Catalytic Activation of the Plant MAPK Phosphatase NtMKP1 by Its Physiological Substrate Salicylic Acid-induced Protein Kinase but Not by Calmodulins*

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MAPK phosphatases (MKPs) are negative regulators of MAPKs. Previously, we identified NtMKP1 as a novel calmodulin (CaM)-binding protein (Yamakawa, H., Katou, S., Seo, S., Mitsuhara, I., Kamada, H., and Ohashi, Y. (2004) J. Biol. Chem. 279, 928–936). In this study, we characterized the interaction of NtMKP1 with substrate MAPKs and CaM. NtMKP1 (produced by in vitro transcription/translation) interacted with salicylic acid-induced protein kinase (SIPK) through dephosphorylation of the TEY motif of SIPK. CaM bound but unexpectedly did not activate the phosphatase activity of NtMKP1. NtMKP1 has four characteristic domains, viz. a dual-specificity phosphatase catalytic domain, a gelsolin homology domain, a CaM-binding domain, and C-terminal domain. Deletion analysis revealed that the N-terminal non-catalytic region of NtMKP1 bound SIPK and was essential for inactivating SIPK, whereas the CaM-binding and C-terminal domains were dispensable. Moreover, the phosphatase activity of NtMKP1 was increased strongly by the binding of SIPK, but weakly by another MAPK, wound-induced protein kinase. Swapping and site-directed mutagenesis of SIPK and wound-induced protein kinase revealed that the strong activation of NtMKP1 phosphatase activity by SIPK partially depended on the putative common docking domain of SIPK. On the other hand, conversion of Lys41 and Arg44 of NtMKP1 to Ala (K41A/R43A) abolished the interaction with SIPK. Expression of constitutively active MAPK kinase in Nicotiana benthamiana induced activation of SIPK and cell death. Simultaneous expression of either NtMKP1 or NtMKP1 L443R, which was unable to bind CaM, compromised the constitutively active MAPK kinase-induced responses, whereas that of NtMKP1 K41A/R43A did not. These results indicate that the regulation of NtMKP1 activity by SIPK binding, but not by CaM binding, is important for the function of NtMKP1.

The MAPKs are key molecules of signal transduction responding to various extracellular stimuli in eukaryotes. The activity of MAPKs is strictly regulated via phosphorylation of the conserved TXY motif by an upstream MAPK kinase (MAPKK) (1, 2). Conversely, MAPKs are dephosphorylated and inactivated by protein phosphatases, including tyrosine-specific phosphatases; serine/threonine-specific phosphatases; and dual-specificity MAPK phosphatases (MKPs), which are highly specific to MAPKs (3–5). In mammals, the 14 members of the MAPK family represent three major subfamilies, viz. ERK, JNK, and p38 (1, 2). On the other hand, the mammalian MKP family has 10 members, which share sequence homology, but differ in substrate specificity and subcellular localization (3–5). Mammalian MKPs interact with their substrate MAPKs via a substrate-binding domain at their N termini, and such an interaction increases the catalytic activity of some MKPs. This “substrate-triggered activation” of MKP phosphatase activity has been shown to play an important role in determining substrate specificity at the subfamily level (6–9).

Plant MAPKs also constitute a large family. However, no obvious INK or p38 type of MAPK has been identified in plants. The Arabidopsis genome contains 20 MAPKs, 12 of which are the ERK type, and the others are the plant-specific type (10). In contrast to many members of the MAPK family, plant MAPKs likely form a small gene family. Only five MKPs are predicted in the Arabidopsis genome, including the previously reported AtMKP1, IBR5, PHS1, and DsPTP1 (11). This disproportionate ratio of MAPK to MKP may suggest that one MKP regulates multiple MAPKs in plants and that other types of protein phosphatases such as protein phosphatase 2C (12) and tyrosine-specific phosphatase (13) also play an important role in the regulation of plant MAPKs. Mutations of the AtMKP1, IBRS, and PHS1 genes causes sensitivity to genotoxic stress, reduced responses to the phytohormones auxin and abscisic acid, and compromised cortical microtubule functions, respectively (14–16). This genetic evidence clearly reveals the important biological role of plant MKPs. However, the biochemical characteristics of plant MKPs are not well understood. Although yeast two-hybrid analysis showed that AtMKP1 specifically interacts with AtMPK3, AtMPK4, and AtMPK6 among AtMPK1–9 (17), the phosphatase activity of AtMPK1 and IBR5 has not been proven. PHS1 has general phosphatase activity as shown by the hydrolysis of an artificial substrate, but its target MAPK is unclear (16). DsPTP1 dephosphorylates and inactivates AtMPK4 in vitro; however, it has been suggested that DsPTP1 is unlikely to inactivate AtMPK4 in vivo (13, 18).

WIPK, wound-induced protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; IPTG, isopropyl-β-D-thiogalactopyranoside; MBP, myelin basic protein; GST, glutathione S-transferase; OMPF, 3-O-methylfluoresceinophosphate; MES, 4-morpholineethanesulfonic acid; WT, wild-type; CD, common docking.

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3 The abbreviations used are: MAPK, mitogen-activated protein kinase; MKP, mitogen-activated protein kinase phosphatase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; CaM, calmodulin; GH, gelsolin homology; SIPK, salicylic acid-induced protein kinase; SIPK, salicylic acid-induced protein kinase/extra-cellular signal-regulated kinase; IPTG, isopropyl-β-D-thiogalactopyranoside; MBP, myelin basic protein; GST, glutathione S-transferase; OMPF, 3-O-methylfluoresceinophosphate; MES, 4-morpholineethanesulfonic acid; WT, wild-type; CD, common docking.

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Previously, we identified a putative tobacco ortholog of AtMKP1 (NtMKP1) as a calmodulin (CaM)-binding protein (19). It has also been reported that CaM binds two different sites of DsPTP1 (20). To date, no MKP from organisms other than plants has been shown to bind CaM. Therefore, interaction with CaM seems to be a unique feature of plant MKPs. Compared with mammalian MKPs, NtMKP1 and AtMKP1 are large proteins carrying several characteristic domains, viz. a dual-specificity phosphatase domain, a gelsolin homology (GH) domain, a CaM-binding domain, and a long C-terminal domain containing a serine-rich region (see Fig. 4A). In tobacco, two MAPKs, salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK), are activated by a variety of extracellular stimuli, including wounding (21, 22). We showed previously that overexpression of NtMKP1 compromises wound-induced activation of SIPK and WIPK, suggesting a role for NtMKP1 in regulating these MAPKs (17). These results indicate that SIPK is a physiological substrate of NtMKP1.

Previously, we failed to detect the phosphatase activity of NtMKP1 (19). To demonstrate that NtMKP1 is an active MAPK phosphatase, we produced the NtMKP1 protein by in vitro transcription/translation. We also prepared phosphorylated SIPK as a substrate of NtMKP1. Here, we show that NtMKP1 is an active phosphatase that dephosphorylates the conserved TEY motif of SIPK in vitro. The N-terminal non-catalytic region of NtMKP1 interacted with SIPK and was essential for inactivating SIPK. Moreover, the phosphatase activity of NtMKP1 was significantly increased by the binding of SIPK. We show that the catalytic activation of NtMKP1 by SIPK, but not by CaM, is important for the function of NtMKP1 both in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Plant Materials—Nicotiana benthamiana was grown in a temperature-controlled growth room maintained at 25 °C with a 16-h light/8-h dark cycle. Four- to five-week-old plants were used for experiments.

Production of Recombinant Proteins of MAPKs and Constitutively Active MAPKKs—The open reading frames of SIPK and WIPK were amplified by PCR with MscI and XhoI sites attached to the 5'- and 3'-ends, respectively, and cloned into the corresponding sites of the pET32a vector (Novagen), allowing the production of each MAPK with thioredoxin tag at the N terminus and His6 tag at the C terminus. The pET32a vector (Novagen), allowing the production of recombinant SIPK and WIPK (0.4 μg each) were incubated at 25 °C for 15 min in buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM MgCl2, 0.25 mg/ml MBP, 50 μM ATP, and 50 μCi/ml [γ-32P]ATP. The reactions were terminated by the addition of SDS sample buffer. Phosphorylated SIPK, WIPK, and MBP were visualized by autoradiography after separation on a 15% SDS-polyacrylamide gel.

Phosphorylation of SIPK by MEK232—Purified SIPK (50 μg/ml) and MEK232 (10 μg/ml) were incubated at 30 °C for 30 min in buffer containing 20 mM HEPES-KOH (pH 7.5), 10 mM MgCl2, 1 mM dithiothreitol, and 500 μM ATP. Two-hundred microliters of 50% S protein-agarose (Novagen) was added to the reaction mixture to remove MEK232. The mixture was rotated at room temperature for 30 min, and the agarose beads were removed by brief centrifugation. The resulting supernatant containing phosphorylated SIPK was concentrated using a Microcon YM-30 concentrator, mixed with an equal volume of glycerol, and stored at −80 °C.

Inactivation of SIPK by NtMKP1 Produced by in Vitro Transcription/Translation—The open reading frame of NtMKP1 was fused at its N terminus to a FLAG epitope (FLAG-MKP1) and cloned into the in vitro translation/translation vector pTNT (Promega). In vitro transcription/translation was performed using a TnT wheat germ extract kit (Promega) at 30 °C for 2 h according to the manufacturer’s recommendations. In vitro translated products were diluted with buffer A (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.5% Triton X-100) containing 1 mM EGTA and 1× Complete (Roche Applied Science); 2 μl of anti-FLAG antibody M2 (Sigma) was added; and the mixture was incubated at 4 °C for 1 h. Then, 20 μl of protein A-Sepharose was added, and the reaction mixture was rotated at 4 °C for 1 h. Note that anti-FLAG antibody M2 is isotype 1 of mouse immunoglobulin G and binds protein G stronger than protein A. However, protein G-Sepharose had greater nonspecific binding in our assay system. Therefore, we used protein A-Sepharose to precipitate anti-FLAG antibody M2. The beads were collected by brief centrifugation and washed three times with 1 ml of buffer A containing 1 mM EGTA and twice with buffer B (20 mM Tris-HCl (pH 7.5) and 1 mM dithiothreitol). Then, the beads were suspended in buffer B containing 5 μl/ml phosphorylated SIPK and incubated at ambient temperature with continuous agitation. At specific times, reaction mixtures were flash-centrifuged, and supernatants containing SIPK were removed. The kinase activities of SIPK were assayed at ambient temperature for 10 min with 0.25 mg/ml MBP, 50 μM ATP, and 5 μCi/ml [γ-32P]ATP. The reactions were terminated by the addition of SDS sample buffer. The phosphorylation of MBP was analyzed by autoradiography after separation on a 15% SDS-polyacrylamide gel. To investigate the effect of CaM, 1 μM NtCaM1, NtCaM3, or NtCaM13 was added to the reaction mixture in the presence (100 μM CaCl2) or absence (5 mM EGTA) of Ca2+.

Interaction of FLAG-MKP1 with NtCaM1—FLAG-MKP1 was produced by in vitro transcription/translation and purified. FLAG-MKP1 bound to Sepharose was suspended in 0.5 μM NtCaM1 in buffer A containing 100 μM CaCl2 or 1 mM EGTA and rotated at 4 °C for 1 h. The beads were collected by brief centrifugation and washed three times with 1 ml of buffer A containing 100 μM CaCl2 or 1 mM EGTA. The beads were suspended in SDS sample buffer and used for immunoblot analyses with anti-NtCaM1 or anti-NtMKP1 antibody, respectively. The CaM overlay assay was performed as described previously (19), but 1 μg/ml alkaline phosphatase-labeled NtCaM1 was used.

Production of Recombinant NtMKP1 Proteins in E. coli—The cDNA fragments corresponding to residues 1–114 and 115–361 of NtMKP1 were amplified by PCR with BamH I and XhoI sites attached to the 5'- and 3'-ends, respectively, and cloned into the corresponding sites of pGEX-4T-1, resulting in glutathione S-transferase (GST)-MKP1-(1–361).
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NtMKP1 Inactivates SIPK via Dephosphorylation of the Conserved TEY Motif in Vitro—To determine the phosphatase activity of NtMKP1, we prepared phosphorylated SIPK as a substrate of NtMKP1. Recombinant SIPK and MEK<sup>DD</sup>, a constitutively active form of the mammalian ERK MAPK, were expressed in E. coli and purified with nickel-affinity chromatography and SDS-PAGE analysis followed by Coomassie Brilliant Blue staining of purified proteins. The production of MEK<sup>DD</sup> was confirmed by immunoblot analysis using anti-phospho-p44/42 MAPK antibody, which specifically recognizes the phosphorylated form of the conserved TEY motif of mammalian ERK MAPK. Although this antibody has been previously used to monitor the phosphorylation of SIPK (30–32), we first confirmed by sequencing.

RESULTS

NtMKP1 Inactivates SIPK via Dephosphorylation of the Conserved TEY Motif in Vitro—To determine the phosphatase activity of NtMKP1, we prepared phosphorylated SIPK as a substrate of NtMKP1. Recombinant SIPK and MEK<sup>DD</sup>, a constitutively active form of the mammalian ERK MAPK, were expressed in E. coli and purified with nickel-affinity chromatography and SDS-PAGE analysis followed by Coomassie Brilliant Blue staining of purified proteins. To determine the phosphatase activity of NtMKP1, a 30 °C in 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1 mM EDTA, and 500 μM OMFP. The amount of 3-O-methylfluorescein was determined from the absorbance at 477 nm. The non-enzymatic hydrolysis of OMFP was corrected by measuring the control or FLAG-MKP1 derivatives were mixed with 0.0526 volume of Agrobacterium cell extract. The production of FLAG-MKP1 was confirmed by immunoblot analysis using anti-FLAG antibody. The activity of SIPK was measured 2 days after infiltration by immunocomplex kinase assay as described previously (21).

Precipitation of SIPK by GST-fused NtMKP1 Derivatives—Total protein was extracted from N. benthamiana leaves infiltrated with Agrobacterium cells carrying SIPK 2 days after infiltration as described previously (21). Total protein was mixed with GST alone or GST-MKP1 derivatives bound to glutathione-Sepharose and rotated overnight at 4 °C. The beads were washed three times with 1 ml of buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, and 0.5% Triton X-100. Precipitated proteins were denatured with SDS sample buffer and used for immunoblotting.

Construction of (S/W)IPK, (W/S)IPK, SIPK<sup>N</sup>, and SIPK<sup>W</sup>IPK and (W/S)IPK were constructed as reported by Zhang and Liu (27). A PstI site introduced into the WIPK gene does not change the amino acid sequence. Various mutations were generated using the Mutan-Super Express Km kit and confirmed by sequencing.
motif of SIPK in vitro. The phosphorylation of the conserved TEY motif of SIPK decreased upon incubation with FLAG-MKP1, whereas the amount of SIPK protein was constant (Fig. 2B). Collectively, these results indicate that NtMKP1 inactivates SIPK via dephosphorylation of its TEY motif.

Calmodulin Binds but Does Not Activate FLAG-MKP1—In a previous study, we identified NtMKP1 as a CaM-binding protein (19). We first confirmed the interaction of FLAG-MKP1 with NtCaM1. NtCaM1 was mixed with FLAG-MKP1 bound to Sepharose beads in the presence of Ca2+/H11001. Precipitated NtCaM1 was detected by immunoblot analysis using antibody against NtCaM1. As shown in Fig. 3A, NtCaM1 was precipitated by FLAG-MKP1 in a Ca2+-dependent manner. Previously, we showed that Trp440 and Leu443, but not Gly450, are essential for the GST-fused CaM-binding domain of NtMKP1 to bind CaM (19). We investigated whether these amino acids are also critical for full-length NtMKP1 to bind CaM (19). We tested whether these amino acids are also critical for full-length NtMKP1 to bind CaM by analyzing the production of FLAG-MKP1 using FLAG-MKP1 W440R, L443R, and G450R recombinant proteins, in which Trp440, Leu443, and Gly450 were converted to Arg, respectively, were produced by in vitro transcription/translation and purified by immunoprecipitation. Ligand binding assay using alkaline phosphatase-labeled NtCaM1 showed that FLAG-tagged

FIGURE 1. NtMKP1 inactivates SIPK in vitro. A, recombinant proteins of SIPK and MEKDD were produced in E. coli and purified by nickel affinity chromatography. The purified proteins (0.5 μg) were separated on an SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue (CBB). The positions of molecular mass markers in kilodaltons are indicated on the left. B, FLAG-MKP1 was produced by in vitro transcription/translation using wheat germ extracts. The production of FLAG-MKP1 was confirmed by immunoblot analyses using anti-FLAG antibody (α-FLAG) or anti-NtMKP1 antibody (α-MKP1). C, FLAG-MKP1 was purified by immunoprecipitation with anti-FLAG antibody and protein A-Sepharose. The precipitated beads were suspended in buffer containing 5 μg/ml phosphorylated SIPK and incubated at ambient temperature with continuous agitation. At the indicated times, the beads were removed by flash centrifugation. The kinase activity of SIPK was assayed with MBP as a substrate, and SIPK protein was detected by immunoblot analysis with anti-SIPK antibody (α-SIPK). The production of FLAG-MKP1 protein was confirmed by immunoblot analysis with anti-NtMKP1 antibody.

FIGURE 2. NtMKP1 dephosphorylates the conserved TEY motif of SIPK in vitro. A, the specificity of anti-phospho-p44/42 MAPK antibody was confirmed by immunoblot analyses with non-phosphorylated and phosphorylated SIPK (p-SIPK). B, FLAG-MKP1 was purified by immunoprecipitation with anti-FLAG antibody and protein A-Sepharose. The precipitated beads were suspended in buffer containing 5 μg/ml phosphorylated SIPK and incubated at ambient temperature with continuous agitation. At the indicated times, the beads were removed by flash centrifugation. The amounts of phosphorylated SIPK and total SIPK protein were determined by immunoblot analyses with anti-phospho-p44/42 MAPK antibody (α-pTEpY) and anti-SIPK antibody (α-SIPK), respectively. The production of FLAG-MKP1 protein was confirmed by immunoblot analysis with anti-NtMKP1 antibody (α-MKP1).
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FIGURE 3. CaM binds but does not activate FLAG-MKP1. A, FLAG-MKP1-bound Sepharose beads were mixed with 1 μM NtCaM1 in the presence (100 μM CaCl2) or absence (1 mM EGTA) of Ca2+. After washing, precipitated proteins were subjected to immunoblot analysis with anti-NtCaM1 antibody (α-NtCaM1) or anti-FLAG-MKP1 antibody (α-MKP1). B, recombinant proteins of FLAG-tagged WT-MKP1 and mutants W440R, L443R, and G450R were produced by in vitro transcription/translation. They were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with alkaline phosphatase-labeled NtCaM1 (AP-NtCaM1). A parallel membrane blot was subjected to immunoblot analysis with anti-NtMKP1 antibody to verify that similar amounts of FLAG-MKP1 proteins were used.

As one well known and characterized effect of CaM binding to targeted proteins is activation of the enzymatic activity of the proteins (33, 34), we investigated the effect of NtCaM1 binding on the phosphatase activity of FLAG-MKP1. However, we found no significant activation of FLAG-MKP1 upon the addition of not only NtCaM1, but also NtCaM3 and NtCaM13 (up to 1 μM),4 which are different types of tobacco CaMs from NtCaM1 (35). As most target proteins are significantly activated by CaM at nanomolar levels (33, 35), and 1 μM NtCaM1 was sufficient to detect interaction between NtCaM1 and NtMKP1 (Fig. 3A). CaM binding might have function other than enzymatic activation of NtMKP1.

The N-terminal 361 Amino Acids of NtMKP1 Are Sufficient to Inactivate SIPK—NtMKP1 has four characteristic domains, viz. a dual-specificity phosphatase domain and a GH domain at its N terminus, a CaM-binding domain at its center, and a C-terminal domain containing a serine-rich region (Fig. 4A). To investigate the role of the CaM-binding and C-terminal domains, the N-terminal 361 amino acids of NtMKP1, including the dual-specificity phosphatase and GH domains, were expressed in E. coli as a GST fusion protein (GST-MKP1-(1–361)) and assayed for SIPK-inactivating activity. As shown in Fig. 4B, GST-MKP1-(1–361) effectively inactivated SIPK, suggesting that the CaM-binding and C-terminal domains of NtMKP1 are dispensable for its activity. Further deletion of either the N-terminal non-catalytic region (residues 1–114) or the GH domain (residues 277–361) of GST-MKP1-(1–361) greatly reduced its activity (Fig. 4C). Similarly, replacement of Cys201 in the putative catalytic center with Ser (C201S) abrogated the activity of GST-MKP1-(1–361) (Fig. 4C). The remaining SIPK activity was estimated by quantifying the intensity of 32P-labeled MBP and SIPK proteins. SIPK activity ([32P]-labeled MBP/SIPK protein) reacted with GST-MKP1-(1–361) was 39% of that reacted with GST. In contrast, SIPK activity reacted with GST-MKP1-(1–276), GST-MKP1-(115–361), or GST-MKP1-(1–361) C201S was 98–109% of that reacted with GST, suggesting that these proteins are inactive.

Binding to SIPK Activates the Phosphatase Activity of NtMKP1—In addition to a catalytic domain, mammalian MKPs have a substrate-binding domain at their N termini (5). In Fig. 4C, deletion of the N-terminal non-catalytic region (residues 1–114) or the GH domain (residues 277–361) of GST-MKP1-(1–361) reduced the phosphatase activity despite the presence of an intact catalytic domain. This result raised the possibility that the N-terminal non-catalytic region or the GH domain binds SIPK. Therefore, the SIPK-binding domain of NtMKP1 was mapped by coprecipitation assay with total protein extracts prepared from tobacco N. benthamiana leaves overexpressing the SIPK gene. SIPK was precipitated by GST-MKP1-(1–361), but not by GST-MKP1-(362–467), GST-MKP1-(468–862), or GST alone (Fig. 5A, left panel). Further deletion analysis confirmed that the N-terminal 114 amino acids of NtMKP1 were required and sufficient to bind and precipitate SIPK (Fig. 5A, right panel). These results indicate that the N-terminal non-catalytic region of NtMKP1 binds SIPK. However, the involvement of the C-terminal domain in the binding to SIPK cannot be ruled out because GST-MKP1-(468–862) was highly unstable in the extracts despite the presence of a protease inhibitor mixture (compare before (Fig. 5A, left panel, lane 5) and after (lane 4) incubation with the extracts).

We tested whether NtMKP1 is activated by the binding of its substrate SIPK using an artificial substrate. Although p-nitrophenyl phosphate is the most commonly used artificial substrate of MKPs, we found no significant dephosphorylation of p-nitrophenyl phosphate by NtMKP1 (19). It has been reported that MKPs prefer bulky polycyclic aryl phosphates such as OMFP (36). Naoi and Hashimoto (16) reported that PHS1, an Arabidopsis MKP involved in microtubule organization, dephosphorylates OMFP, but not p-nitrophenyl phosphate. Therefore, the activity of NtMKP1 was assayed using OMFP as a substrate. We found no significant activity of FLAG-MKP1, again probably because only a small amount of FLAG-MKP1 protein was produced by in vitro transcription/translation. Then, we used partially purified GST-MKP1 as another source of the phosphatase. As shown in Fig. 5B, the partially purified fraction of GST-MKP1 contains a major band of 125 kDa (indicated by the arrowhead), which corresponds to the predicted molecular mass of GST-MKP1 (122.7 kDa). As expected, GST-MKP1 dephosphorylated OMFP in a time- and concentration-dependent manner (Fig. 5B). Moreover, the dephosphorylation of OMFP by GST-MKP1 was significantly increased by the addition of recombinant SIPK in a dose-dependent manner (Fig. 5C). This effect was independent of the kinase activity of SIPK because the reaction mixture contained EDTA, which chelates Mg2+ and inhibits kinase activity. We analyzed the effect of CaM binding on the OMFP phosphate activity of GST-MKP1 in the presence or absence of SIPK, but found no significant change in the dephosphorylation activity of GST-MKP1 (Fig. 5D). As purified GST-MKP1-(1–361) also dephosphorylated OMFP (Fig. 5E) in a similar manner, possible dephosphorylation by other proteins can be ruled out. The OMFP phosphate activity of GST-MKP1-(115–361), which could not inactivate SIPK (Fig. 4B), was also analyzed. Interestingly, purified GST-MKP1-(115–361) dephosphorylated OMFP more effectively than did purified GST-MKP1-(1–361) (Fig. 5E). This result further indicates that the N-terminal non-catalytic region of NtMKP1 is involved in the binding to SIPK, but is not essential for phosphatase activity.

Differential Activation of NtMKP1 by SIPK and WIPK—WIPK is another stress-inducible tobacco MAPK, and some forms of stress, including pathogen-induced cell death, induce activation of both WIPK and SIPK (22). WIPK is highly similar to SIPK in primary structure, and

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4 S. Katou and Y. Ohashi, unpublished data.
they share the same upstream MAPKK, NtMEK2 (37). However, their activities are regulated differentially at both the transcriptional and post-translational levels (27, 38). We tested whether WIPK also activates OMFP hydrolysis of GST-MKP1. The ability of recombinant SIPK and WIPK to activate the OMFP phosphatase activity of GST-MKP1 was compared (Fig. 6A). WIPK also stimulated the dephosphorylation of OMFP by GST-MKP1 in a dose-dependent manner, but its effect was weaker than that of SIPK (Fig. 6C). Because WIPK could phosphorylate more WIPK itself and MBP than did SIPK (Fig. 6B), the possibility that the WIPK protein was folded improperly and is unable to activate NtMKP1 was ruled out. To investigate the role of the CaM-binding and C-terminal domains of NtMKP1 in the catalytic activation of NtMKP1 by SIPK and WIPK, the effect of SIPK and WIPK on the OMFP phosphatase activity of GST-MKP1-(1–361) was analyzed. Like GST-MKP1, the OMFP phosphatase activity of GST-MKP1-(1–361) was activated strongly by SIPK and weakly by WIPK in a dose-dependent manner (Fig. 6D). These results indicate that the CaM-binding and C-terminal domains are dispensable for the catalytic activation of NtMKP1 by SIPK and WIPK. As the expression of GST-MKP1 in E. coli is very weak, we used GST-MKP1-(1–361) in the subsequent experiments.

The Putative Common Docking Domain of SIPK Is Partially Responsible for Activation of NtMKP1 by SIPK—SIPK and WIPK share 72% identity in their amino acid sequences. SIPK is slightly larger than WIPK because of its N-terminal extension (Fig. 7A). A major difference between SIPK and WIPK exists in the N terminus. To determine whether the N-terminal extension of SIPK is responsible for the strong activation of GST-MKP1-(1–361), two chimeras, (S/W)IPK and (W/S)IPK, were constructed as reported by Zhang and Liu (27). The (S/W)IPK construct has the SIPK N terminus fused to the WIPK C terminus, whereas (W/S)IPK construct has the WIPK N terminus fused to the SIPK C terminus (Fig. 7A). The ability of the recombinant proteins produced to activate the OMFP phosphatase activity of GST-MKP1-(1–361) was compared (Fig. 7B). As shown in Fig. 7C, (W/S)IPK stimulated the dephosphorylation of OMFP by GST-MKP1-(1–361) like SIPK, whereas (S/W)IPK acted like WIPK. This result suggests that the C terminus of SIPK determines the strong activation of NtMKP1 phosphatase activity by SIPK.

It has been demonstrated that the C-terminal acidic motif of mammalian MAPks, known as the common docking (CD) domain, is responsible for the interaction with the upstream MAPK and sub-
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FIGURE 5. Catalytic activation of NtMKP1 by SIPK binding.

A, the N-terminal non-catalytic region of NtMKP1 binds SIPK. Total protein extracted from N. benthamiana leaves overexpressing the SIPK gene was mixed with glutathione-Sepharose-bound GST (lanes 1 and 6) or GST-MKP1 fragments (lanes 2–4 and 7–10) and rotated overnight at 4 °C. After washing, precipitated SIPK was detected with anti-SIPK antibody (α-SIPK). The precipitates were also stained with Coomassie Brilliant Blue (CBB) to verify that similar amounts of GST-fused proteins were used. GST-MKP1-(468–862) before incubation with protein extracts is also shown (lane 5). B, GST-MKP1 dephosphorylates OMFP. Purified GST (0.5 μg) and partially purified GST-MKP1 (2 μg) were separated on an SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. The band corresponding to the predicted molecular mass of GST-MKP1 is indicated by a black arrowhead. The positions of molecular mass markers in kilodaltons are indicated on the left. OMFP was incubated with 40 μg of GST-MKP1 (●) or GST (●) at 30 °C. At the indicated times, the absorbance was measured at 477 nm. C, dose-dependent activation of GST-MKP1 by SIPK. The hydrolysis of OMFP by 20 μg of GST-MKP1 was assayed with 20 μg (●), 10 μg (●), or 5 μg (●) of SIPK or with 10 μg of vector only as a control (●). D, hydrolysis of OMFP by 20 μg of GST-MKP1 as assayed without SIPK and NtCaM1 (●) or with 20 μg of SIPK (●), 7 μg of NtCaM1 (●), or 20 μg of SIPK and 7 μg of NtCaM1 (●). E, GST-MKP1-(1–361) and GST-MKP1-(115–361) dephosphorylate OMFP. The hydrolysis of OMFP was assayed with 20 μg of purified GST-MKP1-(1–361) (●) or purified GST-MKP1-(115–361) (●).

strates as well as negative regulator MKPs (39). The CD domain has two invariable acidic amino acids that are also conserved in plant MAPKs. A Drosophila melanogaster sevenmaker gain-of-function mutant, in which the second invariable acidic amino acid (Asp) of the CD domain is replaced with Asn, shows significantly reduced sensitivity to MKP (40, 41). SIPK also has a putative CD domain, in which Asp350 and Asp352 correspond to invariable acidic amino acids. To investigate the importance of these acidic amino acids, either the second or both Asp residues of SIPK were replaced with Asn, resulting in SIPK(N) and SIPK(NN), respectively (Fig. 7A). Recombinant proteins were then produced (Fig. 7D), and their ability to activate the OMFP phosphatase activity of GST-MKP1-(1–361) was compared. Both SIPK(N) and SIPK(NN) stimulated the dephosphorylation of OMFP by GST-MKP1-(1–361) in a concentration-dependent manner (Fig. 7E), although their efficiency was about a half that of SIPK.

The N-terminal Non-catalytic Region, but Not CaM Binding, Is Required for NtMKP1 to Inactivate SIPK in Vivo—Transient expression of MEKDD, a constitutively active form of StMEK1 MAPK, in tobacco N. benthamiana induces cell death probably through activation of endogenous SIPK (23, 27–29). Because NtMKP1 inactivates SIPK in vitro (Fig. 1C) and its overexpression compromises wound-induced activation of SIPK (19), we expected the simultaneous expression of NtMKP1 to compromise MEKDD-induced cell death. Agrobacterium cells carrying FLAG-MEKDD were mixed with Agrobacterium cells carrying either the vector control or FLAG-MKP1 and then infiltrated into N. benthamiana leaves (agroinfiltration). As expected, expression of WT MKP1 compromised both the cell death (Fig. 8C) and SIPK activation (Fig. 8B, lane 3) induced by MEKDD. In contrast, expression of MKP1 C201S, in which the putative catalytically essential Cys residue was replaced with Ser, failed to suppress either response (Fig. 8, B, lane 13; and F), suggesting the significance of the phosphatase activity of NtMKP1. To investigate the significance of the N-terminal non-catalytic region of NtMKP1 and CaM binding in vivo, we first compared protein accumulation of NtMKP1 derivatives in N. benthamiana. FLAG-MKP1 derivatives were transiently expressed in N. benthamiana by agroinfiltration, and their accumulation was detected by immunoblot analysis with anti-NtMKP1 antibody. Compared with WT MKP1, the level of MKP1 L443R was slightly but reproducibly low (Fig. 8A, lane
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SIPK and WIPK (0.4 μg each) were incubated with MBP in the presence of 32P-ATP. Phosphorylated SIPK, WIPK, and MBP were visualized by autoradiography. C, the hydrolysis of OMFP by 20 μg of GST-MKP1 was assayed with increasing amounts of SIPK (●) or WIPK (○). D, the hydrolysis of OMFP by 10 μg of GST-MKP1-(1–361) was assayed with increasing amounts of SIPK (●) or WIPK (○).

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FIGURE 7. Catalytic activation of NtMKP1 by SIPK partially depends on the putative CD domain of SIPK. A, shown is a schematic representation of SIPK and WIPK chimeras. A PstI site was introduced into the WIPK gene, which does not change the amino acid sequence. The amino acid sequences of the putative CD domains of SIPK, SIPK\(^N\), and SIPK\(^{NN}\) are shown, with two conserved Asp residues in boldface. The second Asp residue alone or both Asp residues were replaced with Asn in SIPKN and SIPKNN, respectively. Trx, thioredoxin. 

B, recombinant SIPK, (S/W)IPK, (W/S)IPK, and WIPK were produced in \(E.\) \(coli\) and purified by nickel affinity chromatography. The purified proteins (1 \(\mu\)g) were separated on an SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue (CBB). The asterisk indicates contaminated \(E.\) \(coli\) protein. 

C, the hydrolysis of OMFP by 10 \(\mu\)g of GST-MKP1-(1–361) was assayed in the absence \(\bullet\) or presence of 10 \(\mu\)g of SIPK \(\bullet\), (S/W)IPK \(\Delta\), (W/S)IPK \(\bullet\), or WIPK \(\circ\). D, recombinant SIPK, SIPK\(^N\), and SIPK\(^{NN}\) were produced in \(E.\) \(coli\) and purified by nickel affinity chromatography. The purified proteins (1 \(\mu\)g) were separated on an SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. E, the hydrolysis of OMFP by 10 \(\mu\)g of GST-MKP1-(1–361) was assayed with increasing amounts of SIPK \(\circ\), SIPK\(^N\) \(\bullet\), or SIPK\(^{NN}\) \(\Delta\).
whereas we found no significant difference between the vector control and MKP1 K41A/R43A, suggesting that Lys41 and Arg43 are major and essential SIPK-interacting sites of NtMKP1. Immunoblot analysis with anti-FLAG antibody confirmed the expression of MEKDD (Fig. 9C, lower panel). The decreased mobility of MEKDD upon SDS-PAGE again correlated with the activation of SIPK (Fig. 9C, lower panel). The effect of mutations on the interaction of the N-terminal non-catalytic region of NtMKP1 with SIPK was investigated by coprecipitation analysis with total protein extracts prepared from N. benthamiana leaves overexpressing the SIPK gene. Consistent with the effect on the cell death-suppressing activity of FLAG-MKP1 (Fig. 9, D–G), the K41A/R43A mutation abolished the interaction of GST-MKP1-(1–114) with SIPK, whereas the R56A mutation decreased it (Fig. 9H, upper panel), indicating that Lys41 and Arg43 are essential for NtMKP1 to interact with SIPK. The direct interaction of GST-MKP1-(1–114) derivatives with purified recombinant SIPK (Fig. 1A) was investigated. Although the interaction of GST-MKP1-(1–114) with recombinant SIPK was greatly diminished by the K41A/R43A mutation, it was still detected (Fig. 9H, middle panel). These results indicate that Lys41 and Arg43 of NtMKP1 are major SIPK-interacting sites and are required for high affinity binding to SIPK.
FIGURE 9. Lys41 and Arg43 of NtMKP1 are essential for its interaction with and inactivation of SIPK. A, shown is a schematic representation of NtMKP1 derivatives. The positions of Ala (A) mutations introduced into the N-terminal non-catalytic region of NtMKP1 are indicated. The ability of NtMKP1 derivatives to suppress the MEKDD-induced cell death and activation of SIPK is summarized. DSP, dual-specificity phosphatase domain. B, N-terminally FLAG-tagged NtMKP1 derivatives were transiently expressed in N. benthamiana by agroinfiltration. The accumulation of MKP1 derivatives was detected by immunoblot analysis with anti-NtMKP1 antibody (α-MKP1). The nonspecific staining of the ribulose-bisphosphate carboxylase/oxygenase (Rubisco) large subunit is shown to verify equal loading in each lane. C, N. benthamiana leaves were infiltrated with Agrobacterium cells carrying the indicated constructs. Two days later, SIPK activity was measured by immunocomplex kinase assay using MBP as a substrate (upper panel). Expression of FLAG-MEKDD was detected by immunoblot analysis with anti-FLAG antibody (α-FLAG; lower panel). D–G, N. benthamiana leaves were infiltrated with Agrobacterium cells carrying the indicated constructs. The photographs were taken 84 h after agroinfiltration. H, the binding of GST-MKP1-(1–114) derivatives to SIPK was analyzed with total protein extracted from N. benthamiana leaves overexpressing the SIPK gene as described in the legend to Fig. 5A (upper panel) or with purified SIPK (middle panel). GST and GST-MKP1-(1–114) derivatives bound to glutathione-Sepharose were detected by Coomassie Brilliant Blue (CBB) staining (lower panel). α-SIPK, anti-SIPK antibody.
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DISCUSSION

Catalytic Activation of NtMKP1 by SIPK but Not by CaM—In this study, we have demonstrated that NtMKP1 is an active phosphatase that inactivates its physiological substrate SIPK by dephosphorylating the conserved TETY motif of SIPK (Figs. 1 and 2). We further showed that the catalytic activity of NtMKP1 was increased by the binding of SIPK, but not by CaM (Fig. 5, C and D), showing for the first time that substrate-triggered activation is an evolutionarily conserved mechanism that regulates the activity of mammalian and plant MKPs. The N-terminal non-catalytic region of NtMKP1 was found to interact with SIPK and to be essential for the inactivation of SIPK both in vitro and in vivo (Figs. 4, 5, and 8). The interaction between mammalian MKPs and their substrate MAPKs is mediated via the interaction between a cluster of basic amino acids at the N terminus of MKP and two invariable acidic amino acids within the CD domain located at the C terminus of MAPK (5). However, NtMKP1 has no such cluster in its N-terminal non-catalytic region. Alternatively, we identified Lys44 and Arg43 as important amino acids of NtMKP1 for interaction with and inactivation of SIPK (Fig. 9, C–H). These amino acids are well conserved among putative NtMKP1 orthologs of other plant species and therefore represent a novel MAPK-docking site of MKP. On the other hand, the CD domain and two invariable acidic amino acids are conserved in SIPK. However, mutation of both invariable acidic amino acids of SIPK only partially compromised the activation of MKP1 by SIPK (Fig. 7E). Zhang and Liu (27) showed by transient expression that such a mutation of SIPK does not enhance its activity, but rather weakens it. It is therefore likely that a novel mechanism underlies the interaction of SIPK with NtMKP1.

The activation of SIPK, as well as the accumulation of NtMKP1 mRNA, is induced by both wounding and a hypersensitive reaction against tobacco mosaic virus infection (19, 43). However, the accumulation of NtMKP1 mRNA is much delayed compared with the activation of SIPK (19, 43). Similarly, activation of AtMKP6 (an Arabidopsis SIPK ortholog) is rapidly induced by UVC treatment, whereas the AtMKP1 mRNA level remains constant (17). These results suggest the importance of post-transcriptional regulation of plant MKPs. The catalytic activation of NtMKP1 by its substrate MAPK shown in this study may represent such a regulatory mechanism of NtMKP1.

The catalytic activity of NtMKP1 was increased strongly by the binding of SIPK, but weakly by the binding of WIPK (Fig. 6). AtMKP1 was also shown to interact strongly with AtMKP6 (an Arabidopsis SIPK ortholog), but weakly with AtMKP3 (an Arabidopsis WIPK ortholog) (17). In mammalian MKPs, it has been shown that substrate-triggered activation plays an important role in determining substrate specificity at the subfamily level (6–9). Previously, we found that wound-induced activation of SIPK is more frequently compromised than that of WIPK in NtMKP1-overexpressing plants, although wounding induces much stronger activity of SIPK compared with WIPK (19). This correlation suggests that substrate-triggered activation seems to play an important role in determining the substrate specificity of NtMKP1. However, WIPK activity was suppressed to a similar extent as SIPK activity in one line of our NtMKP1-overexpressing plants (19). Several lines of evidence show that SIPK and WIPK activities are not independent. SIPK and WIPK share the same upstream MAPKK, NtMEK2, and silencing of the SIPK gene by RNA interference leads to enhanced activation of WIPK by ozone (32, 37). Therefore, SIPK and WIPK activities should be carefully compared. The correlation of substrate-triggered activation with substrate specificity of mammalian MKPs has been shown at the subfamily level (ERK, JNK, and p38). In contrast, most plant MAPKs, including SIPK and WIPK, are the ERK type. It would be interesting to investigate whether substrate-triggered activation plays a role in determining the substrate specificity of MKP within the same MAPK sub-family. We will investigate the correlation between the substrate-triggered activation and substrate specificity of NtMKP1 using tobacco MAPKs, including SIPK and WIPK.

Role of the CaM-binding and C-terminal Domains of NtMKP1—NtMKP1 was identified as a CaM-binding protein, but our results demonstrated that CaM binding was not essential for NtMKP1 to inactivate SIPK in vitro or in vivo (Figs. 3, 4, and 8). Moreover, neither the SIPK-inactivating activity nor the OMFP phosphatase activity of NtMKP1 was stimulated by the addition of CaM (Fig. 5) (4). The cell death-suppressing activity of NtMKP1 derivatives seems to correlate with their protein expression level. The level of NtMKP1 L443R protein was slightly but reproducibly lower than that of NtMKP1, and its cell death-suppressing activity was weaker than that of NtMKP1 (Fig. 8). These results suggest that CaM binding might not stimulate NtMKP1 activity. Nevertheless, we cannot rule out the possibility that CaM binding activates NtMKP1 because it has been reported that the native forms of catalase and NADK2 are activated by CaM, but the recombinant forms are not (44, 45). In contrast to NtMKP1, a recent report showed that the phosphatase activity of DsPTP1 is regulated by CaM binding (20). Interestingly, DsPTP1 activity for p-nitrophenyl phosphate is stimulated by CaM, whereas that for tyrosine-phosphorylated MBP is inhibited by CaM (20). This result suggests that the effect of CaM binding may depend on the type of substrate. It is also of interest whether the phosphatase activity of DsPTP1 is activated not only by CaM, but also by its substrate MAPK(s).

NtMKP1 and its putative orthologs from other plant species have a long C-terminal domain, which is not present in most mammalian MKPs. The C-terminal domain is less conserved compared with the N-terminal domain, but the serine-rich region is a conserved feature of the C-terminal domain. In human MKPs, only VHR and MKP7 have a C-terminal stretch containing PEST sequences (5). PEST sequences are abundant in Pro, Glu, Ser, and Thr residues and are frequently found in unstable proteins (46). In addition to PEST sequences, MKP7 has a serine-rich region in its C-terminal stretch, and deletion of the C-terminal stretch stabilizes MKP7 (9). These results suggest that the C-terminal stretch of mammalian MKPs is involved in protein stability. Although no apparent PEST sequence was found in the C-terminal domain of NtMKP1, our results suggest that the C-terminal domain also contributes to the stability of the NtMKP1 protein. Immunoblot analysis of N-terminally FLAG-tagged NtMKP1 expressed in N. benthamiana detected multiple truncated proteins (Fig. 8A). Like MKP7, the level of NtMKP1-(1–361), which lacks the C-terminal domain, was much higher than that of full-length NtMKP1 (Fig. 8A). The GST-fused C-terminal domain was unstable in the protein extracts from N. benthamiana (Fig. 5A). Some mammalian MKPs are phosphorylated by MAPK, and such phosphorylation regulates degradation of MKPs by the proteasome (47, 48). In addition, one effect of CaM binding to targeted proteins is stabilization of the proteins (49). Therefore, the degradation mechanism of NtMKP1 in relation to phosphorylation and CaM binding should be a subject of further analysis.

In this study, we have demonstrated that NtMKP1 is an active phosphatase that inactivates SIPK. Although NtMKP1 is quite different from mammalian MKPs in primary structure, the catalytic activity of NtMKP1 was significantly increased by substrate MAPKs, similar to mammalian MKPs. Our results indicate that the activities of mammalian and plant MKPs are regulated, at least in part, by an evolutionarily conserved mechanism. On the other hand, CaM binding is a unique feature of plant MKPs, although the role of CaM binding to NtMKP1 is still unclear. By elucidating the regulatory mechanism of MKP, an
important negative regulator of MAPK, we will understand how the activity of MAPK is regulated.

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