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Response regulator–mediated MAPKKK heteromer promotes stress signaling to the Spc1 MAPK in fission yeast

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ABSTRACT The Spc1 mitogen-activated protein kinase (MAPK) cascade in fission yeast is activated by two MAPK kinase kinase (MAPKKK) paralogues, Wis4 and Win1, in response to multiple forms of environmental stress. Previous studies identified Mcs4, a "response regulator" protein that associates with the MAPKKKs and receives peroxide stress signals by phosphorelay from the Mak2/Mak3 sensor histidine kinases. Here we show that Mcs4 has an unexpected, phosphorelay-independent function in promoting heteromer association between the Wis4 and Win1 MAPKKKs. Only one of the MAPKKKs in the heteromer complex needs to be catalytically active, but disturbing the integrity of the complex by mutations to Mcs4, Wis4, or Win1 results in reduced MAPKKK–MAPKK interaction and, consequently, compromised MAPK activation. The physical interaction among Mcs4, Wis4, and Win1 is constitutive and not responsive to stress stimuli. Therefore the Mcs4–MAPKKK heteromer complex might serve as a stable platform/scaffold for signaling proteins that convey input and output of different stress signals. The Wis4–Win1 complex discovered in fission yeast demonstrates that heteromer-mediated mechanisms are not limited to mammalian MAPKKKs.

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INTRODUCTION

Mitogen-activated protein kinase (MAPK) cascades transmit signals in the form of sequential activation of three different kinases: MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK. In eukaryotic cells from yeast to human, this conserved signaling module is placed downstream of disparate types of receptors and sensors that detect a variety of extracellular stimuli. Multiple MAPKKKs are often hooked up to fewer MAPKKs and MAPKs, forming signaling networks that determine the specificity of individual MAPK activation (Cuevas *et al.*, 2007). In particular, numerous MAPKKKs have been identified as activators of the stress-responsive JNK and p38 MAPKs in mammals, which are often referred to as stress-activated protein kinases (SAPKs; Kyriakis and Avruch, 2012). The osmosensing HOG pathway in budding yeast, a prototypical SAPK cascade (Brewster *et al.*, 1993), uses three different MAPKKKs. Paralogous Ssk2 and Ssk22 MAPKKKs (Maeda *et al.*, 1995) receive signals from the Sln1–Ypd1–Ssk1 phosphorelay (Posas *et al.*, 1996), whereas Ste11 MAPKKK is shared with the mating-pheromone MAPK cascade (Posas and Saito, 1997). Sln1 and Ssk1 are structurally related to the sensor kinase and the response regulator of bacterial two-component systems (Maeda *et al.*, 1994). Phosphorelay to Ssk1, which binds to Ssk2 MAPKKK, regulates the activity of Ssk2 and its downstream kinase cascade (Posas and Saito, 1998; Horie *et al.*, 2008).

The Spc1 (also known as Sty1) MAPK cascade in the fission yeast *Schizosaccharomyces pombe* (Figure 1A) is highly homologous to the HOG pathway (Millar *et al.*, 1995; Shiozaki and Russell, 1995), but, like mammalian SAPKs, stress stimuli that activate the Spc1 MAPK are not limited to osmostress (Degols *et al.*, 1996). Consistent with its role in sensing diverse environmental conditions, genetic studies of the Spc1 cascade have uncovered multiple regulatory roles of the SAPK in cell physiology. First, Spc1 regulates expression

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Abbreviations used: CMiN, conserved motif in N-terminus; GST, glutathione *S*-transferase; HA, hemagglutinin; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase; SAPK, stress-activated protein kinase; TAP, tandem-affinity purification; Y2H, yeast two-hybrid.
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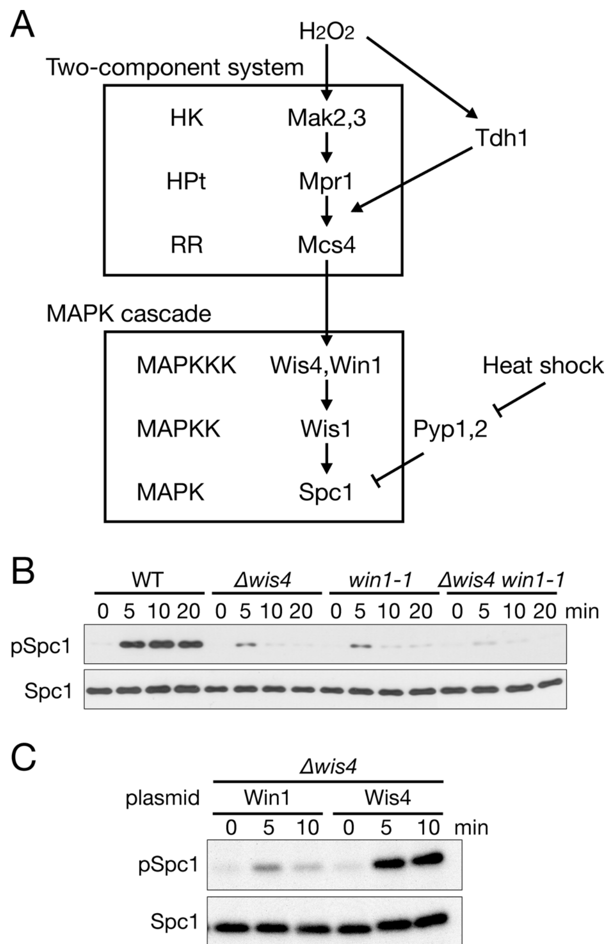

FIGURE 1: (A) The Spc1 signaling pathway in the fission yeast *S. pombe*. See *Introduction* for details. It remains to be determined how osmolarity stress signals are transmitted to the Spc1 MAPK cascade. HK, histidine kinase; HPt, histidine phosphotransferase; RR, response regulator. (B) Both Wis4 and Win1 MAPKKKs are required for activation of the Spc1 MAPK cascade in response to osmostress. Wild-type, Δ*wis4*, *win1-1*, and Δ*wis4 win1-1* strains were exposed to high-osmolarity stress of 0.6 M KCl, and their cell lysate was analyzed by immunoblotting to detect the phosphorylated, active Spc1 (pSpc1) and the total Spc1 protein level. (C) Overexpressed Win1 MAPKKK is unable to complement the Δ*wis4* defect in osmostress-induced activation of the Spc1 MAPK. Δ*wis4* strains carrying a multicopy plasmid to express either Win1 or Wis4 were exposed to osmostress of 0.6 M KCl, and their cell lysate was analyzed by immunoblotting with anti-Spc1 and anti–phospho-Spc1 (pSpc1) antibodies.

of genes required for cellular survival from diverse environmental stress (Degols *et al.*, 1996; Shiozaki and Russell, 1996; Wilkinson *et al.*, 1996; Chen *et al.*, 2003; Wang *et al.*, 2005; Wang and Shiozaki, 2006). Second, Spc1 interacts with a Hal4 protein kinase, which increases cellular resistance to toxic cations through regulation of cellular K^+ uptake (Wang *et al.*, 2005). Third, Spc1 modulates timing of mitotic initiation through a Polo-like kinase in response to nutritional conditions sensed by target-of-rapamycin kinases (Petersen and Hagan, 2005; Petersen and Nurse, 2007). Finally, the Spc1 cascade interacts with Tea4 (also known as Wsh3), a cell-end protein required for the maintenance of cell polarity under environmental stress (Tatebe *et al.*, 2005). Therefore the Spc1 pathway in *S. pombe* serves as a model system to elucidate how multiple stress stimuli are funneled into a SAPK that controls diverse cell physiology.

Heat shock induces activation of the Spc1 MAPK through inactivation of the Pyp1 and Pyp2 tyrosine phosphatases that dephosphorylate Spc1 (Figure 1A; Shiozaki *et al.*, 1998; Nguyen and Shiozaki, 1999). The Mak2/Mak3–Mpr1–Mcs4 phosphorelay is homologous to the Sln1–Ypd1–Ssk1 system in budding yeast but responsible for transmitting signals of peroxide stress, rather than osmostress, to the Spc1 cascade (Nguyen *et al.*, 2000; Buck *et al.*, 2001; Morigasaki *et al.*, 2008). Thus mutational inactivation of the Mak2 and Mak3 sensor kinases or the Mpr1 histidine phosphotransferase leads to a specific defect in peroxide-induced activation of Spc1 (Nguyen *et al.*, 2000; Buck *et al.*, 2001; Quinn *et al.*, 2002). On the other hand, strains lacking the Mcs4 response regulator of the phosphorelay fail to activate the Spc1 MAPK not only under peroxide stress but also other stress conditions, such as osmostress (Shieh *et al.*, 1997; Shiozaki *et al.*, 1997). It is likely that Mcs4 has another function essential for activation of the Spc1 cascade in addition to its role in the peroxide-signaling phosphorelay.

The Mcs4 response regulator binds to Win1 and Wis4, two MAPKKK paralogues in the Spc1 MAPK cascade (Buck *et al.*, 2001; Morigasaki *et al.*, 2008). It was previously proposed that those two MAPKKKs have distinct biological functions, with Win1 responsible for osmostress signaling (Samejima *et al.*, 1998). This model was based on the observations that osmostress-induced activation of Spc1 is severely compromised in strains carrying a *win1*-null mutation, *win1-1*, and that the *win1-1* defect in osmoresponse is not complemented by overexpression of Wis4. However, strains lacking Wis4 (also called Wik1 or Wak1) also fail to activate the Spc1 cascade upon osmostress (Shiozaki *et al.*, 1997), implying a significant contribution of this second MAPKKK to osmostress signaling. Thus both Win1 and Wis4 MAPKKKs may be required for transmitting osmostress signals, although such a notion is incongruous with observations in the budding yeast HOG cascade, where the Wis4/Win1 orthologues, Ssk2 and Ssk22, appear to be redundant (Maeda *et al.*, 1995).

In this study, we further characterize the roles of Wis4 and Win1 MAPKKKs in osmostress signaling and find that the two MAPKKKs function as a heteromer. In the absence of the Mcs4 response regulator, the MAPKKK heteromer complex is destabilized, resulting in reduced MAPKKK–MAPKK interaction and compromised MAPK activation. Thus Mcs4 seems to be a unique response regulator protein with dual roles in stress signaling. One is to receive peroxide stress signals from the Mak2/Mak3–Mpr1 phosphorelay and activate the Wis4 and Win1 MAPKKKs. The other is to stabilize the MAPKKK heteromer complex, whose integrity promotes physical interaction with the Wis1 MAPKK and, thus, signaling to the Spc1 MAPK. These results offer novel molecular insights into the signaling mechanisms that operate in the stress MAPK cascade responsive to multiple environmental inputs.

RESULTS

The Wis4 and Win1 MAPKKKs function as a complex

The *wis4*-null (Δ*wis4*; Shiozaki *et al.*, 1997) and *win1-1* (Samejima *et al.*, 1998) mutants show greatly compromised Spc1 activation in response to high osmolarity stress. Indeed, our side-by-side experiments confirmed that Δ*wis4* and *win1-1* mutants are similarly defective in osmostress-induced activation of the Spc1 MAPK (Figure 1B); only brief, weak activation of Spc1 was observed in either mutant, and the Δ*wis4 win1-1* double mutant showed very little Spc1 activation. Thus both Wis4 and Win1 MAPKKKs are required for robust osmostress signaling to the Spc1 MAPK, and therefore these MAPKKK paralogues do not appear to be redundant. Indeed, a previous study found that overexpression of Wis4 cannot complement

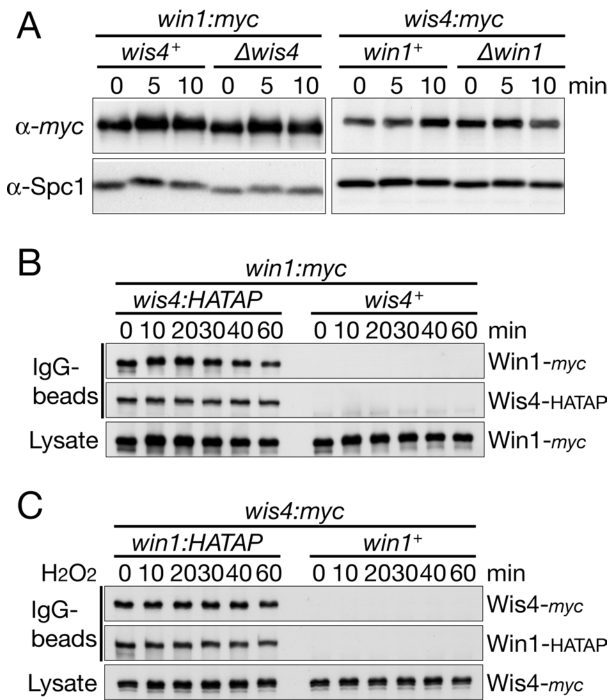

FIGURE 2: The Wis4 and Win1 MAPKKKs form a heteromer complex. (A) *win1:myc* and *wis4:myc* strains were used to monitor the expression levels of the MAPKKKs before and after osmostress of 0.6 M KCl by immunoblotting with anti-*myc* antibodies (α-*myc*). The Spc1 protein in the samples was detected with anti-Spc1 antibodies (α-Spc1) as an internal control. (B, C) Association between the Wis4 and Win1 MAPKKKs before and after stress. Strains that express one MAPKKK tagged with the *myc* epitope and the other tagged with HATAP were exposed to osmostress of 0.6 M KCl (B) or oxidative stress of 0.73 mM H_2O_2 (C), and their cell lysate was subjected to affinity purification with IgG–Sepharose, followed by immunoblotting with anti-*myc* and anti-HA antibodies. The strains expressing no HATAP-tagged MAPKKK served as negative controls (*wis4*+ and *win1*+). Bottom, levels of *myc*-tagged Win1 (B) and Wis4 (C) in the cell lysate used for the affinity purification.

the *win1-1* defect in osmostress response (Samejima *et al.*, 1998). We also performed a reciprocal experiment, which showed that ectopically expressed Win1 does not complement the defective Spc1 activation in Δ*wis4* cells after osmostress (Figure 1C).

One possible explanation for this phenomenon is that expression of Wis4 and Win1 MAPKKKs is interdependent, and the loss of either one leads to a reduced level of the other. Using strains in which the chromosomal *wis4*+ or *win1*+ genes are fused with a sequence encoding the *myc* epitope (Morigasaki *et al.*, 2008), we demonstrated that the Δ*win1* and Δ*wis4* mutations did not affect the expression level of Wis4-*myc* and Win1-*myc*, respectively (Figure 2A). Because dimerization of MTK1, a mammalian homologue of Wis4 and Win1, is critical for its activation (Miyake *et al.*, 2007; Arimoto *et al.*, 2008), we examined an alternative model of whether Wis4 and Win1 form a heteromer complex to function cooperatively. To test the interaction between the two MAPKKKs, a sequence encoding the hemagglutinin (HA) epitope followed by the tandem-affinity purification tag (HATAP) was inserted immediately before the termination codon of the chromosomal *wis4*+ gene in the *win1:myc* strain. Affinity purification of Wis4-HATAP from the cell lysate resulted in coprecipitation of Win1-*myc* (Figure 2B). A reciprocal experiment using a *wis4:myc* strain expressing Win1-HATAP also detected the Wis4–Win1 heteromer complex (Figure 2C). The observed Wis4–Win1 interaction showed little change before and after osmostress and oxidative stress, suggesting constitutive interaction of the two MAPKKKs.

Catalytic activity of only one MAPKKK is required for stress signaling

Activation of the human MTK1 MAPKKK involves *trans* autophosphorylation between the homodimerized MAPKKK molecules (Miyake *et al.*, 2007). Therefore we next examined whether both Wis4 and Win1 MAPKKKs need to be catalytically active to transmit stress signals to the Spc1 MAPK. Lys-1066 in the ATP-binding site of Wis4 was substituted with methionine in the *wis4:myc* strain to create the *wis4KM:myc* strain, which expresses catalytically inactive Wis4KM-*myc* at a level comparable to active Wis4-*myc* (Figure 3A, bottom). Spc1 activation upon osmostress was only slightly reduced in the *wis4KM* strain and was stronger and more prolonged than that in Δ*wis4* cells (Figure 3A, top). This strong Spc1 activation observed in the *wis4KM* strain is dependent on the functional Win1 MAPKKK (Supplemental Figure S1), confirming that Wis4KM is catalytically inactive. We also introduced a similar mutation, methionine substitution of Lys-1149, to the Win1 MAPKKK to test whether Win1 plays a more important role in osmostress signaling than Wis4 (Samejima *et al.*, 1997, 1998). The strain expressing the catalytically inactive Win1KM showed Spc1 activation equal to that in the *wis4KM* strain, and only the *wis4KM win1KM* double mutant failed to induce activation of Spc1 after osmostress (Figure 3B). The KM mutations in Wis4 and Win1 did not affect the Wis4-Win1 interaction, and formation of Wis4-Win1KM (Figure 3C) and Wis4KM-Win1 (Figure 3D) heteromers was observed.

Together the Wis4 and Win1 MAPKKKs form a stable complex whose disruption results in defective osmostress signaling. The MAPKKK complex is largely functional as long as either of the two MAPKKKs is catalytically active. Although less crucial, the MAPKKK heteromer also appears to contribute to prompt activation of the MAPK cascade upon oxidative stress (Supplemental Figure S2; Shiozaki *et al.*, 1998).

The N-terminal segment of Wis4 interacts with the central region of Win1

The experiments described so far revealed that heteromer formation between the Wis4 and Win1 MAPKKKs plays an important role in activation of the Spc1 MAPK cascade. Therefore we further characterized the heteromer interaction between Wis4 and Win1 by testing truncated Wis4 fragments for their interaction with Win1 in a yeast two-hybrid assay. We found that the N-terminal region (amino acid residues 1–300) of Wis4 binds Win1 (Figure 4A). This Wis4(1-300) fragment was subsequently tested for interaction with a series of truncated Win1 fragments, revealing that the central, noncatalytic domain of Win1 (residues 558–1024) binds the Wis4 N-terminal fragment (Figure 4B).

To test whether such interaction between Wis4 and Win1 is indeed important for stress signaling in vivo, we constructed chimera MAPKKKs that have the N-terminal Wis4(1-300) segment followed by the central region of Win1 (chimeras 4-1-1 and 4-1-4 in Figure 4C). Based on the Wis4–Win1 interaction analysis described earlier, these chimeras should allow homophilic interactions. Their ability to induce activation of the Spc1 MAPK cascade was assayed in the absence of the endogenous Wis4 and Win1 MAPKKKs. We found that both 4-1-1 and 4-1-4 chimeras induce strong activation of Spc1 upon osmostress in the Δ*wis4* Δ*win1* strain, whereas expression of wild-type Win1 does not (Figure 4, D and E). No significant

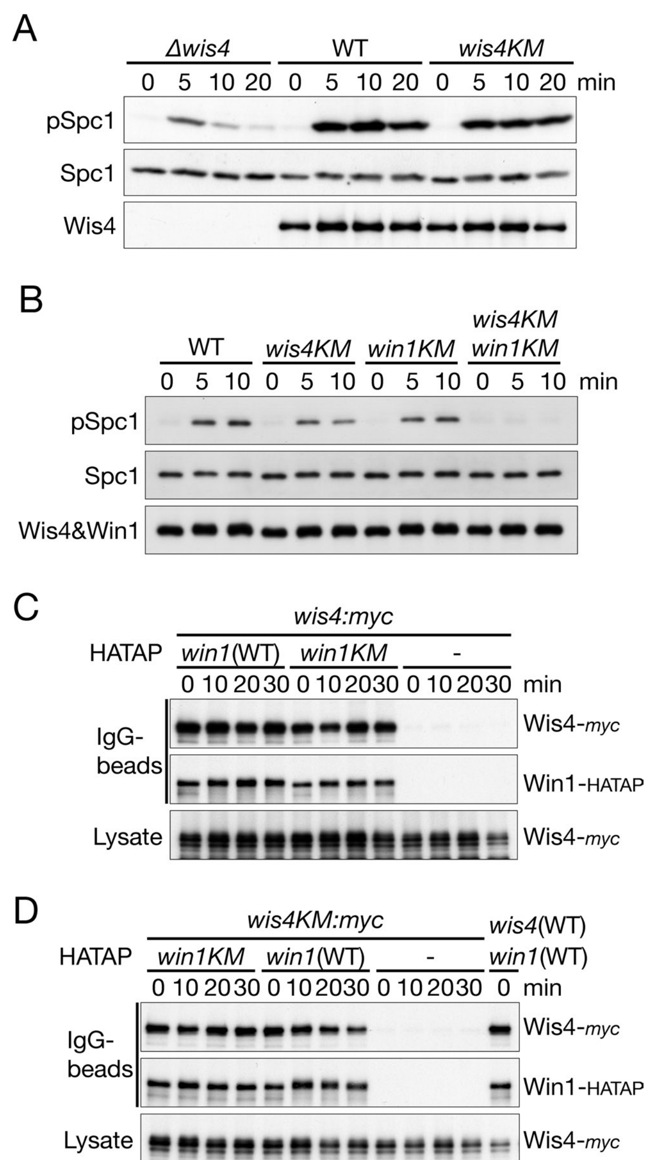

FIGURE 3: A Wis4–Win1 heteromer with only one active MAPKKK can induce activation of the Spc1 cascade. (A) The Δ*wis4* mutant, as well as strains expressing *myc*-tagged wild-type or KM mutants of the Wis4 MAPKKK, were exposed to osmostress of 0.6 M KCl and activation of the Spc1 MAPK was monitored by immunoblotting. The expression levels of Wis4-*myc* and Wis4KM-*myc* were determined by anti-*myc* antibodies (bottom). (B) A strain expressing the *myc*-tagged wild-type Wis4, as well as *wis4KM:myc*, *win1KM:myc*, and *wis4KM:myc win1KM:myc* strains, was exposed to osmostress of 0.6 M KCl and analyzed as in A. (C, D) Strains that express *myc*-tagged Wis4 (C) or Wis4KM (D), together with Win1 or Win1KM with the HATAP tag, were exposed to osmostress of 0.6 M KCl. The cell lysate was subjected to affinity purification with IgG–Sepharose, followed by immunoblotting using anti-*myc* and anti-HA antibodies. The strains expressing no HATAP-tagged MAPKKK served as negative controls (–). Bottom, levels of Wis4-*myc* (C) and Wis4KM-*myc* (D) in the cell lysate used for the affinity purification.

difference was observed between 4-1-1 and 4-1-4 carrying the kinase domain derived from Win1 and Wis4, respectively, and there may be no significant functional difference between the kinase domains of the two MAPKKKs. On the other hand, chimera MAPKKKs with the Win1 N-terminal segment followed by the central region of Wis4 (chimeras 1-4-1 and 1-4-4, Figure 4C) failed to activate the MAPK cascade (Figure 4E and Supplemental Figure S3). Thus, consistent with the results from the yeast two-hybrid assays (Figure 4A and our unpublished results), reciprocal interaction between the Win1 N-terminal segment and the Wis4 central region may not occur.

The Wis4 N-terminal segment contains a sequence that binds the Mcs4 response regulator

Although no function has been assigned to the Win1 central region, we noticed that the N-terminal region of Wis4 contains an ~25–amino acid sequence highly conserved among fungal orthologues of the Wis4 and Win1 MAPKKKs, including Ssk2 in budding yeast (Figure 5A). We refer to this sequence element (Figure 5A) as conserved motif in N-terminus (CMiN) hereafter. The CMiN sequence in Ssk2 (amino acid residues 385–409) is within its N-terminal region required for binding (residues 294–413) and activation (residues 381–440) by the Ssk1 response regulator (Posas and Saito, 1998; Horie *et al.*, 2008). Therefore we first examined whether the Mcs4 response regulator, an Ssk1 orthologue in *S. pombe*, binds to the Wis4 N-terminus that also contains the CMiN. Amino acid residues 120–320 of Wis4 were expressed as a fusion with glutathione *S*-transferase (GST) in the Δ*wis4* Δ*win1* double mutant. Isolation of the GST-fusion protein by glutathione beads copurified Mcs4 (Figure 5B), indicating that this CMiN-containing fragment of Wis4 is sufficient to bind the Mcs4 response regulator. To test whether the CMiN in the Wis4 MAPKKK is indeed required for interaction with Mcs4, we constructed strains in which the sequence encoding the CMiN is deleted from the chromosomal *wis4:HATAP* gene. Immunoglobulin G (IgG)–bead precipitation of Wis4-HATAP resulted in copurification of Mcs4 (Morigasaki *et al.*, 2008), but their association was significantly compromised when the CMiN was deleted (Figure 5C, compare lanes 2 and 4). In the absence of Win1, no Mcs4 was coprecipitated with Wis4-HATAP lacking the CMiN (lane 5), and therefore a fraction of Mcs4 is likely to associate with Win1 in the complex with Wis4. Indeed, similar experiments with Win1-HATAP detected Mcs4 that associates with Win1 in a manner dependent on its CMiN (Figure 5D). These results strongly suggest that the CMiN sequence in the Wis4 and Win1 MAPKKKs is required for their association with the Mcs4 response regulator. Strains expressing Wis4 or Win1 lacking their CMiN exhibited significantly compromised activation of the Spc1 MAPK by high osmolarity stress (Figure 5, E and F), suggesting the importance of Mcs4–MAPKKK association.

Mcs4 is required for stable heteromer formation between the Wis4 and Win1 MAPKKKs

We noticed that deleting the CMiN from the Wis4(1-300) fragment abrogates its interaction with Win1 in Y2H assays (Supplemental Figure S4), implying involvement of the CMiN in Wis4–Win1 heteromer formation. Because the CMiN is required for the binding of Mcs4 response regulator to those MAPKKKs, we tested whether the Mcs4 response regulator affects the Wis4–Win1 association. Affinity purification of Wis4-HATAP from *mcs4*$^{+}$ cells with IgG beads resulted in coprecipitation of Win1-*myc* (Figure 6A, lane 2), which was dramatically reduced in the Δ*mcs4* background (lane 3). Because the Δ*mcs4* mutation appeared not to affect the expression levels of the Wis4-HATAP and Win1-*myc* proteins, the results indicate that the Mcs4 response regulator is required for stable interaction between the Wis4 and Win1 MAPKKKs. We also found that deletion of the CMiN in Wis4 decreased the amount of associated Win1 (lane 4), which was further reduced by the Δ*mcs4* mutation (lane 5). Thus the contribution of Mcs4 to the Wis4–Win1 association is not solely

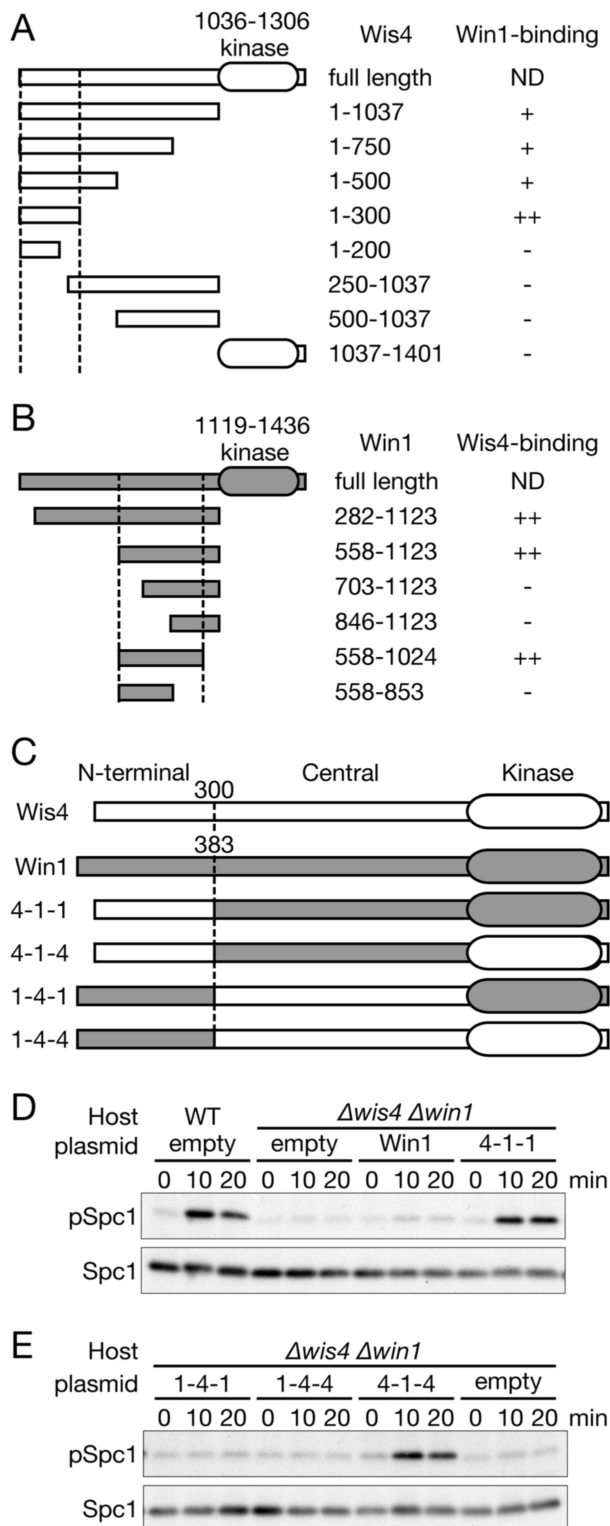

FIGURE 4: The N-terminal segment of Wis4 and the central region of Win1 are important for physical interaction between the MAPKKKs and for activation of the Spc1 cascade. (A) Yeast two-hybrid assays to detect interaction of amino acid residues 282–1123 of Win1 (bait) with a series of truncated Wis4 fragments of indicated amino acid residues (prey). ND, not determined. (B) Yeast two-hybrid assays to detect interactions of the Wis4 fragment of amino acid residues 1–300 (prey) with a series of truncated Win1 fragments as indicated (bait). (C) Designs of chimera MAPKKKs. Wis4 and Win1 MAPKKKs were divided into three segments: the N-terminal region with the CMiN, the central, noncatalytic domain, and the kinase domain. Chimeras were constructed by different combinations of the three segments as schematically shown. (D, E) In a Δ*wis4* Δ*win1* strain, chimera MAPKKKs shown in C were expressed at low levels from plasmids using the thiamine-repressible *nmt1* promoter (Maundrell, 1990) in the presence of 2 μM thiamine. Activation of the Spc1 MAPK was monitored by immunoblotting along the time course after exposure to osmostress of 0.6 M KCl. Wild-type and Δ*wis4* Δ*win1* strains carrying the empty vector plasmid were included as controls.

through the Wis4 CMiN. Indeed, deletion of the CMiN from Win1 also compromises the Wis4–Win1 interaction (compare lanes 7 and 9), suggesting a role of Mcs4 bound to the Win1 CMiN.

Because Wis4–Win1 interaction is stable before and after stress (Figure 2), we examined whether association of Mcs4 with the MAPKKKs is also constitutive. The amount of Mcs4 coprecipitated with Wis4-HATAP (Figure 6B) or Win1-HATAP (Figure 6C) during IgG-bead purification did not significantly change throughout the time course of osmostress. Thus the Mcs4 response regulator constitutively binds to the MAPKKKs, suggesting a stable ternary complex composed of Mcs4 and the Wis4–Win1 heteromer.

The response regulator–MAPKKK heteromer complex interacts with the downstream MAPKK

Osmostress-induced activation of the Spc1 MAPK cascade is significantly compromised when the Mcs4–Wis4–Win1 complex is disturbed by the Δ*mcs4* (Shieh *et al.*, 1997), Δ*wis4*, and *win1-1* (Figure 1) mutations or by deletion of the CMiN from Wis4 or Win1 (Figure 5, E and F). In addition, the functional chimera MAPKKKs with the Wis4 N-terminal segment and the Win1 central region (4-1-1 and 4-1-4 in Figure 4C) also require Mcs4 for robust Spc1 activation upon osmostress (Supplemental Figure S5). Thus it is likely that formation of the response regulator–MAPKKK complex is important for receiving osmostress stimuli and/or activating the downstream MAPKK. Because the mechanism that senses osmostress upstream of the Spc1 cascade is unknown, we examined physical interaction of the Wis4 and Win1 MAPKKKs with the downstream Wis1 MAPKK. IgG-bead precipitation of Win1-HATAP detected association of the Wis1 MAPKK (Figure 7A, lane 4), whereas MAPKKK and MAPKK dissociate immediately after exposure to osmolarity stress (lanes 5 and 6). However, isolation of Win1-HATAP from the Δ*mcs4* strain copurified no detectable Wis1 MAPKK (lane 7), indicating that the MAPKKK–MAPKK association is significantly compromised in the absence of Mcs4. Similarly, binding of the Wis4 MAPKKK to the Wis1 MAPKK was detected in the absence of stress stimuli, but their association is disrupted in the Δ*mcs4* as well as in the Δ*win1* strain (Figure 7B). These results suggest that the Wis4–Win1 MAPKKK heteromer stabilized by the Mcs4 response regulator physically associates with the Wis1 MAPKK in the absence of stress stimuli. This prestress complex between MAPKKK and MAPKK is likely to be crucial for robust activation of the MAPK cascade.

To test whether the stress-induced dissociation of the Wis1 MAPKK from the MAPKKKs is dependent on activation of the MAPK cascade, we examined the MAPKKK–MAPKK interaction in a *wis4KM win1KM* strain. The KM mutations in Wis4 and Win1 did not affect their heteromer formation (Figure 3D), and association of the Wis1 MAPKK with Wis4KM was detectable in the absence of stress (Figure 7C). Of interest, Wis1 was released from the mutant MAPKKK upon osmostress, as in wild-type cells. Thus dissociation of the Wis1 MAPKK from the MAPKKK complex appears to be triggered by a

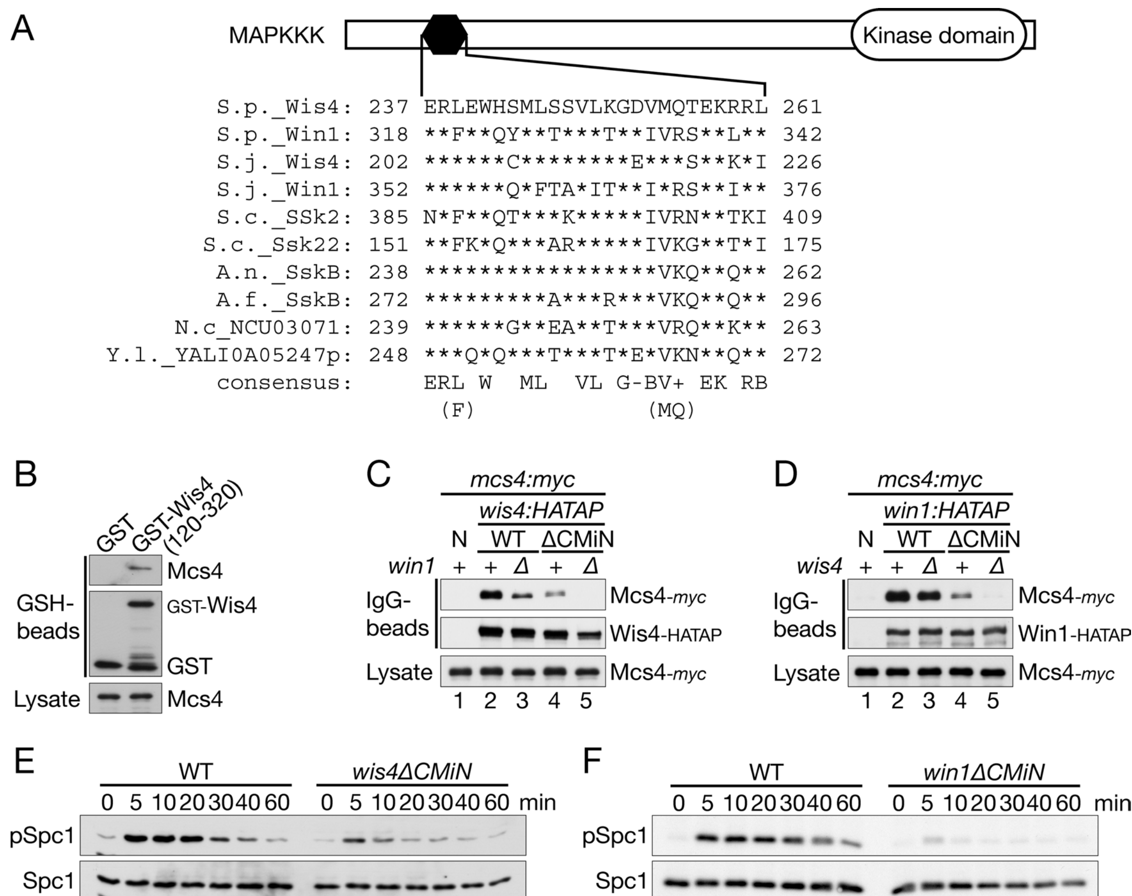

FIGURE 5: The conserved motif in N-terminus (CMiN) of the Wis4 and Win1 MAPKKKs is required for interaction with the Mcs4 response regulator. (A) Alignment of the CMiN sequences of fungal stress MAPKKKs using ClustalW2 (Goujon *et al.*, 2010). A.f., *Aspergillus fumigatus*; A.n., *Aspergillus niger*; N.c., *Neurospora crassa*; S.j., *Schizosaccharomyces japonicus*; S.p., *Schizosaccharomyces pombe*; Y.l., *Yarrowia lipolytica*. Asterisks indicate amino acid residues identical to those in *S. pombe* Wis4. The consensus sequence is shown at the bottom. –, acidic residues; +, basic residues; B, branched-chain residues. (B) Mcs4 binds to the N-terminal region of Wis4 MAPKKK. GST and GST fused to amino acid residues 120–320 of Wis4 were expressed in a *mcs4:myc* strain and affinity purified using glutathione–Sepharose. Proteins bound to the beads and Mcs4-*myc* in the crude lysate were detected with anti-*myc* and anti-GST antibodies. (C, D) Deletion of the CMiN (ΔCMiN) in the MAPKKKs impairs their association with the Mcs4 response regulator. Cell lysate from *mcs4:myc* strains expressing Wis4-HATAP (C) or Win1-HATAP (D) with or without CMiN were subjected to IgG–Sepharose affinity purification, and proteins bound to the beads and Mcs4-*myc* in the lysate were detected by immunoblotting. N, negative controls with strains expressing no HATAP-tagged MAPKKK. (E, F) Osmostress-induced activation of the Spc1 MAPK in cells expressing Wis4ΔCMiN-HATAP (E) or Win1ΔCMiN-HATAP (F) was compared with that in wild-type cells (WT). Spc1 phosphorylation was determined as in Figure 1C.

mechanism independent of the stress-induced phosphorylation of Wis1 by the MAPKKKs.

DISCUSSION

In this study, we demonstrated that osmotic activation of the Spc1 MAPK cascade is mediated by the Wis4–Win1 MAPKKK heteromer complex stabilized by the Mcs4 response regulator. Of interest, only one of the MAPKKKs in the complex needs to be catalytically active, but the integrity of the complex (Figure 8) is important for physical interaction with the downstream Wis1 MAPKK. MAPKKK–MAPKK interaction promoted by formation of MAPKKK heteromer may represent a novel signaling mechanism within a MAPK cascade.

It was previously proposed that Win1, but not Wis4, is responsible for osmostress signaling to the Spc1 cascade, because increased expression of Wis4 was unable to complement the defective osmostress response of the Δ*win1* mutant (Samejima *et al.*, 1998). However, this phenomenon is now explained by the essential role of the Wis4–Win1 MAPKKK heteromer in osmostress signaling. In contrast, the two MAPKKK paralogues, Ssk2 and Ssk22, in the HOG cascade of *Saccharomyces cerevisiae* are redundant, and Ssk2 alone is sufficient to transmit osmolarity signals from the Sln1–Ypd1–Ssk1 phosphorelay (Maeda *et al.*, 1995). Nevertheless, recent large-scale interaction mapping detected physical association between Ssk2 and Ssk22 (Breitkreutz *et al.*, 2010). Although the functional significance of their association is unknown, stress-activated MAPKKK heteromer may also be conserved in *S. cerevisiae*. On the other hand, many fungal species other than *Saccharomyces* and *Schizosaccharomyces* have only a single MAPKKK structurally related to Ssk2/Ssk22/Wis4/Win1 (Supplemental Table S1 and Supplemental Figure S6). It is conceivable that the stress-activated MAPKKKs in those species function as a homodimer, which might be a prototype of the Wis4–Win1 interaction evolved in fission yeast.

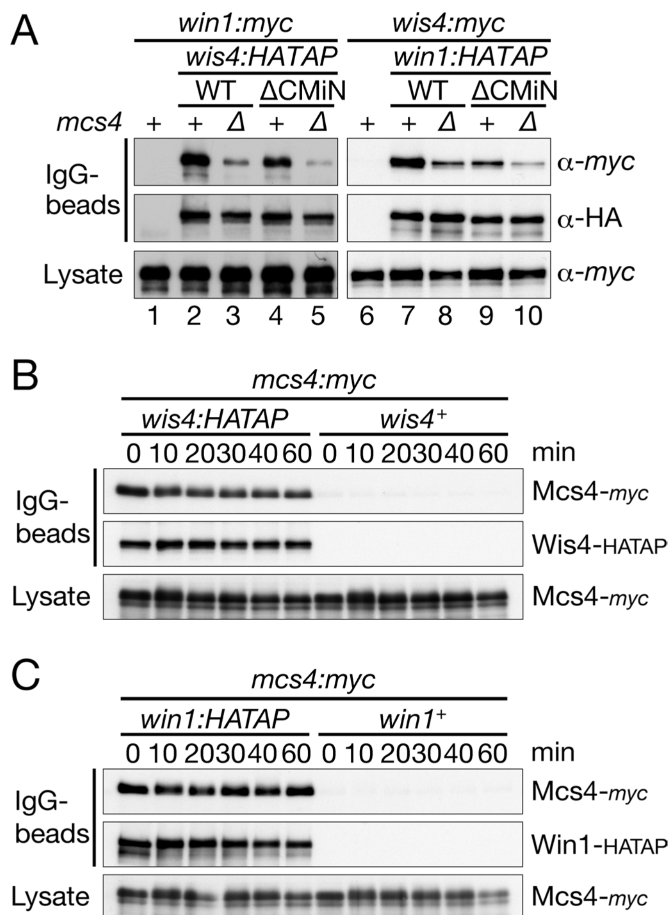

FIGURE 6: The Mcs4 response regulator constitutively binds to the Wis4 and Win1 MAPKKKs and promotes their association. (A) Mcs4-dependent interaction between Wis4 and Win1. Association between Wis4 and Win1 was detected as in Figure 2, B and C, using strains expressing HATAP-tagged, wild-type, and ΔCMiN mutant MAPKKKs, both in the *mcs4*+ (+) and Δ*mcs4* (Δ) backgrounds. Lanes 1 and 6, negative controls with strains expressing no HATAP-tagged MAPKKK. (B, C) Mcs4 constitutively associates with the Wis4 and Win1 MAPKKKs. Cell lysate from *mcs4:myc* strains expressing HATAP-tagged Wis4 (B) or Win1 (C) was subjected to IgG–Sepharose affinity purification, and proteins bound to the beads and Mcs4-*myc* in the lysate were detected by immunoblotting. Strains expressing no HATAP-tagged MAPKKK (*wis4*+ and *win1*+) were used as negative controls.

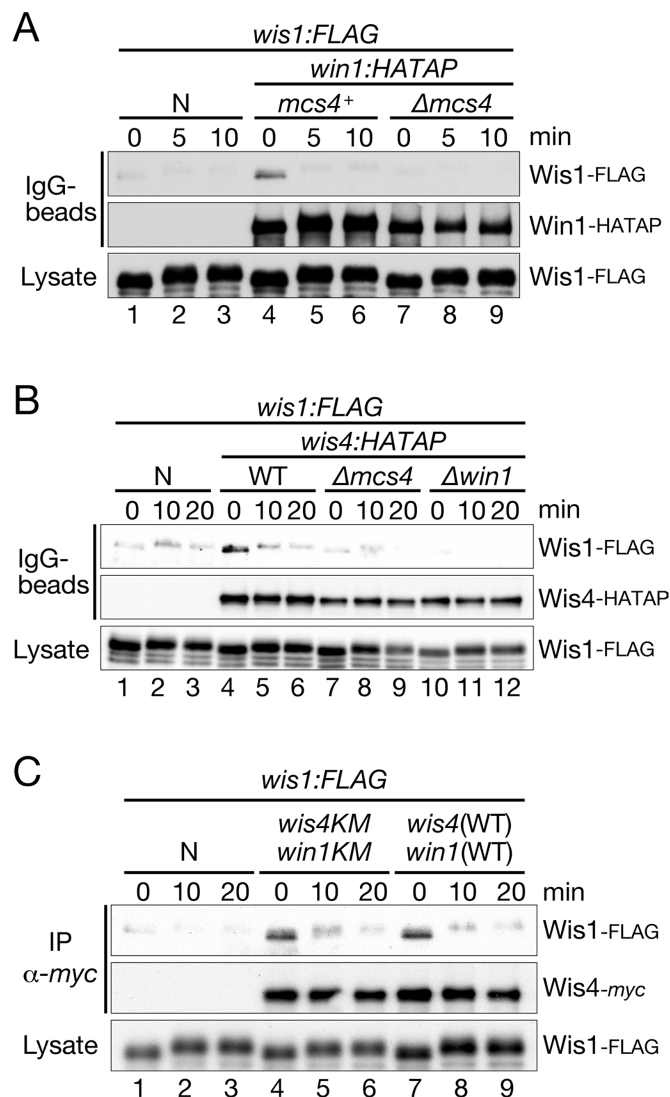

FIGURE 7: Mcs4-dependent interaction of the Wis4 and Win1 MAPKKKs with the Wis1 MAPKK in the absence of stress stimuli. (A, B) *wis1:FLAG* strains expressing HATAP-tagged Win1 (A) or Wis4 (B) MAPKKKs were exposed to osmostress of 0.6 M KCl, and association between Wis1-FLAG and the HATAP-tagged MAPKKKs was examined by IgG–Sepharose affinity purification followed by immunoblotting. Experiments were performed in the wild-type, Δ*mcs4*, and Δ*win1* backgrounds. N, negative controls with strains expressing no HATAP-tagged MAPKKK. Bottom, expression level of Wis1-FLAG in the cell lysate used for the immunoprecipitation. (C) *wis1:FLAG* strains expressing *myc*-tagged Wis4 and HATAP-tagged Win1 with or without the KM mutations (Figure 3) were exposed to osmostress of 0.6 M KCl, and association between Wis1-FLAG and Wis4-*myc* was examined by anti-*myc* immunoprecipitation followed by immunoblotting. N, negative controls with a *wis1:FLAG win1:HATAP* strain expressing untagged Wis4.

Our results strongly suggest that interaction between the N-terminal segment of Wis4 and the central region of Win1 is the molecular basis for heterologous association between the two MAPKKKs. Stable Wis4–Win1 interaction is dependent on the Mcs4 response regulator; the MAPKKK heteromer complex is undermined in the absence of Mcs4, resulting in compromised signaling to the Spc1 MAPK. The Δ*mcs4* phenotype was not complemented by artificial ligand-induced dimerization (Ho *et al.*, 1996) by fusing FKBP12 and the FKBP-rapamycin binding domain to the N-termini of Win1 and Wis4, respectively (our unpublished results). Therefore the Wis4–Win1 heteromer may need to be assembled by Mcs4 in a specific configuration. The Mcs4 response regulator is part of the Mak2/3–Mpr1–Mcs4 phosphorelay that transmits peroxide stress signals and activates the Wis4 and Win1 MAPKKKs (Nguyen *et al.*, 2000; Buck *et al.*, 2001; Morigasaki *et al.*, 2008). However, the Mcs4 function manifested in osmostress signaling is independent of the phosphorelay, because mutations to the phosphor-acceptor site in Mpr1 or Mcs4 do not impair activation of the Spc1 MAPK cascade by high osmolarity (Nguyen *et al.*, 2000; Buck *et al.*, 2001). It is likely that the defects of *mcs4* mutants in Spc1 activation by diverse stress (Shieh *et al.*, 1997; Shiozaki *et al.*, 1997) are due to destabilized MAPKKK heteromer and compromised signaling to the Wis1 MAPKK. The phosphorelay-independent function of Mcs4 seems to be quite

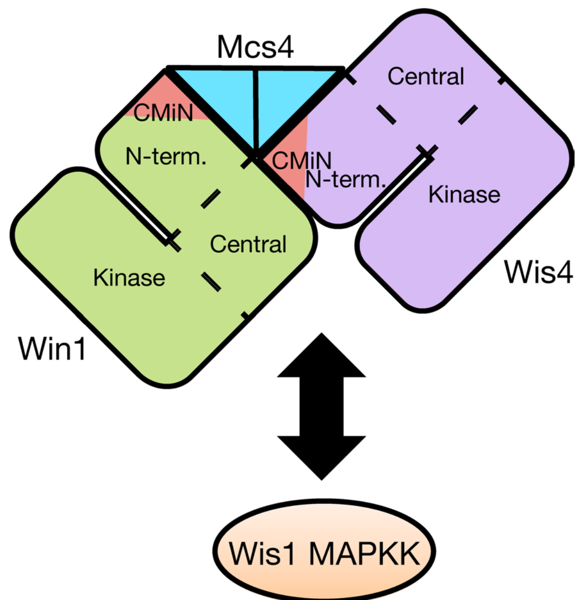

FIGURE 8: Schematic model of the Wis4–Win1 MAPKKK heteromer complex.

unique among response regulators, which normally act as terminal effectors of phosphorelay signaling (Gao and Stock, 2010). The dual functionality of Mcs4 might have evolved as a part of the mechanism to transmit multiple stress stimuli to the Spc1 MAPK cascade.

The Mcs4 response regulator binds to the Wis4 and Win1 MAPKKKs within their N-terminal region that contains CMiN, a sequence motif conserved among fungal Wis4/Win1 orthologues. We noticed a CMiN-like sequence in the N-terminal region of MTK1 and MEKK4, mammalian orthologues of Wis4/Win1 (Supplemental Figure S7). Our results and those of others strongly suggest pivotal roles of the CMiN sequence in the regulation of the stress-responsive MAPKKKs. First, the CMiN is within the binding site for activator proteins, the Mcs4 response regulator in *S. pombe*, the Ssk1 response regulator in *S. cerevisiae* (Posas and Saito, 1998), and GADD45 in mammals (Takekawa and Saito, 1998). We show that Mcs4 cannot bind to the Wis4 and Win1 MAPKKKs when their CMiN is deleted. Second, eliminating CMiN from Ssk2 (Horie *et al.*, 2008), Wis4, and Win1 (Figure 5) abrogates activation of those MAPKKKs. Third, the Wis4 CMiN is essential for the interaction between the Wis4 N-terminal segment and the central region of Win1 (Supplemental Figure S4). Although the N-terminus of Win1 including CMiN does not appear to be directly involved in the interaction with Wis4, deletion of CMiN from Win1 compromises its association with Wis4, as well as activation of the Spc1 MAPK (Figure 5). Because many response regulator proteins, including Ssk1, dimerize (Horie *et al.*, 2008; Gao and Stock, 2010), it is possible that Mcs4 bound to the CMiN regions of Win1 and Wis4 dimerizes to form the stable Mcs4–Wis4–Win1 complex (Figure 8). Another possibility is that binding of Mcs4 alters the conformation of Win1 and Wis4 and thus promotes their association; it has been proposed that binding of GADD45 to the human MTK1 MAPKKK unmasks its coiled-coil domain, leading to homodimer formation (Miyake *et al.*, 2007; Arimoto *et al.*, 2008). Obviously, these possibilities are not mutually exclusive.

Binding of GADD45 to MTK1 around its CMiN-like sequence is also believed to dismiss autoinhibitory interaction within MTK1 and trigger activation of the MAPKKK (Takekawa and Saito, 1998; Mita *et al.*, 2002). In contrast, association of the Mcs4 response regulator to the Wis4–Win1 heteromer is constitutive, and therefore binding of Mcs4 itself is not a switch to turn on the Wis4 and Win1 activity in response to stress. However, deletion of the N-terminal noncatalytic domain of Wis4 and Win1, including the CMiN region, results in activation of the MAPKKKs even in the absence of Mcs4 (Shiozaki *et al.*, 1997; our unpublished results). Thus it is likely that the N-terminal regions of Wis4 and Win1 are also inhibitory to their kinase activity, and binding of Mcs4 to the CMiN sequence may play an important role in relieving the autoinhibition of the MAPKKKs upon stress stimuli. In addition, we found that the intact Mcs4–Wis4–Win1 complex is required for physical association between the MAPKKKs and Wis1 MAPKK in the absence of stress (Figure 7). The stable Mcs4–MAPKKK heteromer complex may serve as a scaffold that assembles the MAPKK and additional signaling components for stress-induced activation of the Spc1 cascade. Scaffold proteins play key roles in specific activation and signal transmission of MAPK cascades in diverse eukaryotes (Dhanasekaran *et al.*, 2007). Indeed, our experiments using the catalytically inactive Wis4 and Win1 MAPKKKs (Figure 3) imply their structural role, which may bear some resemblance to the function of kinase suppressor of Ras, a metazoan scaffold protein with inactivating mutations to its Raf-like kinase domain (Morrison and Davis, 2003).

In response to high osmolarity stress, the Wis1 MAPKK dissociates from the Mcs4–Wis4–Win1 complex, even when both MAPKKKs are catalytically inactive and cannot phosphorylate Wis1. The Mcs4–MAPKKK complex might be subjected to a conformational change that triggers the release of Wis1 from the complex. It is also worth noting that osmostress induces a shift in the electrophoretic mobility of the Wis1 MAPKK even in the Δ*mcs4*, Δ*win1*, and *wis4KM win1KM* strains (lysate panels in Figure 7). Thus, in addition to activating phosphorylation by the MAPKKKs, Wis1 might receive some osmostress signal input, resulting in its release from the MAPKKKs. Our future studies will focus on the identification of such a signaling mechanism to the Wis1 MAPKK.

In mammals, several heterologous MAPKKK interactions have been identified, including Ras-induced heteromer formation between B-Raf and C-Raf (Raf-1; Weber *et al.*, 2001; Garnett *et al.*, 2005) and heteromeric association of Ask1 and Ask2, MAPKKK paralogues involved in oxidative stress response (Takeda *et al.*, 2007). As far as we know, the Wis4–Win1 association discovered in this study is the first example of a functional MAPKKK heteromer complex in yeast. We expect that further genetic and biochemical characterization of the Wis4–Win1 complex as prototypical MAPKKK heteromer will offer new insights into evolutionarily conserved principles of MAPKKK regulation.

MATERIALS AND METHODS

General *S. pombe* methods

The *S. pombe* strains used in this study are listed in Supplemental Table S2. Growth media and basic techniques for fission yeast were previously described (Alfa *et al.*, 1993). Epitope tagging and disruption of chromosomal genes were performed by the PCR-based method (Bähler *et al.*, 1998). Phenotypic analysis confirmed that the tag sequences did not affect the function of the proteins used in this study. Stress treatment of *S. pombe* cells was carried out as previously described (Shiozaki and Russell, 1997). For oxidative stress, H_2O_2 was used at the final concentration of 0.73 mM.

Construction of yeast strains expressing a mutant MAPKKK

For kinase-dead forms of the MAPKKKs, Lys-1066 in Wis4 and Lys-1149 in Win1 were mutated to methionine in *wis4KM* and *win1KM*, respectively. The site-directed mutagenesis was carried out using

plasmids for *myc*-tagging, pBSII-wis4(2206-4203):*myc* and pBSII-win1(3196-4308):*myc*, and the primer sets wis4KM and win1KM, respectively (Supplemental Table S3), and the resulting plasmids were integrated into the *wis4*$^+$ and *win1*$^+$ loci. To remove the CMiN-coding sequence from the *wis4*$^+$ locus, the CMiN sequence was first replaced with the *ura4*$^+$ gene by homologous recombination using a PCR fragment amplified by the primer set wis4ΔCMiN::ura4$^+$. The resultant, *S. pombe* strain, *wis4ΔCMiN::ura4*$^+$, was transformed with another PCR fragment consisting of nucleotides +340 to +708 (340–708) directly followed by nucleotides 784–1185 of the *wis4*$^+$ gene (PCR primer set, wis4ΔCMiN). Transformants that had lost the *ura4*$^+$ gene were selected on growth medium with 5-fluoroorotic acid. The Win1ΔCMiN-expressing strain was constructed through a similar procedure using the primer sets win1ΔCMiN::ura4$^+$ and win1ΔCMiN. The nucleotide sequences of the mutated loci were confirmed by DNA sequencing.

Construction of plasmids

The plasmids used in this study are listed in Supplemental Table S4. Site-directed mutagenesis was performed using the QuikChange Kit (Agilent Technologies, Santa Clara, CA) and confirmed by DNA sequencing.

For the construction of pREP1-wis4:FLAG, two base substitutions, T1782C and C3690T, were introduced within the *wis4*$^+$ open reading frame (ORF) by site-directed mutagenesis in order to eliminate *Nde*I restriction sites. These substitutions are silent and do not alter the encoded amino acid sequence. The resultant *wis4*$^+$ gene fragment was PCR amplified using the primer set wis4 full length, which adds *Nde*I and *Not*I restriction sites to the 5′ and 3′ termini of the ORF, respectively. Subsequently, the *Nde*I–*Not*I fragment of *wis4*$^+$ was cloned into the pREP1 vector (Maundrell, 1990) carrying three copies of the FLAG epitope sequence immediately after the *Not*I cloning site.

The full-length, wild-type *win1*$^+$ gene cannot be cloned in *Escherichia coli* (Samejima *et al.*, 1998). For the construction of pREP1-win1:FLAG, the following nucleotide changes were introduced to the *win1*$^+$ ORF by site-directed mutagenesis without altering the encoded amino acids; T1278G, A1281G (Tatebe *et al.*, 2005), C1005A, T1011G, T1599C, and A2442G. The *win1*$^+$ ORF was cloned between the *Nde*I and *Not*I sites in the described pREP1-FLAG vector.

The chimera MAPKKK genes (Figure 4C) were constructed in the pBluescript II SK- (pBS) plasmid vector through PCR-mediated gene fusion and subcloning. Subsequently, each chimera was cloned into the pREP1-FLAG vector as *Nde*I–*Not*I fragments and expressed in *S. pombe* in the presence of 2 μM thiamine, which allows leaky, low-level expression from the thiamine-repressible *nmt1*$^+$ promoter (Maundrell, 1990). The chimera genes are structured as follows (nucleotide numbers of the *wis4*$^+$ and *win1*$^+$ ORFs are shown). chimera 4-1-1, wis4(1-900)–win1(1150-4308); chimera 4-1-4, wis4(1-900)–win1(1150-3354)–wis4(3106-4203); chimera 1-4-1, win1(1-1149)–wis4(901-3105)–win1(3355-4308); chimera 1-4-4, win1(1-1149)– wis4(901-4203).

Detection of protein–protein interactions

Yeast two-hybrid assays to determine the regions involved in the interaction between Wis4 and Win1 were performed as described previously (Tatebe *et al.*, 2005).

For detection of protein interactions by coimmunoprecipitation experiments (Morigasaki and Shiozaki, 2010), two different pairs of lysis and wash buffers were used: lysis A (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40, 10% glycerol, 1 mM 2-mercaptoethanol, 5 mM EDTA, 1 mM ethylene glycol tetraacetic acid, 50 mM NaF, 0.1 mM Na_3VO_4, 60 mM β-glycerophosphate, 15 mM *p*-nitrophenyl phosphate) and wash A (lysis A without β-glycerophosphate and *p*-nitrophenyl phosphate) to examine the interaction between Mcs4, Wis4, and Win1; lysis B (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–KOH, pH 7.8, 500 mM potassium glutamate, 0.1% NP-40, 10% glycerol, 0.1 mM Na_3VO_4, 15 mM *p*-nitrophenyl phosphate) and wash B (same as lysis B without protease inhibitors) for the interaction between MAPKKK and Wis1 MAPKK. Protease inhibitors, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and the Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO), were added to all lysis buffers. Harvested cells (~2×10^8) were disrupted in 400 μl of the lysis buffer, and after centrifugation, the supernatant was incubated for 1 h with IgG–Sepharose (GE Healthcare, Piscataway, NJ) for HATAP-tagged proteins or with anti–c-*myc* agarose conjugate (Sigma-Aldrich) for Wis4-*myc*. After washing four times with the Wash buffer, purified proteins were eluted with the SDS–PAGE sample buffer and analyzed by immunoblotting.

Detection of phosphorylated Spc1 and epitope-tagged proteins

Activation of Spc1 MAPK was monitored by immunoblotting with anti–phospho-p38 (Cell Signaling Technology, Beverly, MA) or anti–phospho-Spc1 polyclonal antibodies (Tatebe and Shiozaki, 2003). The total amount of Spc1 in the cell lysate was monitored using rabbit polyclonal antibodies raised against the Spc1 C-terminal peptide (CSFHNMDNELQS). To detect epitope-tagged proteins, immunoblotting was performed using anti-HA (12CA5; Roche, Indianapolis, IN), anti-FLAG (M2; Sigma-Aldrich), anti-*myc* (9E10 sc-40; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-GST (goat serum; GE Healthcare).

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REFERENCES

Alfa C, Fantes P, Hyams J, Warbrick E (1993). Experiments with Fission Yeast : A Laboratory Course Manual, Plainview, NY: Cold Spring Harbor Laboratory Press.

Arimoto K, Fukuda H, Imajoh-Ohmi S, Saito H, Takekawa M (2008). Formation of stress granules inhibits apoptosis by suppressing stress-responsive MAPK pathways. Nat Cell Biol 10, 1324–1332.

Bähler J, Wu JQ, Longtine MS, Shah NG, McKenzie A, Steever AB, Wach A, Philippsen P, Pringle JR (1998). Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. Yeast 14, 943–951.

Breitkreutz A *et al.* (2010). A global protein kinase and phosphatase interaction network in yeast. Science 328, 1043–1046.

Brewster JL, de Valoir T, Dwyer ND, Winter E, Gustin MC (1993). An osmosensing signal transduction pathway in yeast. Science 259, 1760–1763.

Buck V, Quinn J, Soto Pino T, Martin H, Saldanha J, Makino K, Morgan BA, Millar JB (2001). Peroxide sensors for the fission yeast stress-activated mitogen-activated protein kinase pathway. Mol Biol Cell 12, 407–419.

Chen D, Toone WM, Mata J, Lyne R, Burns G, Kivinen K, Brazma A, Jones N, Bähler J (2003). Global transcriptional responses of fission yeast to environmental stress. Mol Biol Cell 14, 214–229.
Cuevas BD, Abell AN, Johnson GL (2007). Role of mitogen-activated protein kinase kinase kinases in signal integration. Oncogene 26, 3159–3171.
Degols G, Shiozaki K, Russell P (1996). Activation and regulation of the Spc1 stress-activated protein kinase in *Schizosaccharomyces pombe*. Mol Cell Biol 16, 2870–2877.
Dhanasekaran DN, Kashef K, Lee CM, Xu H, Reddy EP (2007). Scaffold proteins of MAP-kinase modules. Oncogene 26, 3185–3202.
Gao R, Stock AM (2010). Molecular strategies for phosphorylation-mediated regulation of response regulator activity. Curr Opin Microbiol 13, 160–167.
Garnett MJ, Rana S, Paterson H, Barford D, Marais R (2005). Wild-type and mutant B-RAF activate C-RAF through distinct mechanisms involving heterodimerization. Mol Cell 20, 963–969.
Goujon M, McWilliam H, Li W, Valentin F, Squizzato S, Paern J, Lopez R (2010). A new bioinformatics analysis tools framework at EMBL-EBI. Nucleic Acids Res 38, W695–W699.
Ho SN, Biggar SR, Spencer DM, Schreiber SL, Crabtree GR (1996). Dimeric ligands define a role for transcriptional activation domains in reinitiation. Nature 382, 822–826.
Horie T, Tatebayashi K, Yamada R, Saito H (2008). Phosphorylated Ssk1 prevents unphosphorylated Ssk1 from activating the Ssk2 mitogen-activated protein kinase kinase kinase in the yeast high-osmolarity glycerol osmoregulatory pathway. Mol Cell Biol 28, 5172–5183.
Kyriakis JM, Avruch J (2012). Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update. Physiol Rev 92, 689–737.
Maeda T, Takekawa M, Saito H (1995). Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. Science 269, 554–558.
Maeda T, Wurgler-Murphy SM, Saito H (1994). A two-component system that regulates an osmosensing MAP kinase cascade in yeast. Nature 369, 242–245.
Maundrell K (1990). nmt1 of fission yeast. A highly transcribed gene completely repressed by thiamine. J Biol Chem 265, 10857–10864.
Millar JB, Buck V, Wilkinson MG (1995). Pyp1 and Pyp2 PTPases dephosphorylate an osmosensing MAP kinase controlling cell size at division in fission yeast. Genes Dev 9, 2117–2130.
Mita H, Tsutsui J, Takekawa M, Witten EA, Saito H (2002). Regulation of MTK1/MEKK4 kinase activity by its N-terminal autoinhibitory domain and GADD45 binding. Mol Cell Biol 22, 4544–4555.
Miyake Z, Takekawa M, Ge Q, Saito H (2007). Activation of MTK1/MEKK4 by GADD45 through induced N-C dissociation and dimerization-mediated trans autophosphorylation of the MTK1 kinase domain. Mol Cell Biol 27, 2765–2776.
Morigasaki S, Shimada K, Ikner A, Yanagida M, Shiozaki K (2008). Glycolytic enzyme GAPDH promotes peroxide stress signaling through multistep phosphorelay to a MAPK cascade. Mol Cell 30, 108–113.
Morigasaki S, Shiozaki K (2010). Two-component signaling to the stress MAP kinase cascade in fission yeast. Methods Enzymol 471, 279–289.
Morrison DK, Davis RJ (2003). Regulation of MAP kinase signaling modules by scaffold proteins in mammals. Annu Rev Cell Dev Biol 19, 91–118.
Nguyen AN, Lee A, Place W, Shiozaki K (2000). Multistep phosphorelay proteins transmit oxidative stress signals to the fission yeast stress-activated protein kinase. Mol Biol Cell 11, 1169–1181.
Nguyen AN, Shiozaki K (1999). Heat-shock-induced activation of stress MAP kinase is regulated by threonine- and tyrosine-specific phosphatases. Genes Dev 13, 1653–1663.
Petersen J, Hagan IM (2005). Polo kinase links the stress pathway to cell cycle control and tip growth in fission yeast. Nature 435, 507–512.
Petersen J, Nurse P (2007). TOR signalling regulates mitotic commitment through the stress MAP kinase pathway and the Polo and Cdc2 kinases. Nat Cell Biol 9, 1263–1272.
Posas F, Saito H (1997). Osmotic activation of the HOG MAPK pathway via Ste11p MAPKKK: scaffold role of Pbs2p MAPKK. Science 276, 1702–1705.
Posas F, Saito H (1998). Activation of the yeast SSK2 MAP kinase kinase kinase by the SSK1 two-component response regulator. EMBO J 17, 1385–1394.
Posas F, Wurgler-Murphy SM, Maeda T, Witten EA, Thai TC, Saito H (1996). Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 "two-component" osmosensor. Cell 86, 865–875.
Quinn J, Findlay VJ, Dawson K, Millar JB, Jones N, Morgan BA, Toone WM (2002). Distinct regulatory proteins control the graded transcriptional response to increasing H2O2 levels in fission yeast *Schizosaccharomyces pombe*. Mol Biol Cell 13, 805–816.
Samejima I, Mackie S, Fantes PA (1997). Multiple modes of activation of the stress-responsive MAP kinase pathway in fission yeast. EMBO J 16, 6162–6170.
Samejima I, Mackie S, Warbrick E, Weisman R, Fantes PA (1998). The fission yeast mitotic regulator win1$^+$ encodes an MAP kinase kinase kinase that phosphorylates and activates Wis1 MAP kinase kinase in response to high osmolarity. Mol Biol Cell 9, 2325–2335.
Shieh JC, Wilkinson MG, Buck V, Morgan BA, Makino K, Millar JB (1997). The Mcs4 response regulator coordinately controls the stress-activated Wak1-Wis1-Sty1 MAP kinase pathway and fission yeast cell cycle. Genes Dev 11, 1008–1022.
Shiozaki K, Russell P (1995). Cell-cycle control linked to extracellular environment by MAP kinase pathway in fission yeast. Nature 378, 739–743.
Shiozaki K, Russell P (1996). Conjugation, meiosis, and the osmotic stress response are regulated by Spc1 kinase through Atf1 transcription factor in fission yeast. Genes Dev 10, 2276–2288.
Shiozaki K, Russell P (1997). Stress-activated protein kinase pathway in cell cycle control of fission yeast. Methods Enzymol 283, 506–520.
Shiozaki K, Shiozaki M, Russell P (1997). Mcs4 mitotic catastrophe suppressor regulates the fission yeast cell cycle through the Wik1-Wis1-Spc1 kinase cascade. Mol Biol Cell 8, 409–419.
Shiozaki K, Shiozaki M, Russell P (1998). Heat stress activates fission yeast Spc1/StyI MAPK by a MEKK-independent mechanism. Mol Biol Cell 9, 1339–1349.
Takeda K, Shimozono R, Noguchi T, Umeda T, Morimoto Y, Naguro I, Tobiume K, Saitoh M, Matsuzawa A, Ichijo H (2007). Apoptosis signal-regulating kinase (ASK) 2 functions as a mitogen-activated protein kinase kinase kinase in a heteromeric complex with ASK1. J Biol Chem 282, 7522–7531.
Takekawa M, Saito H (1998). A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK. Cell 95, 521–530.
Tatebe H, Shimada K, Uzawa S, Morigasaki S, Shiozaki K (2005). Wsh3/Tea4 is a novel cell-end factor essential for bipolar distribution of Tea1 and protects cell polarity under environmental stress in *S. pombe*. Curr Biol 15, 1006–1015.
Tatebe H, Shiozaki K (2003). Identification of Cdc37 as a novel regulator of the stress-responsive mitogen-activated protein kinase. Mol Cell Biol 23, 5132–5142.
Wang LY, Shimada K, Morishita M, Shiozaki K (2005). Response of fission yeast to toxic cations involves cooperative action of the stress-activated protein kinase Spc1/Sty1 and the Hal4 protein kinase. Mol Cell Biol 25, 3945–3955.
Wang LY, Shiozaki K (2006). The fission yeast stress MAPK cascade regulates the pmp3$^+$ gene that encodes a highly conserved plasma membrane protein. FEBS Lett 580, 2409–2413.
Weber CK, Slupsky JR, Kalmes HA, Rapp UR (2001). Active Ras induces heterodimerization of cRaf and BRaf. Cancer Res 61, 3595–3598.
Wilkinson MG, Samuels M, Takeda T, Toone WM, Shieh JC, Toda T, Millar JB, Jones N (1996). The Atf1 transcription factor is a target for the Sty1 stress-activated MAP kinase pathway in fission yeast. Genes Dev 10, 2289–2301.