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Phosphorelay-dependent and -independent regulation of MAPKKK by the Mcs4 response regulator in fission yeast

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin epitope; HK, histidine kinase; HPt, histidine phosphotransferase; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase; RR, response regulator; SAPK, stress-activated protein kinase; TAP, tandem affinity purification

In a "two-component system," extracellular stimuli are transmitted by the transfer of a phosphoryl group from a sensor histidine kinase to a response regulator (RR), a mechanism referred to as phosphorelay. In the fission yeast *Schizosaccharomyces pombe*, peroxide stress signals are transmitted by phosphorelay to the Mcs4 RR, which activates the Spc1 MAP kinase (MAPK) cascade. We previously demonstrated that a glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) physically interacts with Mcs4 and promotes phosphorelay signaling to Mcs4. Independently of the phosphorelay mechanism, Mcs4 also plays a critical role in osmostress signaling, as a part of the stable ternary complex with the Wis4 and Win1 MAPK kinase kinases (MAPKKKs). Interestingly, GAPDH dissociates from Mcs4 upon osmostress, while oxidative stress promotes their association. The Mcs4 RR may serve as a switching hub that mediates activation of the Wis4-Win1 MAPKKK heteromer in response to different forms of stress.

The two-component system is a cellular signaling module composed of two proteins, a sensor histidine kinase and a response regulator (RR)[1] and it has been identified in both prokaryotes and eukaryotes except the animal kingdom. In response to stimuli, the sensor kinase auto-phosphorylates its histidine residue and the phosphoryl group is in turn transferred to an aspartic acid residue of the RR. This signaling mechanism is called "His-Asp phosphorelay." Phosphorylation modulates the activity of RR proteins that carry an output domain of diverse functions, such as DNA binding, protein binding and enzymatic activity.[2] Some two-component systems utilize a third factor, histidine phosphotransferase (HPt) that mediates phosphorelay from sensor kinase to RR and such "three-component" systems are often referred to as "multi-step phosphorelays."

The genome of the fission yeast *Schizosaccharomyces pombe* encodes two RRs, Prr1 and Mcs4. While Prr1 has a DNA-binding domain and functions as a transcription factor, Mcs4 regulates a MAP kinase (MAPK) cascade composed of Spc1 MAPK, Wis1 MAPK kinase (MAPKK) and two MAPKK kinases (MAPKKKs), Wis4 and Win1.[3] Spc1 belongs to the stress-activated protein kinase (SAPK) family, whose members include Hog1 in budding yeast and mammalian p38.[4] The Spc1 MAPK cascade is activated by diverse environmental stress such as high osmolarity, oxidative stress and heat shock, and controls a gene expression program that confers cellular stress resistance.[5]

Disruption of the *mcs4*[+] gene drastically compromises activation of the Spc1 MAPK in response to multiple forms of stress.[6,7] On the other hand, mutations to the phosphor-acceptor Asp in the Mcs4 RR or loss of upstream phosphorelay components, such as the Mpr1 HPt, only compromise oxidative stress signaling but not osmostress signaling to Spc1.[8,9] These observations unexpectedly suggested that the Mcs4 RR protein functions independently of the two-component phosphorelay to induce activation of the Spc1 MAPK cascade in response to osmostress.

The Mcs4 RR is an Integral Constituent of the MAPKKK Heteromer Complex

It was previously reported that Mcs4 physically interacts with the Wis4 MAPKKK of the Spc1 MAPK cascade.[9] In addition, we recently discovered that Wis4 and its paralog, Win1, form a stable heteromer complex.[10] The following observations strongly

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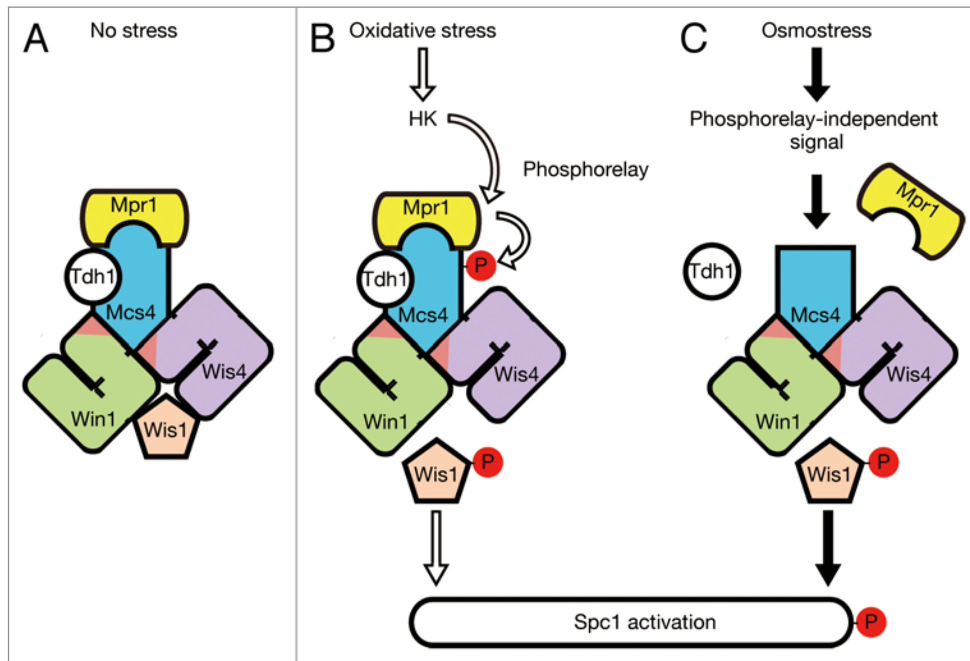

Figure 1. Environmental stress signaling via the Mcs4 RR-MAPKKK complex. (**A**) The Mcs4 RR forms a stable complex with the Wis4-Win1 MAPKKK heteromer. The Wis1 MAPKK and the Tdh1 GAPDH are also found on the complex, and the Mpr1 HPt-Mcs4RR association is detectable in the absence of stress. (**B**) Phosphorelay through the two-component system transmits oxidative stress signals to the Spc1 MAPK cascade. Oxidative stress enhances the Tdh1-Mcs4 association, which is critical for the interaction of the Mpr1 HPt with the Mcs4 RR and successful phosphorelay between them.[11] (**C**) High osmolarity stress signals are transmitted to the Mcs4-MAPKKK complex by an unknown mechanism independent of the two-component system. Tdh1 is released from Mcs4 in response to osmostress. Because Tdh1 is required for the Mpr1-Mcs4 association, Mpr1 is likely to dissociate from Mcs4 upon osmostress. Stress-induced activation of the Mcs4-MAPKKK complex results in phosphorylation and activation of the Wis1 MAPKK and its release from the complex.

suggest that the Mcs4 RR is an integral component of the ternary complex with the Wis4 and Win1 MAPKKKs (**Fig. 1A**). First, constitutive Mcs4-MAPKKK association is detectable both before and after stress stimuli.[10] Second, co-precipitation assay (**Fig. 2A**) found that heteromer association between the Wis4 and Win1 MAPKKKs is not affected by stress treatments. Third, disruption of the *mcs4⁺* gene (Δ*mcs4*) compromises the interaction between the MAPKKKs both in the presence and absence of stress (**Fig. 2A**, lanes 7–11).

The integrity of the Mcs4 RR-MAPKKK complex appears to be important for its physical interaction with the downstream Wis1 MAPKK, because the MAPKKK-MAPKK interaction is not detectable in strains lacking Mcs4 or one of the MAPKKKs.[10] Thus, it is very likely that the Δ*mcs4* defect in osmostress-induced activation of the Spc1 cascade[6] is due to the compromised interaction between the MAPKKKs and the Wis1 MAPKK. The Wis1 MAPKK is released from the Mcs4-MAPKKK complex in response to phosphorelay-dependent (H_2O_2) and -independent (osmostress) signals (**Fig. 2B**).

Regulation of the Mcs4 RR by the Glycolytic Enzyme GAPDH

While it remains unknown how osmostress is sensed and transmitted to the MAPKKK complex, the peroxide stress sensor linked to the Spc1 MAPK cascade is the multi-step phosphorelay composed of the Mak2/Mak3 histidine kinases, the Mpr1 HPt and the Mcs4 RR (**Fig. 1B**).[3] We previously reported that glyceraldehyde-3-phosphoate dehydrogenase (GAPDH), a glycolytic enzyme, is involved in the Mcs4-mediated, peroxide signaling in fission yeast.[11] Disruption of the *tdh1⁺* gene encoding GAPDH (Δ*tdh1*) significantly compromises the physical association between the Mcs4 RR and Mpr1 HPt, suggesting that the Tdh1 GAPDH promotes phosphorelay signaling to the Mcs4 RR. As a consequence, the Δ*tdh1* mutant is defective in activation of the Spc1 MAPK by peroxide stress but not other forms of stress. Although we initially identified Tdh1 as a protein co-purified with the Wis4 MAPKKK and Mcs4,[11] association of Tdh1 to Mcs4 is detectable even in the strain lacking both Wis4 and Win1 MAPKKKs (**Fig. 2C**). Consistent with its role in peroxide stress signaling, binding of Tdh1 to the Mcs4 RR is enhanced after H_2O_2-treatment.[11] Intriguingly, we found that the Tdh1 GAPDH promptly dissociates from the Mcs4 RR in response to high osmolarity stress (**Fig. 2D**). Because Tdh1 is required only for peroxide signaling to the MAPK cascade,[11] detachment of Tdh1 from the Mcs4-MAPKKK complex might represent conversion of the complex to the osmosignaling form (**Fig. 1C**).

Concluding Remarks

Our studies[10,11] garnered evidence for protein-protein interactions that build a multi-protein complex assembled around the

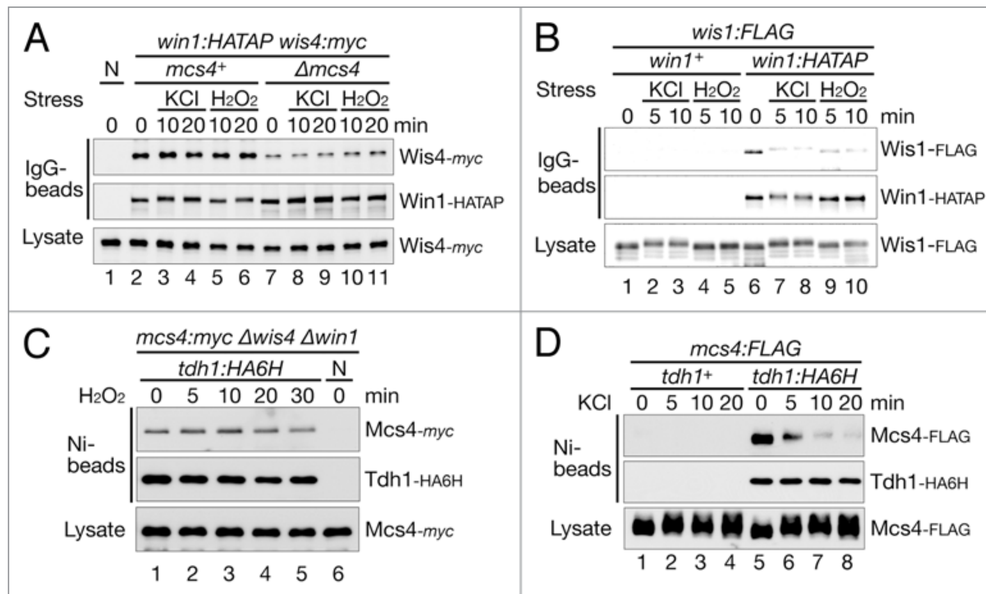

Figure 2. Protein-protein interactions with and within the Mcs4-MAPKKK complex. Wis4, Win1, Wis1, Mcs4 and Tdh1 were expressed from their own genomic loci with carboxyl terminal epitope tags or the HA epitope tag followed by the tandem affinity purification tag (HATAP) or six consecutive histidine residues (HA6H).[10,11] Cells in early log-phase were treated with 0.6 M KCl (osmostress) or 0.73 mM H_2O_2 (oxidative stress) for the indicated times. The cell lysate supernatant after high-speed centrifugation was subjected to the co-precipitation assay to monitor protein-protein interactions.[12] (**A**) Disruption of the *mcs4*+ gene (Δ*mcs4*) weakens the Wis4-Win1 MAPKKK heteromer association. Win1-HATAP was purified with IgG-Sepharose from lysate of cells co-expressing Wis4-*myc*, and the proteins in the purified fractions were detected by immunoblotting. The levels of Wis4-*myc* in the lysate used for the affinity purification are shown in the bottom panel. Lane 1, a strain expressing untagged Win1 as a negative control; lanes 2–6, *mcs4*+ cells; lanes 7–11, Δ*mcs4* cells. (**B**) The Wis1 MAPKK dissociates from the MAPKKK complex in response to both phosphorelay-dependent and -independent signals. The Win1-HATAP was purified with IgG-Sepharose from lysate of cells co-expressing Wis1-FLAG (lanes 6–10), and the proteins in the purified fractions were detected by immunoblotting. A strain expressing untagged Win1 was used as a negative control (lanes 1–5). The Wis1-FLAG levels in the lysate are shown in the bottom panel. (**C**) The Tdh1 GAPDH physically associates with the Mcs4 RR independently of the Wis4 and Win1 MAPKKKs. Lysate of the double gene disruptant (Δ*wis4* Δ*win1*) expressing Tdh1-HA6H and Mcs4-*myc* was subjected to affinity-purification of Tdh1 with Ni-NTA-agarose (lanes 1–5), and the proteins in the purified fractions were detected. The amounts of Mcs4-*myc* in the lysate were determined in the bottom panel. A strain expressing untagged Tdh1 was used as a negative control (lane 6). (**D**) Tdh1 is detached from the Mcs4 RR in response to osmotic stress. Tdh1 was purified as described in (**C**) from a strain expressing Mcs4-FLAG (lanes 5–8). A strain expressing untagged Tdh1 was used as a negative control (lanes 1–4). The proteins in the purified fractions and Mcs4-FLAG in the lysate were analyzed.

Wis4-Win1 MAPKKK heteromer (**Fig. 1**). We are beginning to understand the mechanics of this elaborate apparatus that mediates activation of the *S. pombe* Spc1 MAPK cascade in response to different stress stimuli. Of particular note is the dual role of the Mcs4 RR in the MAPKKK complex. Oxidative stress stimuli sensed by the Mak histidine kinases initiate phosphorelay to the Mcs4 RR via the Mpr1 HPt, and the phosphorylation of Mcs4 triggers activation of the MAPKKK complex (**Fig. 1B**). On the other hand, Mcs4 is essential for the integrity of the MAPKKK complex, and the loss of Mcs4 destabilizes the Wis4-Win1 heteromer association, which appears to be important for the MAPKKK-MAPKK interaction. Unexpectedly, the glycolytic enzyme GAPDH may serve as a modulator of the Mcs4 function. The Tdh1 GAPDH bound to Mcs4 promotes physical interaction between the Mpr1 HPt and Mcs4 RR, thus accelerating the phosphorelay to transmit oxidative stress signals.[11] Like in the Δ*tdh1* mutant,[11] dissociation of Tdh1 from Mcs4 upon osmostress (**Fig. 2D**) may destabilize the Mpr1-Mcs4 interaction and convert the MAPKKK complex to a configuration dedicated for osmostress signaling (**Fig. 1C**).

No osmostress sensor has been identified in fission yeast, and it remains to be discovered how the stress signals are transmitted to the MAPKKK complex. Although the Mcs4 RR may not be the receiver of osmostress signals within the complex, it is conceivable that the Mcs4-MAPKKK interaction plays similar, pivotal roles in both osmostress- and oxidative stress-induced activation of the MAPKKKs.

Disclosure of Potential Conflicts of Interest

There is no potential conflict of interest to disclose.

Acknowledgments

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