, we performed a similar experiment to that described in A. Kdx1 up-regulated *RCK1*. (C) To identify the effects of Rck1 on MAP kinases, the expression levels of Bck1, Mkk1, and Mkk2 were investigated. Total proteins were purified from the indicated cells, and Western blotting was performed with the indicated antibodies.

RT and twice with 0.2X SSC containing 0.1% SDS at 65 °C; after washing, the membranes were exposed to X-ray film. Total RNA and specific genes were probed using radiolabeled PCR fragments containing the ORFs of *KDX1*, *GPD1*, *CWP1, SLT2, CTT1,* and *ACT1*. The following list presents the genes and the forward and reverse primers used to create them, respectively: *KDX1*, 5′-CTTGGTAGGAAACCAATG-3′ and 5′-TGGCTGGGTTGTAGTTAGTG-3′; *GPD1*, 5-TACCCGACAATTTGGTTG-3′ and 5′-GACGTTTCTACCACCAGC-3′; *CWP1*, GGCAGCGGTAGTGGCTCA-3′ and 5′-ACAGATCCGCTCTTACT-3′; *SLT2,* 5-ATGGCTGATAAGATAGAGAG-3′ and 5′-ACAGGATTCTCCGAATAACC-3′; *CTT1*, 5′-ATGAACGTGTTCGGTAAAAAAG-3′ and 5′-CACCACCAACGGTGGAAAAACG-3′; *ACT1*, 5′-ACACGGTATTGTCACCAACTGGG-3′ and 5′-AGGACAAAACGGCTTGGAGG-3′.

2.7. Immunoblot analysis

Cells were grown in SD-medium until an optical density at 600 nm (OD600) of approximately 0.5 was attained. The cells were incubated with zymolyase (5 u/ml) for 2 h to induce cell wall damage and harvested by centrifugation. Cell pellets were resuspended and lysed by vortexing with glass beads in 250 μl of cold lysis buffer (50 mM Tris–HCl, pH 7.5, 10% glycerol, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 50 mM NaF, 1 mM sodium orthovanadate, 50 mM β-glycerol phosphate, 5 mM sodium pyrophosphate, 5 mM EDTA, 1 mM phenylmethylsulfonylfluoride, and protease inhibitors) [1]. The proteins (50 μg) were resolved on SDS–polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were then immunoblotted using antibodies directed against anti-phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) (1:2000 dilution) and anti-phospho-p38 (1:2000 dilution) (Cell Signaling), anti-HA (1:5000 dilution) (C29F4 Cell Signaling), Pgk1 (1: 50,000) (Invitrogen), and Mpk1 (Y-244) (1:5000 dilution) and Hog1 (Y-215) (1:5000 dilution) (Santa Cruz Biotechnology).

3. Results

3.1. Rck1 down-regulates KDX1 gene expression

We previously reported that Kdx1 up-regulates *RCK1* gene expression [32]. To identify the functions of Rck1, we subjected a strain over-expressing *RCK1* to microarray analysis to determine the identity of the genes regulated by Rck1. Full-length *RCK1* was cloned into a multicopy plasmid and transformed into wild-type cells. As shown in Fig. 1A, we found that genes involved in the pheromone response were down-regulated by *RCK1* over-expression. In addition, genes regulated by Msn2/Msn4 (transcription factors that are regulated by Hog1 in response to many types of stresses) were up-regulated. However, genes that are regulated by Hot1 (another Hog1-regulated transcription factor) were down-regulated. The activities of Msn2/Msn4 and Hot1 are regulated by Hog1 [33]. These results indicate that genes that are regulated by Rck1 are also regulated by Hog1 and that Rck1 and Hog1 share the same stress-response targeting pathway. Interestingly, we found that the expression of *KDX1*1 was down-regulated by Rck1. The *KDX1* expression pattern was confirmed by Northern blot analysis. Total RNAs were purified from wild type, *RCK1*

deletion, and *RCK1* over-expression strains, and Northern blot analysis was performed (Fig. 1B). *KDX1* was detected when the cells were treated with zymolyase, and the *RCK1* deletion strain showed the same pattern as the wild type cells. However, when *RCK1* was over-expressed, the amount of *KDX1* mRNA dramatically decreased; the same result was found in the microarray data. We then addressed how *KDX1* is down-regulated by *RCK1*. In addition, we confirmed the expression of *GPD1* and *CWP1*, which are regulated by Hot1 [33]; *GPD1* and *CWP1* were down-regulated by Rck1, and this result was confirmed using microarray analysis.

3.2. MAK kinase proteins are down-regulated by RCK1 over-expression

To identify the mechanism of Rck1-mediated *KDX1* down-regulation, we examined the function of Slt2, which is a positive regulator of Rlm1 and Kdx1. To identify the function of Slt2, Slt2-P was detected using western blot analysis. Total protein samples were purified from wild type and *RCK1* over-expressing cells, and Western blotting was performed (Fig. 2A). The level of Slt2-P decreased when *RCK1* was over-expressed, and this result indicates that Rck1 affects the MAP kinase pathway that targets the stress response. Furthermore, we tested the effect of Kdx1 on Slt2 activity because Kdx1 up-regulates Rck1. As shown in Fig. 2B, *KDX1* over-expression down-regulated Slt2-P in a manner similar to *RCK1* over-expression, and this result is consistent with the results presented in Fig. 2A. To further elucidate the role of Rck1 in the MAP kinase pathway, we examined the function of MAP kinases that are located upstream of Slt2. Mkk1 and Mkk2 phosphorylate Slt2 and are redundant during stress. In addition, Bck1 is upstream of Mkk1 and Mkk2 and phosphorylates Mkk1 and Mkk2 [14]. We found large amounts of the phosphorylated forms of Mkk1 and Mkk2 in the wild type and *RCK1* deletion strains (Fig. 2C). However, when *RCK1* was over-expressed, the phosphorylated form of Mkk2 was not detected. This result indicates that *RCK1* over-expression affects Mkk2 (which is located upstream of Slt2) and then regulates Slt2 and subsequently Kdx1.

3.3. Rck1 down-regulates Ptp2, a negative regulator of Hog1

The expression of *KDX1* is regulated by Rlm1, a transcription factor that is directly regulated by Slt2 in response to cell wall stress [10–12]. Expression of the phosphatase Ptp2 is regulated by Rlm1. Ptp2 dephosphorylates Hog1-P and inactivates Hog1 [34–36]. We tested the effect of Rck1 on the activity of Ptp2, which is an Rlm1 target gene. As shown in Fig. 3A, a *PTP2* deletion strain demonstrated a higher level of the phosphorylated form of Hog1 than did wild type cells, and the level of Hog1-P increased with zymolyase treatment. In addition, *PTP2* deletion resulted in the up-regulation of *KDX1* (Fig. 3B). This result might be explained by the up-regulation of Hog1, which is a positive regulator of *KDX1*. In addition, we tested the effects of Ptp2 on zymolyase stress by measuring growth; *PTP2* deletion resulted in resistance to zymolyase stress (Fig. 3C). Based on these results, we hypothesized that Rck1 down-regulates the Slt2 MAP kinase pathway and that Ptp2, subsequently activates Hog1. To confirm our hypothesis, we investigated the levels of Hog1-P using western blot analysis. A higher basal level of Hog1-P was detected in *RCK1* over-expressing cells (Fig. 3D). In contrast, a lower level of Ptp2-P was detected in *RCK1* over-expressing cells. These results confirm our hypothesis. Furthermore, we examined the effects of Slt2 on Ptp2-P because Slt2 is located upstream of Rlm1. As shown in Fig. 3E, *SLT2* deletion resulted in the down-regulation of Ptp2. Taken together, these results demonstrate that Rck1 down-regulates Ptp2 and up-regulates Hog1, with Rck1 ultimately providing stress resistance.

3.4. Mutation of Rck1 lysine 152 to arginine fails to lead to Hog1 up-regulation

Rck2, a paralog of Rck1, contains a kinase region, and point mutation of the kinase region results in failure to perform its function [37–38]. We found that Rck1 has a putative active site including lysine 152 and that the same amino acids are found at the corresponding site in Rck2. We mutated lysine 152 to arginine and introduced the resulting mutant RCK1 into a *RCK1* deletion strain. As shown in Fig. 4A, zymolyase up-regulated Hog1-P in the wild type and *RCK1* deletion strains. In addition, a higher basal level of Hog1-P was found in *RCK1* over-expressing cells. However, the K152R mutant of RCK1 failed to activate Hog1-P. This result

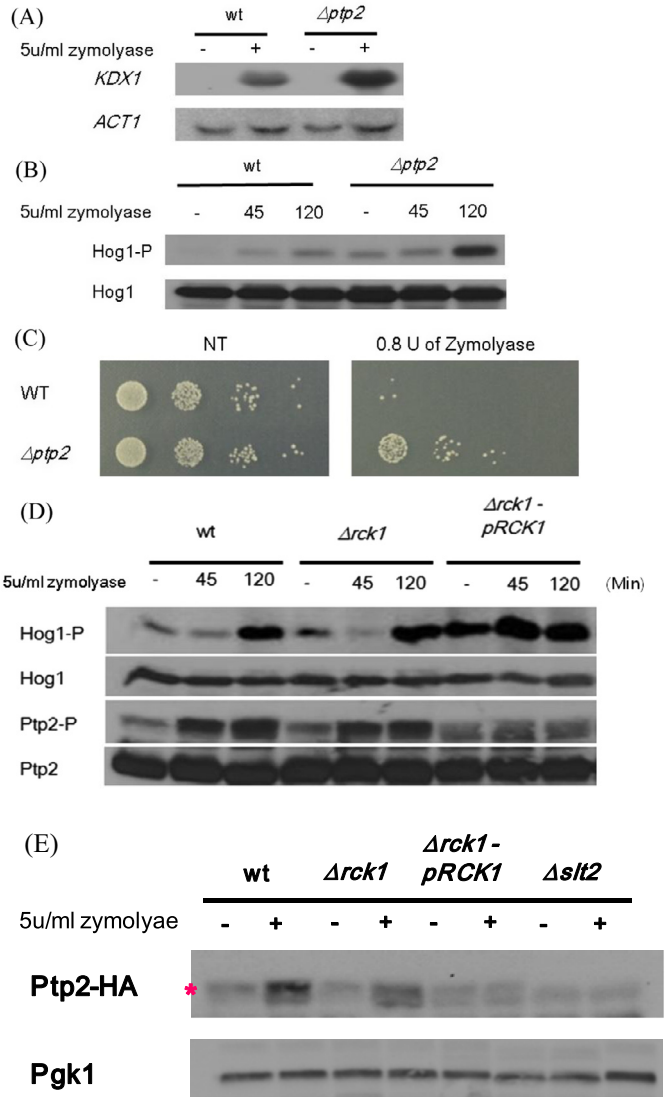

Fig. 3. Rck1 down-regulates Ptp2, a negative regulator of Hog1. (A) The effect of Ptp2 on KDX1 gene expression was investigated. Total RNA was purified from the indicated strains, and Northern blotting was performed. (B) In addition, the effects of Ptp2 on Hog1 activity i.e., Hog1-P expression, was detected using a Hog1-P specific antibody. Total proteins were purified from the indicated cells and subjected to western blotting. (C) The effect of Ptp2 on zymolyase resistance was investigated. Non-treated and 0.8-U-zymolyase-treated cells were 10-fold serially diluted, and 10 ml of cells were spotted onto YPD media. (D) The effects of Rck1 on Ptp2 and Hog1 activity were then investigated. Zymolyase was used to treat the indicated cells for 45 or 120 min; then, total proteins were purified and subjected to western blotting. Hog1-P and Ptp2-P were detected using Hog1-P and Ptp2-P specific antibodies, respectively. Hog1 and Ptp2 were used as loading controls. (E) The effect of Slt2 on Ptp2 activity was investigated and asterisk indicates Ptp2-P.

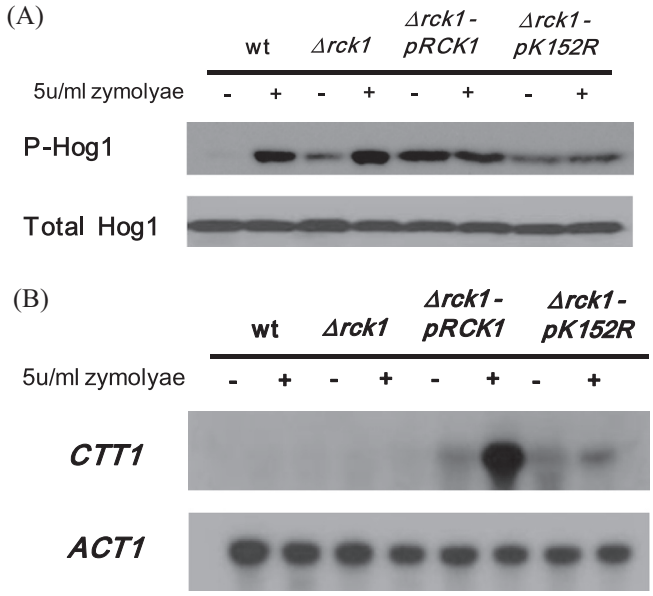

Fig. 4. A lysine to arginine mutant of Rck1 at residue 152 failed to up-regulate Hog1. (A) Lysine 152 of Rck1 was mutated to arginine and used to construct a plasmid encoding K152R Rck1. The mutated RCK1 protein was introduced into a RCK1 deletion strain, and Hog1-P was detected. (B) The transcript of CTT1 was detected using Northern blot analysis. Total RNA was purified from the indicated cells and subjected to Northern blotting using a probe recognizing the CTT1 internal region.

indicates that Rck1 contains a catalytic site including lysine 152. To identify the importance of the Rck1 catalytic site, we investigated the expression of *CTT1,* which is an Msn2/4 target gene. Msn2 and Msn4 are transcriptional factors, and their activity is dependent on Hog1 [33]. As shown in Fig. 4B, *CTT1* was up-regulated when *RCK1* was over-expressed. However, K152R RCK1 failed to up-regulate *CTT1*. These results indicate that Rck1 regulates Hog1 activity via the catalytic site at lysine 152.

4. Discussion

Rck1 and Rck2 were identified as Radiation sensitivity Complementation Kinases from *S. cerevisiae* [39]. In *S. pombe*, Sty1 (a Hog1 homolog) regulates the activity of the Rck1 homologues Mkp1 and Mkp2. In *S. pombe*, Mkp1 and Mkp2 work as negative regulators in the meiosis pathway [28,29,38,39]. In *S. cerevisiae*, the exact functions of Rck1 and Rck2 have not been identified. Rck2 has been studied more extensively than Rck1 and was found to interact with Hog1 and is phosphorylated in a Hog1-dependent manner to regulate gene expression during osmotic stress [18,30,28,29].

Rck1 is a homolog of Rck2 in *S. cerevisiae* [31], and we previously reported that *RCK1* gene expression is up-regulated by Kdx1 (a component of the Slt2 MAP kinase pathway) [32]. However, the physiological functions of Rck1 have not been studied; thus, we partially examined the functions of Rck1 in this study. As shown in Fig. 1, microarray data provide clues for the function of Rck1 because *RCK1* over-expression up- or down-regulated genes that are regulated in a Hog1-dependent manner. The microarray data imply that Rck1 may be involved in a Hog1-dependent pathway. Interestingly, Rck1 up-regulated general stress response genes, such as Msn2- and Msn4-dependent genes, indicating that Rck1 is involved in the stress response pathway in a Hog1-dependent manner because Msn2 and Msn4 are Hog1-dependent transcription factors [33]. As shown in Fig. 3, Rck1 activated Hog1 activity, resulting in resistance to zymolyase stress. Our data showed that Rck1 is involved in the Hog1-dependent stress response pathway.

It is unclear how Rck1 activates Hog1 activity. Rck2 is a mediator that is known to transfer signals from Hog1 to downstream elements, and RCK2 over-expression results in resistance to osmotic stress [30]. Rck1 is paralog of Rck2, and the over-expression of RCK1 activates Hog1. RCK1 over-expression down-regulated Ptp2, a negative regulator of Hog1. It has been reported that Ptp2 is regulated by Rlm1, which is regulated in turn by Slt2 [34]. As shown in Fig. 2, several MAP kinases located up-stream of Slt2 in the MAP kinase pathway were down-regulated by Rck1, which subsequently down-regulated Ptp2. Interestingly, Mkk2 was regulated by Rck1, and Mkk2-P was not detected when RCK1 was over-expressed (Fig. 2C). We found a lysine residue in Rck1 that is proposed to have a catalytic activity. A similar lysine residue in Rck2 also plays an important role in signaling pathways [30–37]. As shown in Fig. 4, point mutation of the lysine residue to arginine failed to up-regulate *CTT1*, which is a known Hog1 target gene; this implies that the lysine residue is important for the catalytic activity. Taken together, the findings suggest that Rck1 affects Mkk2 phosphorylation by an unknown mechanism, the resulting signal is transferred to Slt2 and inhibits Ptp2, ultimately activating Hog1.

The mechanism of Mkk2 dephosphorylation requires further study. Bck1 phosphorylates Mkk2 [14]. We tested components that lie upstream of Mkk2 and found that RCK1 over-expression results in the down-regulation of Bck1 and Pkc1 at the protein level. However, Bck1 and Pkc1 protein levels were decreased when RCK1 was deleted. This result indicates that additional factors are involved in the regulation of Pkc1 and Bck1, and these factors should be identified.

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