In fission yeast, the Sty1/Spcl/Phh1 mitogen-activated protein kinase (MAPK) pathway is known to be involved in multiple-stress responses. It is currently thought that the Sty1 MAPK cascade is mediated by histidine kinases and phosphorylray proteins in response to oxidative stress signals. However, studies of the exact transduction mechanism of multiple-stress responses are lacking. Thus, in response to various stimuli, we monitored the Sty1 MAPK pathway through the downstream transcription factor Atlf1 in living cells using a highly sensitive luciferase reporter gene. Surprisingly, in cadmium and low glucose (LG) medium, Atlf1 activation was observed even in the absence of all of the four fission yeast MAPK kinase kinases (MAPKKKs); whereas in osmotic stress, Atlf1 activation was abolished. Thus, the osmotic stress likely mediates the MAPK activation via MAPKKKs, whereas a cadmium or LG condition activates the MAPK in a MAPKK-independent manner. On the other hand, knockout of tyrosine phosphatase gene pypl abolishes the Atlf1 response to cadmium and LG, but not to osmotic stress, suggesting that Pyp1 is a sensor for cadmium and LG.

The MAPK signaling pathways are critical for the response of eukaryotic cells to adapt to external environment conditions (1). A MAPK module is a three-kinase cascade, consisting of three protein kinases: a MAPKKK2 that activates a MAPK enzyme. In the fission yeast Schizosaccharomyces pombe, the Sty1 MAPK pathway is known to be involved in multiple-stress responses such as high temperature, osmotic stress, oxidative stress, nutrient limitation, and heavy metal toxicity (2–5). In general, signals induced by these multiple stress agents are relayed from the membrane or the cytoplasm to the nucleus to regulate gene expression for stress adaptation.

To date, histidine-to-aspartate (His-to-Asp) phosphorylray signaling systems are thought to be involved in the signal transduction implicated in oxidative stress response: activated histidine kinases Mak1, Mak2, and Mak3 (6) mediate their effects through phosphorylray protein Mrp1/Spy1 and Mcs4 response regulator (6–8). Mcs4 then in turn regulates MAPK module by activating the MAPKKKs Wis4/Wak1/Wik1 and Win1 (3, 9), which are functionally redundant in activating MAPKKK Wis1. Wis1 is required for activation of the MAPK Sty1 following osmotic, heat, or oxidative stresses (10, 11). Besides, Pyp1 phosphotyrosine-specific phosphatase that dephosphorylates and inactivates the MAPK (2, 3, 12, 13) was also reported to regulate Sty1 activation upon heat shock (14). Activated Sty1 accumulates in the nucleus (15, 16), where it activates transcription factors, such as bZIP transcription factor Atf1, and regulates a large set of genes that are relevant to the stress adaptation (see Fig. 4A).

In an attempt to identify the mechanisms of signal transduction and the sensing systems of each distinct stress factor in fission yeast, we monitored the transcriptional activity of Atf1 in living cells using a destabilized luciferase reporter gene fused to three tandem repeats of cAMP response elements (CRE)-like sequence under the conditions of osmotic stress KCl, heavy metal CdCl2, and low-glucose (LG) medium. We have investigated the knock-out strains known to be involved in the Sty1 MAPK pathway to see if there are defects in signal transduction accompanying the gene knockout. Here, we show evidence for the presence of MAPKKK-dependent and -independent activation of the MAPK. We also show that Pyp1 plays a role for sensing heavy metal CdCl2 and LG.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Genetic and Molecular Biology Methods**—S. pombe strains used in this study are listed in supplementary Table S1. The media, notation, and genetic methods have been described previously (17, 18).

**Construction of Reporter Plasmid**—A multicopy plasmid (pKB5760) containing the nmt1 promoter without its cis element, three tandem repeats of CRE-like sequence (TGACG-TAG or CTAGCGTCA) which is the binding core of the Atf1-Pcr1 heterodimeric transcriptional activator identified in the fbp1 promoter (19), and the destabilized luciferase from pGL3(R2.2) version containing PEST, CL1, and AU-rich repeats was constructed as described previously (20) except that the CCRE oligonucleotides were replaced by the CRE-like oligonucleotides (sense, 5’-GGC TTT GAC GTA GAT ACA TGA CGT AGA TAC ACA TGA CGT AGA TGC AC-3’; antisense, 5’-TCG AGT GCA TCT ACG TCA TGT GTA TCT

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1 The on-line version of this article (available at http://www.jbc.org) contains supplementary Fig. 1, Tables S1 and S2, and additional references.

2 The abbreviations used are: MAPKKK, MAPK kinase kinase; CRE, cAMP response element; EMM, Edinburgh minimal medium; LG, low glucose.
ACG TCA TGT ATC TAC GTC AAA GCC TGC A-3', CRE-like sequence underlined). Similarly, an integration vector used to stably integrate 3×CRE::luc(R2.2) constructs into the arg1+ locus was constructed by inserting the CRE-like oligonucleotides and arg1+ into pBC SK(+)(Stratagene) to give pKB5810 (21). These two reporter vectors were used for live-cell monitoring of Atf1-mediated transcriptional activity in living cells.

Real-time Monitoring Assay of Atf1-mediated Transcriptional Activity—The above constructed multicopy reporter plasmid was transformed into fission yeast cells for reporter assays, and an integration reporter plasmid was used to construct stable integration strains as described previously (20). For the experiments with the stress agents, cells as indicated were either untreated (water as control) or treated with KCl, CdCl2, and LG, respectively. For the experiments with LG, cells were cultured at 27 °C in EMM containing 2% glucose overnight to mid-log phase and then recovered by filtration and resuspended in EMM containing 0.1% glucose. The assay is based on the interaction of luciferase with substrate luciferin, and yielding luminescence was detected using a luminometer (AB-2300; ATTO Co., Tokyo, Japan) at 1-min intervals and reported as relative light units.

Immunoblot Analyses—An integration vector containing Sty1–2×HA-His6, construct (5) was generously provided by J. Kanoh and was used for transformation of various strains. Immunoblot analyses were performed as described previously (22) using anti-phosphorylated p38 (Thr180/Tyr182) MAPK antibody (Cell Signaling) and anti-HA antibody (Roche). Antibody to fission yeast Cdc4 protein was prepared by immunizing the rabbit with purified Cdc4 protein and was used for the detection of endogenous Cdc4 as a loading control.

Construction of Inducible Expression Plasmid of Sty1 MAPK—For inducible ectopic expression of proteins, we used the thiamine-repressible nmt1 promoter (23). The open reading frame (ORF) of sty1+ was amplified by PCR primers (sense, 5'-CGG GAT CCC ATG GCA GAA TTT ATT CGT AC-3'; antisense, 5'-CGG AAT TCG CGG CCG GAT TGC AGT CTA TTA GCC-3') and subcloned into the BglII site of the ORF of the BY4743 genomic DNA. The ORF was cloned into the BamHI site of pGEM-7Zf(+) (Promega) to give pKB7953. Gene disruption constructs were prepared by inserting lys3+ flanked by EcoRV site into the HincII/EcoRV site of the ORF of the byr2+ gene that was subcloned into pKB7955 and transformed into lys3+-deleted cells to disrupt the byr2+ gene by homologous recombination. The mkh1+ gene was similarly disrupted (24): a 3323-bp fragment (9–3331 base) was amplified by PCR using the following primers: 5'-AAA ACT GCA GCG ATA TCG ACG TCA TGT ATC TAC GTC AAA GCC TGC A-3', CRE-like sequence underlined). Similarly, an integration vector used to stably integrate 3×CRE::luc(R2.2) constructs into the arg1+ locus was constructed by inserting the CRE-like oligonucleotides and arg1+ into pBC SK(+)(Stratagene) to give pKB5810 (21). These two reporter vectors were used for live-cell monitoring of Atf1-mediated transcriptional activity in living cells.

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**Figure 1.** Real-time monitoring of Atf1 activity in living cells. A, live-cell monitoring of Atf1 activity in wild-type cells. Wild-type cells transformed with the multicopy plasmid (3×CRE::luc(R2.2) reporter plasmid) were incubated with β-luciferin and treated with KCl (0.075–0.3 M), CdCl2 (0.5–2 mM), or EMM with LG (0.1% glucose). Relative light units are expressed as the ratio of light emission of each sample to the basal (without stimulation) light emission of wild-type cells in EMM at 180 min. The data shown are representative of multiple experiments. B, reporter assay reflecting Sty1 activity as well as Atf1 activity in living cells. The cells as indicated were transformed with the reporter plasmid, cultured in EMM at 27 °C, and treated with KCl (0.3 M), CdCl2 (2 mM), or LG (0.1% glucose), as described under “Experimental Procedures.” C, detection of phosphorylated Sty1 by immunoblotting. Cells were grown in YPD medium at 27 °C and exposed to LG medium, 0.3 M KCl, or 2 mM CdCl2 for 30 min. Phosphorylated Sty1 protein (Sty1-P) was analyzed by immunoblotting with anti-phosphorylated p38 (Thr180/Tyr182) MAPK antibody and anti-HA antibody as described under “Experimental Procedures.” D, real-time monitoring of integration reporter activities in wild-type and Δsty1 cells. Δsty1 cells harboring the stable integration reporter vector transformed with an inducible-expression plasmid of Sty1 in the presence or absence of thiamine (see “Experimental Procedures”) were treated as indicated. Error bars, mean ± S.D.
Construction of Δpp1 Cells Containing Integrated pREP1-pyp1C470S-GFP—A mutant of wild-type pyp1C470S containing Ser instead of Cys at amino acid 470 (pyp1C470S) was generated using a QuikChange® Site-directed Mutagenesis kit (Stratagene). The mutation was confirmed by DNA sequencing, and the fragment was subcloned into the C-terminal GFP fusion pREP1 vector. The pREP1-pyp1C470S-GFP was linearized and transformed into Δpp1 cells. Integration of pREP1-pyp1C470S-GFP was confirmed by Southern hybridization.

RESULTS AND DISCUSSION

Wild-type cells, Δatf1 cells, and Δsty1 cells were subjected to osmotic stress (0.075—0.3 M KCl), heavy metal stress (0.5—2 mM CdCl2), and LG (0.1% glucose), respectively. In wild-type cells, elevated extracellular KCl caused a continuous rise in a dose-dependent manner in the Atf1 transcriptional activity (Fig. 1A, left panel). CdCl2 also caused a dose-dependent increase (Fig. 1A, center panel); however, it should be noted that there was a delayed onset of increasing response compared with that by KCl. LG showed a steady increase in response, with a peak rise at about 120 min (Fig. 1A, right panel). In Δatf1 cells, there was no response upon treatment with KCl, CdCl2, or LG (Fig. 1B), indicating that the 3×CRE::luc(R2.2) reporter assay reflects the Atf1 activity. In Δsty1 cells, the 3×CRE::luc(R2.2) reporter activity was hardly detected (Fig. 1B), indicating that the reporter assay also reflects the Sty1 activity.

As a further test, we examined the level of dual phosphorylation on threonine and tyrosine of Sty1 by immunoblotting under the treatment with KCl, CdCl2, or LG (Fig. 1C). The immunoblot detected a substantial increase in the phosphorylation level caused by each stress factor, which is consistent with the results of 3×CRE::luc(R2.2) reporter assay.

Also, the integrated 3×CRE::luc(R2.2) reporter activities were examined in Δsty1 cells that were transformed with an inducible Sty1 on an expression plasmid. When the Sty1 expression was repressed by thiamine, there was an extremely low response upon stimulation, and in the absence of the thiamine the reporter activities returned to almost the same level as that in wild-type cells (Fig. 1D and supplemental Table S2). These results again indicate that the luciferase reporter reflects Sty1 activity in living cells.

The integrated 3×CRE::luc(R2.2) reporter showed much lower sensitivity than the multicopy reporter, and it failed to detect the activity in some knock-out cells. Therefore, we used the multicopy reporter for most of knock-out cells showing low Atf1 activity. Although the copy number of plasmids in fission yeast is variable, the S.D. obtained with the multicopy reporter suggests that the experiments are reliable.

To investigate the role of Mcs4 in multiple-stress-induced responses, we monitored the reporter activities in Δmcs4 cells.

| Cell types | Basal | KCl (0.3 M) | CdCl2 (2 mM) | LG (2% → 0.1%) |
|------------|-------|------------|-------------|----------------|
| Wild type  | 1.0 ± 0.096 | 3.5 ± 0.31 | 2.1 ± 0.20 | 3.3 ± 0.48 |
| Δmcs4      | 0.048 ± 0.016 | 0.095 ± 0.035 | 0.34 ± 0.13 | 0.82 ± 0.49 |
| Δwis      | 0.078 ± 0.014 | 0.19 ± 0.041 | 0.55 ± 0.068 | 0.66 ± 0.17 |
| ΔwisΔwin1  | 0.15 ± 0.013 | 0.26 ± 0.028 | 1.0 ± 0.17 | 0.80 ± 0.18 |
| ΔwisΔwin1Δhis2Δmkhl | 0.017 ± 0.0016 | 0.017 ± 0.0033 | 0.15 ± 0.032 | 0.10 ± 0.010 |
| Δpp1ΔC470S | 3.18 ± 0.11 | 6.73 ± 0.64 | 3.22 ± 0.37 | 2.91 ± 0.59 |
| pyp1C470S | 3.06 ± 0.15 | 5.62 ± 0.38 | 2.81 ± 0.56 | 2.85 ± 0.14 |
CdCl2 was not detected in Δwis4Δwin1 double knock-out cells by immunoblotting (Fig. 2D), further indicating that the 3×CRE::lacZ reporter assay has a much higher sensitivity than the immunoblot detection. Our findings of the reporter assay suggest that KCl-stimulated Sty1 activation is dependent on MAPKKKs Wis4 and Win1, whereas both CdCl2- and LG-stimulated activations are independent. Possibly, two other known fission yeast MAPKKKs, Byr2 and Mkh1, compensate for the loss of Wis4 and Win1.

Byr2 is required for mating and sporulation (27), and Mkh1 regulates morphogenesis and cell wall integrity (28). We constructed the strains lacking all of the four MAPKKKs and tested them using this reporter assay. In Δwis4Δwin1Δbyr2Δmkh1 tetra knock-out cells, similar reporter activities were observed compared with those in Δwis4Δwin1 double knock-out cells upon the treatment with CdCl2 or LG (Fig. 2E and Table 1). This further suggests that both CdCl2- and LG-stimulated MAP kinase activations are MAPKKK-independent.

These results led us to look into the negative regulators of the Sty1 MAPK cascade. The protein-tyrosine phosphatases Pyp1 and Pyp2 are found to function by inactivating Sty1 (12). We then monitored the reporter activities in Δpyp1 and Δpyp2 cells. Higher basal reporter activity was observed in Δpyp1 cells than in wild-type or Δpyp2 cells (Fig. 3, A–C). This is consistent with the notion that Pyp1 and Pyp2 dephosphorylate and inhibit the Sty1 MAPK (12) and that Pyp1 is a major tyrosine phosphatase for Sty1 dephosphorylation (3). In Δpyp1 cells, notably, the promoter activity stimulated by the treatment with CdCl2 or LG was not or was barely observed, whereas the stimulation by KCl was clearly observed (Fig. 3B). In Δpyp2 cells, the reporter activity stimulated by the three stress agents, respectively, was similar to that in wild-type cells (Fig. 3C). These results suggest that Pyp1, but not Pyp2, is responsible for sensing and transmitting the signals to Sty1 MAPK upon treatment with CdCl2 or LG.

Notably, Δpyp1 cells showed a higher basal reporter activity than wild-type cells (Fig. 3, A and B), suggesting that even in the absence of extracellular stresses, Sty1 is still phosphorylated by MAPKKK Wis1. Consistently, Δwis1Δpyp1 double knock-out cells showed extremely low basal and response reporter activities, that were similar to those of Δwis1 single knock-out cells (data not shown), indicating that high basal reporter activity in Δpyp1 cells is dependent on the activity of Wis1 and on its upstream MAPKKKs. Consistent with this notion, a previous article by Samejima et al. showed that Wis1AA (unphosphorylated form) still phosphorylates Sty1 (3). This seems to be the reason why Wis1 is absolutely required for Sty1 activation by CdCl2 and LG. Also, morphological comparison between Δwis1 cells and wild-type cells overexpressing pyp1+ has been performed. Similar to Δwis1 cells, overexpression of pyp1+ in a way was analyzed by immunoblotting with anti-phosphorylated p38 (Thr180/Tyr182) MAPK antibody (Sty1-P) and anti-HA antibody (Sty1-HA) as described under "Experimental Procedures." E, CdCl2- and LG-stimulated activations are independent of MAPKKKs. Δwis4Δwin1 cells or Δwis4Δwin1Δbyr2Δmkh1 cells transformed with the reporter plasmid were untreated (basal) or treated with CdCl2 (2 mM) or LG. Data were analyzed and plotted as in Fig. 1D.

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**FIGURE 2.** KCl-stimulated Sty1 activation is dependent on MAPKKKs, whereas both the CdCl2- and LG-stimulated activations are independent of MAPKKKs. A, both CdCl2- and LG-stimulated activations of Sty1 are dependent on MAPKK Wis1 but not MAPKKKs Wis4 and Win1. Wild-type, Δwis4, Δwin1, Δwis4Δwin1, and Δwis1 cells, respectively, transformed with the reporter plasmid were treated as indicated. Data were analyzed and plotted as in Fig. 1D, and the inset is a magnification of the results obtained in Δwis4Δwin1 cells and Δwis1 cells. B, MAPKKKs Wis4 and Win1 are required for KCl-stimulated Sty1 activation but not for CdCl2- or LG-stimulated activations. The Δwis4Δwin1 cells transformed with the reporter plasmid were treated as indicated. C, MAPKKK Wis1 is absolutely required for the activation of Sty1. The Δwis1 cells transformed with the reporter plasmid were treated with the three agents, respectively, as described in Fig. 1A. D, detection of phosphorylated Sty1 by immunoblotting under the treatment with CdCl2, is shown. Cells were grown in YPD medium at 27°C. CdCl2 was added to a final concentration of 2 mM at time 0, and aliquots of cells were harvested every 60 min. Sty1 protein...
wild-type background led to significant cell elongation (supplementary Fig. 1), indicating that Pyp1 is actually opposing the Wis1 activity.

Recent studies in mammalian cells have reported that CdCl₂ induces the formation of reactive oxygen species (29–31) and that LG causes a metabolic oxidative stress (32, 33). The highly conserved Cys residue located within the core catalytic domains of all known protein-tyrosine phosphatases is essential for the catalytic activity in vitro (34–36), and it has been reported that the conserved Cys residue in PTPα is sensitive to reactive oxygen species and that it may function as a redox sensor (37). In fission yeast, to determine whether this Cys residue (Cys470) is also crucial for the ability of Pyp1 to sense the signals induced by the treatment with CdCl₂ and LG, respectively, the pREP1-pyp1C470S-GFP was integrated into the chromosome of Δpyp1 cells. The mutation of this Cys residue showed similar reporter activity compared with that in Δpyp1 cells upon the treatment with the three stress agents, respectively (Fig. 3D and Table 1). This suggests that alteration of the Cys residue in the catalytic site abolishes the ability to sense and to transmit the signals to Sty1 MAPK upon the treatment with CdCl₂ and LG, respectively, indicating that Cys470 may function as a sensor for these two stress agents.

In summary, in contrast with the hypothesis that all of the stresses are mediated by the two MAPKKKs (38) (Fig. 4A), here we show evidence in favor of the conclusion shown in Fig. 4B: KCl stimulates the Sty1 MAPK pathway through MAPKKKs, whereas CdCl₂ and LG stimulate the pathway through the inhibition of Pyp1 independently of MAPKKKs. This is in contrast with a previous hypothesis that insights on the mechanisms of stress response and signal transduction in fission yeast may help in studying similar mechanisms in higher eukaryotes.

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