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.

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, 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, 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, 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, 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, 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, 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, 1997).
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 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 de-phosphorylate Spc1 (Figure 1A; Shiozaki et al., 1998; Nguyen and Shiozaki, 1999). The Mak2/Mak3–Mpr1–Mcs4 phosphorelay is homologous to the Sin1–Ypd1–Ssk1 system in budding yeast but responsible for transmitting signals of peroxide stress, rather than osmotic stress, 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 under other stress conditions, such as osmotic stress (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 osmotic stress signaling (Samejima et al., 1998). This model was based on the observations that osmotic-stress-induced activation of Spc1 is severely compromised in strains carrying a win1-null mutation, win1-1, and that the win1-1 defect in osmoreponse is not complemented by overexpression of Wis4. However, strains lacking Wis4 (also called Wik1 or Wak1) also fail to activate the Spc1 cascade upon osmotic stress (Shiozaki et al., 1997), implying a significant contribution of this second MAPKKK to osmotic signaling. Thus both Win1 and Wis4 MAPKKKs may be required for transmitting osmotic 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 osmotic stress 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 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 (Δwis4; 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 Δwis4 and win1-1 mutants are similarly defective in osmotic-stress-induced activation of the Spc1 MAPK (Figure 1B); only brief, weak activation of Spc1 was observed in either mutant, and the Δwis4 win1-1 double mutant showed very little Spc1 activation. Thus both Wis4 and Win1 MAPKKKs are required for robust osmotic stress 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...
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 Δwis4 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 Δwin1 and Δwis4 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 wise4-myc strain to create the wise4KM-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 wise4KM strain and was stronger and more prolonged than that in Δwis4 cells (Figure 3A, top). This strong Spc1 activation observed in the wise4KM 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 wise4KM strain, and only the wise4KM win1KM double mutant failed to induce activation of Spc1 after osmostress (Figure 3B). The KM mutations in Wis4 and Win1 did not affect the Wise4-Win1 interaction, and formation of Wise4-Win1KM (Figure 3C) and Wise4KM-Win1 (Figure 3D) heteromers was observed.

Together the Wise4 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 Wise4 and Win1 MAPKKKs plays an important role in activation of the Spc1 MAPK cascade. Therefore we further characterized the heteromer interaction between Wise4 and Win1 by testing truncated Wise4 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 Wise4 binds Win1 (Figure 4A). This Wise4(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 Wise4 N-terminal fragment (Figure 4B).

To test whether such interaction between Wise4 and Win1 is indeed important for stress signaling in vivo, we constructed chimera MAPKKKs that have the N-terminal Wise4(1–300) segment followed by the central region of Win1 (chimeras 4-1-1 and 4-1-4 in Figure 4C). Based on the Wise4–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 Wise4 and Win1 MAPKKKs. We found that both 4-1-1 and 4-1-4 chimeras induce strong activation of Spc1 upon osmostress in the Δwis4 Δwin1 strain, whereas expression of wild-type Win1 does not (Figure 4, D and E). No significant
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 CMI. Amino acid residues 120–320 of Wis4 were expressed as a fusion with glutathione S-transferase (GST) in the Δwis4 Δwin1 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 Δmcs4 background (lane 3). Because the Δmcs4 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 Δmcs4 mutation (lane 5). Thus, the contribution of Mcs4 to the Wis4–Win1 association is not solely
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 ∆mcs4 (Shieh et al., 1997), ∆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 osmo-stress 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 MAPKK and MAPKK dissociate immediately after exposure to osmolarity stress (lanes 5 and 6). However, isolation of Win1-HATAP from the ∆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 ∆mcs4 as well as in the ∆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 ∆wis4∆win1 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 MAPKKK from the MAPKKK complex appears to be triggered by a

**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 ∆wis4∆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 ∆wis4∆win1 strains carrying the empty vector plasmid were included as controls.
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.

**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 Δwin1 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.
manifested in osmostress signaling is independent of the phospho-relay, 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

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 of 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 Δmcs4 phenotype was not complemented by artificial ligand-induced dimerization (Ho et al., 1998) 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

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 ΔCMI mutant MAPKKKs, both in the mcs4+ (+) and Δmcs4 (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+ strains expressing HATAP-tagged Wis4 (B) or Win1 (C) was subjected to IgG–Sepharose affinity purification, and proteins bound to the beads and Mcs4+ in the lysate were detected by immunoblotting. Strains expressing no HATAP-tagged MAPKKK (wis4+ and win+) 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, Δmcs4, and Δwin1 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.
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; Animoto 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 MAPKKK 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 osmotic stress induces a shift in the electrophoretic mobility of the Wis1 MAPKK even in the Δmcs4, Δwin1, and wis4KM win1KM strains (lysate panels in Figure 7). Thus, in addition to activating phosphorylation by the MAPKKKs, Wis1 might receive some osmotic stress 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, H2O2 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-wis1(3196-4308):myc, and the primer sets wis4KM and win1KM, respectively (Supplemental Table S3), and the resulting plasmids were integrated into the wis4-1 and win1-1 loci. To remove the CMiN-coding sequence from the wis4-4 locus, the CMiN sequence was first replaced with the ura4-1 gene by homologous recombination using a PCR fragment amplified by the primer set wis4ΔCMiN::ura4-1. The resultant, S. pombe strain, wis4ΔCMiN::ura4-1, was transformed with another PCR fragment consisting of nucleotides +340 to +708 (340–708) directly followed by nucleotides 784–1185 of the wis4-4 gene (PCR primer set, wis4ΔCMiN). Transformants that had lost the ura4-1 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::ura4-1 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 the pREP1-wis4:FLAG, two base substitutions, T1782C and C3690T, were introduced within the wis4-4 open reading frame (ORF) by site-directed mutagenesis in order to eliminate Ndel restriction sites. These substitutions are silent and do not alter the encoded amino acid sequence. The resultant wis4-4 gene fragment was PCR amplified using the primer set wis4 full length, which adds Ndel and NotI restriction sites to the 5' and 3' termini of the ORF, respectively. Subsequently, the Ndel–NotI fragment of wis4-4 was cloned into the pREP1 vector (Maundrell, 1990) carrying three copies of the FLAG epitope sequence immediately after the NotI cloning site.

The full-length, wild-type win1-1 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 win1-1 ORF by site-directed mutagenesis without altering the encoded amino acids: T1278G, A1281G (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–phospho-Spc1 polyclonal antibodies (Tatebe and Shiozaki, 2003). For the construction of pREP1-wis4:FLAG, two base substitutions, T1782C and C3690T, were introduced within the wis4-4 reading frame (ORF) by site-directed mutagenesis in order to eliminate the encoded amino acid sequence. The resultant wis4-4 gene was PCR amplified using the primer set wis4 full length, which adds Ndel and NotI restriction sites to the 5' and 3' termini of the ORF, respectively. Subsequently, the Ndel–NotI fragment of wis4-4 was cloned into the pREP1 vector (Maundrell, 1990) carrying three copies of the FLAG epitope sequence immediately after the NotI cloning site.

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 Ndel–NotI 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 wis4-4 and win1-1 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 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). For the construction of pREP1-wis4:FLAG, two base substitutions, T1782C and C3690T, were introduced within the wis4-4 open reading frame (ORF) by site-directed mutagenesis in order to eliminate Ndel restriction sites. These substitutions are silent and do not alter the encoded amino acid sequence. The resultant wis4-4 gene fragment was PCR amplified using the primer set wis4 full length, which adds Ndel and NotI restriction sites to the 5’ and 3’ termini of the ORF, respectively. Subsequently, the Ndel–NotI fragment of wis4-4 was cloned into the pREP1 vector (Maundrell, 1990) carrying three copies of the FLAG epitope sequence immediately after the NotI cloning site.

The full-length, wild-type win1-1 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 win1-1 ORF by site-directed mutagenesis without altering the encoded amino acids: T1278G, A1281G (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 (PE10 sc-40; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-GST (goat serum; GE Healthcare).

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