Investigation of Intracellular Activation of SAPK, Mpk1, in Response to Clotrimazole

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Introduction

Activation of Stress-activated Protein Kinase (SAPK), Mpk1, in the Cell Wall Integrity (CWI) signaling pathway has been associated with anti-fungal drug resistance. However, the molecular mechanism of Mpk1 activation during drug treatment remains poorly understood. In this study, we use the yeast *Saccharomyces cerevisiae* to investigate Mpk1 activation in response to the anti-fungal drug clotrimazole.

Previous results indicate that clotrimazole-induced Mpk1 activation can be detected even without its upstream protein kinases, with the introduction of Mkk1-DD, a phosphomimetic-form of Mkk1, suggesting an intracellular mechanism of Mpk1 activation, in contrast to the established "top-down" mechanism from cell-surface signaling.

The objective of this research is to evaluate and identify additional proteins involved in clotrimazole-induced activation of Mpk1.

We are taking both a candidate approach with a focus on Ste11 and an unbiased mass spectrometric approach.

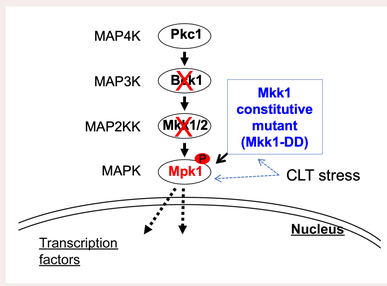

Figure 1. The CWI signaling cascade

Methods

Strain	Genotype
DL100	*MAT*a EG123 *ura3-52 leu2-3, 112 trp1-1 his4 can1*
DL252	*MAT*a EG123 *bck1Δ::URA3*
DL3947	*MAT*α S288c *Ste11Δ::HphMX*
DL4317	*MAT*a EG123 *bck1Δ::URA3 mkk1Δ::LEU2 mkk2Δ::URA3*
DL4626	*MAT*a EG123 *bck1Δ::URA3 Ste11Δ::HphMX*

Table 1. Yeast strains

Plasmid	Description
p118	pRS314
p2283	YEp351-*MPK1-GFP*
p3372	pRS314-*12HA-MKK1*
p3373	pRS314-*12HA-MKK1-DD*

Table 2. Plasmids

Yeast cell growth conditions and transformation: Cells were grown at 30°C in YPD (1% Bacto yeast extract, 2% Bacto Peptone, 2% glucose) and minimal selective medium, SDM (0.67% yeast nitrogen base, 2% glucose). The transformation was carried out using the lithium acetate method. Yeast cells were treated with clotrimazole at 50 µg/mL and calcofluor white at 40 µg/mL.

Genomic deletion

A ~2.7kb PCR fragment including 5' upstream of Ste11 sequence, HPH gene, and 3' downstream of Ste11 was transformed into a bck1Δ strain (DL252). A double delete Ste11Δbck1Δ (DL4626) mutant was generated by replacing Ste11 with HPH using sequence homologous recombination. Double delete was validated by genomic PCR for its 5' and 3' junctions.

Total cell extract preparation

For testing the time course of clotrimazole, sodium hydroxide/boiling method was used. For preparation of mass spectrometry samples, a bead-beating glass beads lysis method was used to break down cells mechanically.

SDS-PAGE and Western blotting analysis

Proteins were separated with SDS-PAGE (8-16% gradient gels, BioRad). Mouse monoclonal α-HA (1:2000), α-GFP (1:5000), and HRP-conjugated Mpk1 (1:10,000) were used. Rabbit polyclonal α-phospho-p44/42 MAPK was used at a dilution of 1:2000.

Immunoprecipitation

Total cell extracts were incubated with 50 µL of GFP trap beads at 4°C overnight, and samples were washed and boiled for SDS-PAGE.

Sample preparation for mass spectrometric analysis

Mpk1 pulldown complex was prepared from DL252 with Mkk1-DD and tested by both Coomassie blue staining for protein quantity and Western blotting for protein expression.

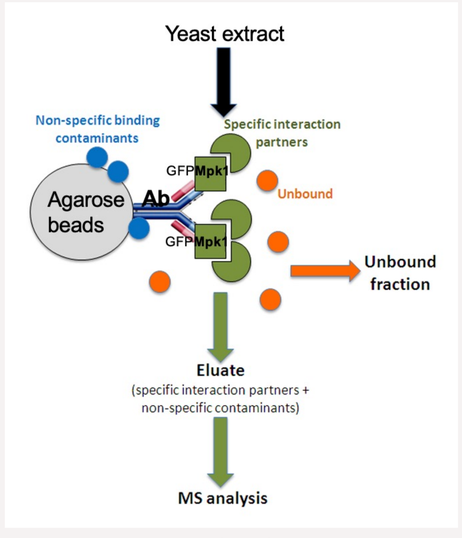

Figure 2. GFP-tagged Mpk1 immuno-precipitation with GFP-trap agarose beads

Results

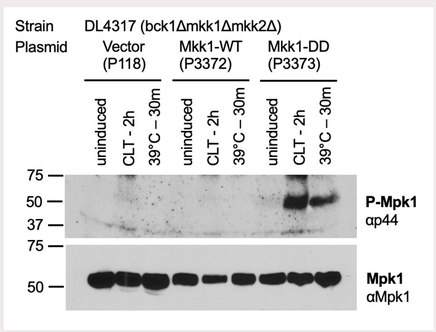

Figure 3. **Mpk1 activation in bck1Δmkk1Δmkk2Δ cells requires Mkk1-DD.** bck1Δmkk1Δmkk2Δ (DL4317) yeast cells were transformed with plasmids of a vector, Mkk1-WT, or Mkk1-DD. Cells that were uninduced, induced with Clotrimazole (CLT) for 2 hours, or 39°C for 30 minutes were harvested for Western analysis. Cells with deletions of upstream kinases Bck1 and Mkk1/2 require the basal signal provided by Mkk1-DD to activate Mpk1.

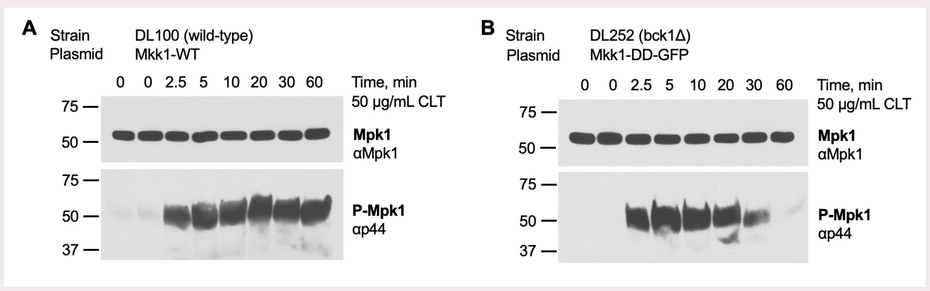

Figure 4. **Testing optimal time points of clotrimazole-induced Mpk1 activation by a time course. (A)** Wild-type (DL100) yeast cells were treated with 50 µg/mL of clotrimazole (CLT) for the times indicated on the top of the figure. Mpk1 activation was seen after 2.5 minutes. **(B)** bck1Δ (DL252) yeast cells transformed with the Mkk1-DD plasmid were treated with 50 µg/mL of CLT. The activation of Mpk1 reached a peak around 10 minutes.

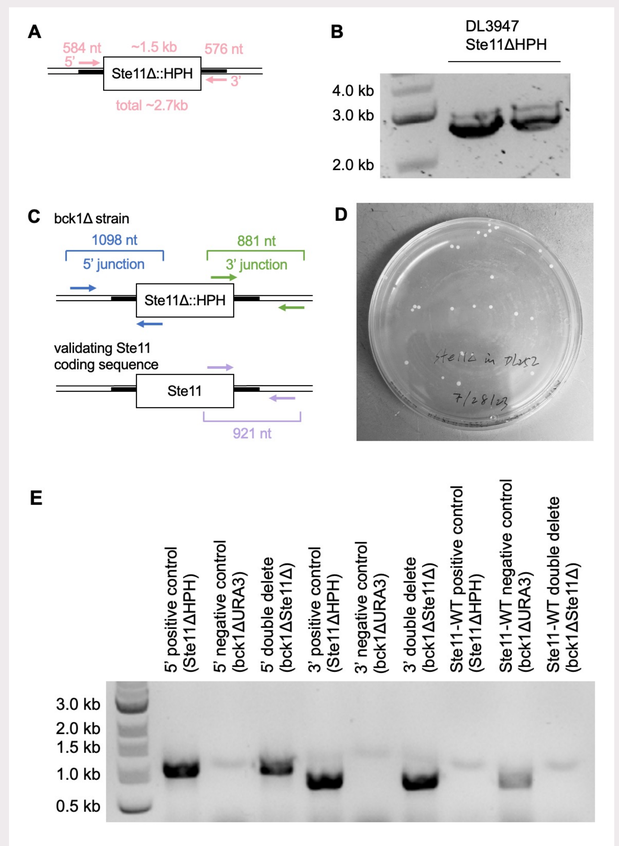

Figure 5. **Generation of a double delete of ste11 and bck1 by homologous sequence recombination. (A)** A diagram for the replacement of Ste11 with HPH gene. Primers used to amplify the PCR deletion fragment are indicated. **(B)** The expected ~2.7kb PCR fragment was detected using agarose gel electrophoresis. **(C)** Schematic for validation of Ste11 deletion, using primers outside of PCR deletion fragment. **(D)** The Ste11Δbck1Δ cells were grown on a plate containing hygromycin to select for the cells carrying the HPH gene. **(E)** Validation of double delete by genomic PCR.

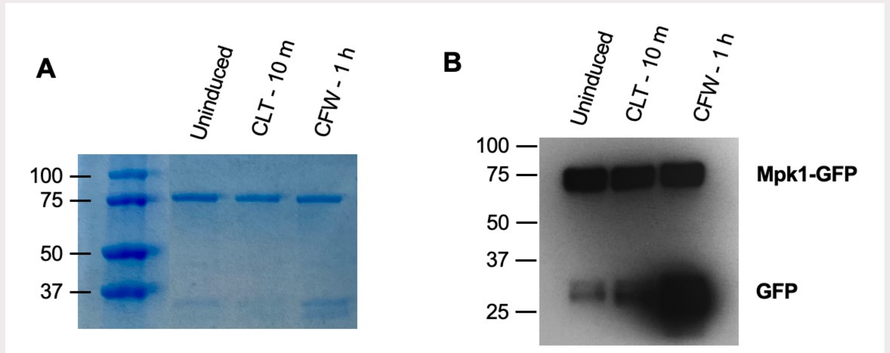

Figure 6. **Pulldown of GFP-tagged Mpk1 and its associated protein complex. (A)** bck1Δ (DL252) cells expressing Mkk1-DD and Mpk1-GFP were treated with clotrimazole (CLT) for 10 minutes and calcofluor white (CFW) for 1 hour at 30°C. The total cell extracts were prepared, and the proteins were separated by SDS-PAGE. The resulting gel was stained with Coomassie blue. **(B)** The samples are as described in (A) and detected by Western blot using an anti-GFP antibody.

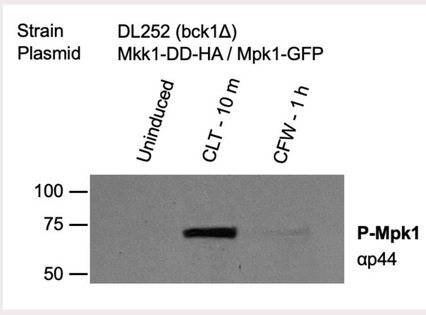

Figure 7. **Activation of Mpk1 in bck1Δ cells with Mkk1-DD under stresses of clotrimazole (CLT) and calcofluor white (CFW).** The samples were prepared as in Figure 6(A). Activation of Mpk1 was detected by Western blotting using an anti-phosphorylated-Mpk1 antibody. Mpk1 was activated only by CLT but not CFW, suggesting an intracellular activation mechanism in contrast to a "top-down" mechanism from cell surface signaling.

Conclusions/ Discussion

- Our preliminary results indicate a potential Mpk1 intracellular activation mechanism. The signal entry points could be Mkk1 or its downstream components in the CWI signaling pathway.
- The optimal time for clotrimazole induction is 10 minutes, which will be used for subsequent studies.
- We made a double deletion strain, which will allow us to test the requirement of Ste11 for the activation of Mpk1.
- We have successfully isolated the Mpk1 protein complex, which will be subjected to mass spectrometry analysis.
- Mass spectrometry results will reveal which proteins are involved in Mpk1 activation, which is important to understanding the intracellular activation mechanisms of the CWI pathway.
- Identification of protein factors involved in the Mpk1 activation process may hold potential as anti-fungal drug targets for future pharmaceutical studies, enhancing the development of effective therapeutic strategies.

References

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