Slingshot-1 (SSH1), a member of a dual-specificity protein phosphatase family, regulates actin dynamics by dephosphorylating and reactivating cofilin, an actin-depolymerizing factor. SSH1 has the SSH family-specific, N-terminal, noncatalytic (SSH-N) domain, consisting of the A and B subdomains. SSH1 is activated by binding to actin filaments. In this study, we examined the mechanisms of SSH1 substrate recognition of phosphocofilin (P-cofilin) and SSH1 activation by F-actin. We found that P-cofilin binds to a phosphatase-inactive mutant, SSH1(CS), in which the catalytic Cys-393 is replaced by Ser. Using a series of deletion mutants, we provided evidence that both the phosphatase (P) domain and the adjacent B domain are indispensable for P-cofilin binding of SSH1(CS) and cofilin-phosphatase activity of SSH1. In contrast, the A domain is required for the F-actin-mediated activation of SSH1, but not for P-cofilin binding or basal cofilin-phosphatase activity. The P domain alone is sufficient for the phosphatase activity toward p-nitrophenyl phosphate (pNPP), indicating that the SSH-N domain is not essential for the basal phosphatase activity of SSH1. Addition of F-actin increased the cofilin-phosphatase activity of SSH1 more than 1200-fold, but the pNPP-phosphatase activity only 2.2-fold, which suggests that F-actin principally affects the cofilin-specific phosphatase activity of SSH1. When expressed in cultured cells, SSH1, but not its mutant deleted of SSH-N, accumulated in the rear of the lamellipodium. Together, these findings suggest that the conserved SSH-N domain plays critical roles in P-cofilin recognition, F-actin-mediated activation, and subcellular localization of SSH1.

Protein phosphorylation is one of the most ubiquitous types of post-translational protein modification and is essential for signaling pathways mediating diverse cellular processes. Protein phosphorylation is regulated by the balanced actions of protein kinases and protein phosphatases. Protein-tyrosine phosphatases (PTPs)² constitute the largest family of phosphatases. These enzymes are defined by their active-site signature motif, (H/V)CX₅C₆R(S/T), in which the nucleophilic Cys residue attacks the phosphate group of target proteins (1–3). Of ~100 phosphatase genes classified into the PTP family in the human genome, the most diverse group, in terms of the substrate specificity, is the group of dual-specificity phosphatases (DSPs) (4). DSPs are further divided into several subgroups, each having a wide range of substrate specificities for phospho-Ser, phospho-Thr, phosphoinositol, and mRNA, in addition to phospho-Tyr (3, 4). These phosphatases play specific roles in decreasing the phosphorylation levels of target molecules and controlling signal transduction pathways that underlie a variety of physiological processes.

Cofilin is a key regulator of actin filament dynamics and functions by stimulating the depolymerization and severance of actin filaments (5). Cofilin activity is regulated by its specific kinases and phosphatases. Cofilin is inactivated by phosphorylation at Ser-3 by LIM-kinases and related testicular protein kinases (6–8) and reactivated by dephosphorylation by Slingshot (SSH) family phosphatases, composed of SSH1, SSH2, and SSH3 (9, 10), and chronophin (11). SSHs are classified into the DSP family, based on the sequence similarity of their phosphatase domains (3, 4, 9, 10). The cofilin-phosphatase activity of SSH1 is extremely enhanced by its association with F-actin (12–15), and F-actin-mediated activation is essential for the function of SSH1 in directional cell migration (13). However, little is known about the molecular mechanisms underlying how SSH1 recognizes its substrate, Ser-3-phosphorylated cofilin (P-cofilin), or how F-actin enhances the cofilin-phosphatase activity of SSH1.

Mitogen-activated protein kinase (MAPK) phosphatases (MKPs) are members of a DSP family that dephosphorylate and inactivate MAPKs (16). Some MKPs, such as MKP-1 and MKP-3, selectively bind to their substrate MAPKs through their N-terminal noncatalytic domains and are activated by interactions with MAPKs (17–20). These selective binding and

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² The abbreviations used are: PTP, protein tyrosine phosphatase; CBB, Coomassie brilliant blue; CFP, cyan fluorescent protein; deP-cofilin, dephosphorylated cofilin; DSP, dual-specificity phosphatase; ERK, extracellular signal-regulated kinase; FL, full-length; GST, glutathione S-transferase; LIMK1, LIM-kinase 1; MAPK, mitogen-activated protein kinase; MKP, MAPK phosphatase; P-cofilin, Ser-3-phosphorylated cofilin; P-coro1B, phospho-coronin 1B; pNPP, p-nitrophenyl phosphate; SSH, Slingshot; SSH1(CS), a Slingshot-1 mutant with replacement of Cys-393 by Ser; VHR, vaccinia H1-related phosphatase; WT, wild-type.
activation mechanisms underlie the substrate specificity of MKPs toward their target MAPKs. SSH1 possesses the SSH family-specific, N-terminal, noncatalytic domain (termed SSH-N domain, consisting of the A and B subdomains). The SSH-N domain is unique to and conserved among SSH family phosphatases and is positioned N-terminal to the phosphatase (P) catalytic domain (9, 10). The N-terminal half of SSH1 (amino acids 1–461, consisting of the A, B, and P domains) is sufficient for both the cofilin-phosphatase activity and F-actin-mediated activation (13), which suggests that the N-terminal half of SSH1 plays important roles for determining the substrate specificity of SSH1 and for conferring F-actin-dependent activation of its cofilin-phosphatase activity.

In the present study, we investigated the molecular mechanisms underlying the substrate specificity and F-actin-mediated activation of SSH1 with a particular focus on the role of the conserved N-terminal A, B, and P domains of SSH1. We provide evidence that both the B and P domains are essential for P-cofilin recognition and cofilin-phosphatase activity of SSH1, while the P domain alone can dephosphorylate an artificial substrate p-nitrophenyl phosphate (pNPP). We also show that F-actin strongly enhances the phosphatase activity of SSH1 toward P-cofilin, but only faintly toward pNPP, and that the A domain is required for this F-actin-induced enhancement of cofilin-phosphatase activity. We also provide evidence that the SSH-N domain is important for the localization of SSH1 in the rear of the lamellipodium. Our results suggest that the N-terminal conserved region of SSH1 plays critical roles in P-cofilin recognition, F-actin-mediated cofilin-phosphatase activity, and subcellular localization of SSH1, and that F-actin primarily affects the cofilin-specific phosphatase activity of SSH1.

EXPERIMENTAL PROCEDURES

**Materials**—Rhodamine-phalloidin and pNPP were purchased from Invitrogen and Sigma, respectively. Rabbit polyclonal antibodies to cofilin and P-cofilin were prepared as previously described (8). Anti-Myc antibody (9E10) and anti-pSerPKC antibody were purchased from Roche Diagnostics and Cell Signaling Technologies, respectively. Rhodamine-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG antibodies were purchased from Chemicon.

**Plasmid Construction**—The plasmids for human SSH1 and its point and deletion mutants were prepared as described (10, 13, 15). Human coronin 1B cDNA was isolated by PCR amplification. For protein expression in a baculovirus system, cDNA inserts were subcloned into the pFastBac1 vector (Invitrogen). For plasmids encoding SSH1 and its mutants that were C-terminally tagged with cyan fluorescent protein (CFP) or (Myc/H11001His)-tag (consisting of a Myc epitope and six His residues), cDNA inserts were subcloned into the pECFP or pcDNA3.1/Myc/H11001His vector (Invitrogen), respectively.

**Proteins**—SSH1 and its mutants were expressed in Sf21 (provided by Dr. K. Nakayama, Tohoku University) or Sf9 insect cells, using the Bac-to-Bac baculovirus expression system (Invitrogen). For proteins encoding SSH1 and its mutants that were C-terminally tagged with cyan fluorescent protein (CFP) or (Myc+His)-tag (consisting of a Myc epitope and six His residues), cDNA inserts were subcloned into the pEFP or pcDNA3.1/Myc+His vector (Invitrogen), respectively.

**Materials**—Rhodamine-phalloidin and pNPP were purchased from Invitrogen and Sigma, respectively. Rabbit polyclonal antibodies to cofilin and P-cofilin were prepared as previously described (8). Anti-Myc antibody (9E10) and anti-pSerPKC antibody were purchased from Roche Diagnostics and Cell Signaling Technologies, respectively. Rhodamine-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG antibodies were purchased from Chemicon.
Mechanism of Cofilin Dephosphorylation by Slingshot

**A**

![Diagram showing binding of SSH1 and P-cofilin](chart)

**B**

![Diagram showing substrate trapping assay](chart)

**C**

![Diagram showing GST-SSH1 and Cofilin](chart)

**D**

![Diagram showing GST-N and Cofilin](chart)

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mM NaCl, 2 mM MgCl₂, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 1 mM dithiothreitol) and clarified by centrifugation. Cell lysate was added to glutathione-Sepharose 4B (GE Healthcare) and incubated for 1 h. After washing with a buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM MgCl₂, and 1 mM dithiothreitol, GST-LIMK1 was eluted with glutathione-Sepharose 4B (20 mM glutathione, 50 mM Tris-HCl, 100 mM NaCl, 2 mM MgCl₂, and 1 mM dithiothreitol, pH 7.5) and dialyzed against a buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, and 1 mM dithiothreitol. Actin was purified from rabbit skeletal muscle as described (21), except that a Superdex 200 column (GE Healthcare) was used for gel filtration. For the phosphorylated coronin 1B (P-coro1B) preparation, coronin 1B-(Myc+His) was co-expressed with GST-PKα in insect cells and purified with Ni-NTA agarose, as described (22).

Preparation of P-cofilin, Dephosphorylated Cofilin (deP-cofilin), and Mutant Cofilin—C-terminally His₆-tagged human cofilin (cofilin-His₆) was expressed in insect cells and affinity-purified with Ni-NTA agarose as described (21). Eluted cofilin-His₆ was applied to a PD-10 column (GE Healthcare), equilibrated with buffer A (20 mM Tris-HCl, pH 7.0, and 1 mM dithiothreitol) at 30 °C for 2 h, and the reaction mixture was incubated with glutathione-Sepharose 4B to deplete GST-LIMK1.

For preparation of deP-cofilin, cofilin-His₆ and GST-SSH1(WT) were separately expressed in insect cells, and these cell lysates were mixed after sonication, incubated on ice for 1 h to dephosphorylate cofilin, and purified through Ni-NTA agarose. Cofilin(S3A)-His₆ and cofilin(S3E)-His₆ were purified with the protocol similar to that for cofilin(WT)-His₆. Purified proteins were dialyzed against a buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 1 mM dithiothreitol.

Substrate Trapping Assay—Sf9 or Sf21 cells expressing GST-tagged SSH1 or its deletion mutants were sonicated in GST lysis buffer, and cell lysates were clarified by ultracentrifugation at 100,000 × g for 30 min at 4 °C. Supernatants were added to glutathione-Sepharose 4B to immobilize GST-fused proteins. After washing with wash buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5% Nonidet P-40, 5% glycerol, and 1 mM dithiothreitol, pH 7.5) and dialyzed against a buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, and 1 mM dithiothreitol, the beads were incubated with 0.5 μM P-cofilin or deP-cofilin in 300 μl of wash buffer for 3 h at 4 °C, washed three times, and sampled for SDS-PAGE. GST-tagged proteins and bound cofilin were stained with Coomassie Brilliant Blue (CBB), and bound cofilin was further analyzed by immunoblotting with anti-cofilin and anti-P-cofilin antibodies.

**Phosphatase Assays**—pNPP-phosphatase assays were performed as follows: GST-tagged SSH1 and its deletion mutants...
immobilized on glutathione Sepharose 4B were incubated with 10 mM pNPP in assay buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM MgCl₂, and 1 mM dithiothreitol) at 30 °C, and the hydrolysis of pNPP was measured by following the absorbance at 405 nm (A₄₀₅). To examine F-actin-mediated activation of the pNPP-phosphatase activity of SSH1, purified SSH1-(Myc+His) was incubated in 200 µl of assay buffer containing 20 mM pNPP with or without F-actin at 30 °C. The changes in A₄₀₅ were recorded every 15 min on a Benchmark plus microplate spectrophotometer (Bio-Rad). Phosphatase assays toward substrates P-cofilin or P-coro1B were performed as described previously (12, 22). Briefly, purified or immobilized SSH1 proteins were incubated in 20 µl of assay buffer containing P-cofilin or P-coro1B at 30 °C. Reaction mixtures were separated on SDS-PAGE, and P-cofilin or P-coro1B was detected with Pro-Q diamond phosphoprotein gel stain (Molecular Probes) or immunoblotting with an anti-pSer⁴⁴⁷ antibody.

**F-actin Cosedimentation Assay**—F-actin cosedimentation assay was performed as described previously (21), except that, in the experiment to examine the effect of phalloidin on the F-actin binding of SSH1 and cofilin, F-actin was preincubated with phalloidin at room temperature for 30 min before use.

**Cell Culture, Transfection, and Microscopic Analysis**—C2C12 mouse myoblast cells and N1E-115 mouse neuroblastoma cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10 and 15% fetal calf serum, respectively. For immunofluorescence analysis, cells were grown on coverslips and transfected with plasmids encoding CFP- or (Myc-His) cofilin, expressed in insect cells using the baculovirus expression system, was subjected to dephosphorylation with GST-LIMK1. After purification, the purity of deP-cofilin and P-cofilin was analyzed by SDS-PAGE followed by CBB staining (Fig. 1A). Immunoblotting with anti-P-cofilin antibody and Pro-Q phosphoprotein staining revealed that the purified P-cofilin, but not deP-cofilin, was phosphorylated (Fig. 1A). We also analyzed the F-actin binding ability of purified deP-cofilin and P-cofilin. F-actin cosedimentation assays revealed that deP-cofilin, but not P-cofilin, coprecipitated with F-actin and increased the amount of actin in the supernatant (supplemental Fig. S1), which indicates that the purified deP-cofilin, but not P-cofilin, has the activity to bind to and depolymerize actin filaments. These results further suggest that both deP- and P-cofilin were purified successfully. These purified proteins were used for the following binding experiments and cofilin phosphatase assays.

PTP and DSP family phosphatases utilize the conserved Cys residues in their active sites as a nucleophile that attacks phosphorus atoms of substrates (1, 2). The phosphatase-inactive CS mutant of each truncated SSH1 was used as a negative control. The reaction mixtures were separated on SDSPAGE and analyzed by CBB and Pro-Q and phosphoprotein gel stain. The bottom panel shows relative P-cofilin levels, with the value of P-cofilin in experiments without GST-N461 taken as 100%. Data are the means ± S.D. of three independent experiments. B, phosphatase activity. GST-N461 and GST-N461N prepared in A were in parallel subjected to pNPP-phosphatase assay, by incubating with pNPP at 30 °C for 1 h. pNPP hydrolysis was analyzed by measuring A₄₀₅. Data are the means ± S.D. of three independent experiments.

**RESULTS**

**P-cofilin Binds to a Phosphatase-inactive CS Mutant of SSH1**—To examine the mechanism by which SSH1 recognizes its substrate P-cofilin, we first prepared dephosphorylated (deP-) and phosphorylated (P-) forms of cofilin. His₆-tagged cofilin, expressed in insect cells using the baculovirus expression system, was subjected to dephosphorylation with GSTSSH1 or phosphorylation with GST-LIMK1. After purification, the purity of deP-cofilin and P-cofilin was analyzed by SDS-PAGE followed by CBB staining (Fig. 1A). Immunoblotting with anti-P-cofilin antibody revealed that the purity of deP-cofilin and P-cofilin was analyzed by SDS-PAGE followed by CBB staining (Fig. 1A).

**SSH-N domain** is required for cofilin-phosphatase activity, but not for pNPP-phosphatase activity, of SSH1. A, cofilin-phosphatase activity of GST-N461 and GST-N461ΔN. Increasing amounts of GST-N461 or GST-N461ΔN were immobilized on glutathione-Sepharose and subjected to the cofilin-phosphatase assay, by incubating with P-cofilin at 30 °C for 2 h. The CS mutant of each truncated SSH1 was used as a negative control. The reaction mixtures were separated on SDSPAGE and analyzed by CBB and Pro-Q staining. The bottom panel shows relative P-cofilin levels, with the value of P-cofilin in experiments without GST-N461 taken as 100%. Data are the means ± S.D. of three independent experiments. B, phosphatase activity. GST-N461 and GST-N461ΔN prepared in A were in parallel subjected to pNPP-phosphatase assay, by incubating with pNPP at 30 °C for 1 h. pNPP hydrolysis was analyzed by measuring A₄₀₅. Data are the means ± S.D. of three independent experiments.
Phosphate Group on Ser-3 of Cofilin Is Required for Its Binding to Slingshot—To define the region(s) of SSH1 involved in P-cofilin binding, we constructed a set of deletion mutants of GST-tagged SSH1(CS) and analyzed their ability to bind P-cofilin by GST pull-down assays (Fig. 2A). P-cofilin coprecipitated with full-length SSH1(CS)-GST, but not with the SSH-N domain-deleted mutant [ΔN(CS)-GST], indicating that the SSH-N domain is required for P-cofilin binding (Fig. 2B). In contrast, a C-terminally deleted mutant [GST-N461(CS)] coprecipitated P-cofilin (Fig. 2C), indicating that the C-terminal region (amino acids 462–1049) is dispensable for P-cofilin binding. Similar results were obtained when we tested the P-cofilin binding ability of the deletion mutants of GST-N461(CS). The A domain-deleted mutant [N461ΔA(CS)] coprecipitated P-cofilin, but neither the B domain-deleted mutant [N461ΔB(CS)] nor the SSH-N domain-deleted mutant [N461ΔN(CS)] coprecipitated P-cofilin (Fig. 2C), indicating that the B domain, but not the A domain, is essential for P-cofilin binding. Similar results were obtained when we tested the P-cofilin binding ability of the deletion mutants of C-terminally GST-tagged N461(CS) [N461(CS)-GST] (supplemental Fig. S2), indicating that the position of the GST tag did not affect the binding ability of the N461(CS) mutants. Since the SSH-N domain alone (GST-N) failed to bind P-cofilin, as demonstrated in Fig. 2D, the phosphatase (P) domain is also required for SSH1(CS) binding to P-cofilin. It is likely that the binding of the phosphate group of P-cofilin to the active site in the catalytic P domain is essential to form the stable enzyme-substrate complex. Taken together, these results indicate that both the noncatalytic B domain and the catalytic P domain are required for SSH1 to recognize P-cofilin as a specific substrate.
Mechanism of Cofilin Dephosphorylation by Slingshot

The SSH-N Domain Is Required for Cofilin-phosphatase Activity, but Not for pNPP-phosphatase Activity—We next examined the role of the N-terminal, noncatalytic region of SSH1 in the phosphatase activity toward P-cofilin, as well as the basal phosphatase activity toward pNPP, an artificial substrate generally used in phosphatase assays. Increasing amounts of GST-tagged N461 and N461ΔN immobilized on glutathione beads were subjected to the phosphatase assay, using P-cofilin or pNPP as substrates. Dephosphorylation of P-cofilin was measured by Pro-Q staining. GST-N461 dose-dependently dephosphorylated P-cofilin, but GST-N461ΔN was unable to dephosphorylate P-cofilin even at the highest dose (Fig. 3A), indicating that the SSH-N domain is essential for the cofilin-phosphatase activity of SSH1. In contrast, both GST-N461 and GST-N461ΔN dephosphorylated pNPP with similar dose-dependent rates (Fig. 3B). This suggests that N461ΔN (consisting of only the catalytic P domain of SSH1) retains basal pNPP-phosphatase activity. Thus, the N domain appears to be involved in the cofilin-specific phosphatase activity of SSH1, but not for the basal pNPP-phosphatase activity.

F-actin Strongly Enhances the Cofilin-phosphatase Activity of SSH1—We previously showed that the cofilin-phosphatase activity of SSH1 was markedly enhanced by association with F-actin (12). To further evaluate this finding quantitatively, we performed kinetic analyses of the cofilin-phosphatase activity of SSH1 in the absence or presence of F-actin, using purified (Myc + His)-tagged SSH1 expressed in insect cells. Consistent with previous reports (12–15), SSH1(WT) displayed weak cofilin-phosphatase activity in the absence of F-actin, however, addition of F-actin dramatically accelerated the rate of SSH1-catalyzed P-cofilin dephosphorylation (Fig. 4A, B, and supplemental Fig. S3). In the absence of F-actin, 500 ng of SSH1 decreased the P-cofilin level to 55–70% after a 60-min reaction, while 50 ng of SSH1 had no detectable effect on P-cofilin (Fig. 4B and supplemental Fig. S3B). In contrast, in the presence of F-actin, 5 ng of SSH1 decreased the P-cofilin level to 52% at 5 min, and 50 or 500 ng of SSH1 almost completely dephosphorylated P-cofilin at 5 min (Fig. 4B and supplemental Fig. S3B). From these kinetic plots, it was estimated that the cofilin-phosphatase activity of SSH1 increased more than 1,200-fold in the presence of F-actin.

To examine the possibility that F-actin might activate the cofilin-phosphatase activity of SSH1 by sequestering the reaction product (deP-cofilin), we tested the effect of phalloidin-decorated F-actin on SSH1-catalyzed cofilin dephosphorylation (Fig. 4, C and D). Phalloidin suppressed the binding of deP-cofilin to F-actin, as reported (27), but had no apparent effect on the SSH1 binding to F-actin (Fig. 4C). Addition of phalloidin did not affect the F-actin-induced enhancement of cofilin-phosphatase activity of SSH1 (Fig. 4D). In addition, G-actin, which also has the cofilin-binding activity, did not affect the cofilin-phosphatase activity of SSH1 is not due to the sequestration of the reaction product (deP-cofilin) by F-actin.

F-actin Only Weakly Increases the pNPP-phosphatase Activity of SSH1—MKP-3, a member of the DSPs, is activated by binding to its substrate extracellular signal-regulated kinase (ERK). Upon binding to ERK, the phosphatase activity of MKP-3 toward pNPP is up-regulated by 30-fold (17). To examine the mechanism of the F-actin-mediated activation of the cofilin-phosphatase activity of SSH1, we analyzed whether or not F-actin affects the pNPP-phosphatase activity of SSH1. When purified SSH1(WT or CS) was subjected to a pNPP-phosphatase assay in the absence of F-actin, SSH1(WT) almost completely dephosphorylated pNPP at 5 min (Fig. 5C), as we reported previously (12). These results indicate that the F-actin-mediated enhancement of cofilin-phosphatase activity of SSH1 is not due to the sequestration of the reaction product (deP-cofilin) by F-actin.

FIGURE 5. Effects of F-actin on the pNPP-phosphatase activity of SSH1. Time-dependent hydrolysis of pNPP by purified SSH1-(Myc + His) was determined by measuring A_{405} every 15 min on a microplate reader. Data points are the means of duplicate determinations. A, dose-dependent hydrolysis of pNPP by SSH1(WT or CS)-(Myc + His). B, activation of the pNPP-phosphatase activity of SSH1(WT) by F-actin. The pNPP-phosphatase activity of 2 μg of SSH1-(Myc + His) with or without F-actin was determined. C, effects of F-actin on the pNPP-phosphatase activity of SSH1(WT) and SSH1(W458A). D, fold-activation of pNPP-phosphatase activity of SSH1(WT) and SSH1(W458A). Fold-activation was calculated by dividing the linear reaction rates in the presence of indicated concentrations of F-actin by those in the absence of F-actin. Data are the means ± S.D. of at least three independent experiments.
concentration increased (Fig. 5, B and D). The fold-activation of the pNPP-phosphatase activity by F-actin was calculated by dividing the linear reaction rate in the presence of F-actin by the rate in the absence of F-actin. The plots of fold-activation against F-actin concentration showed that F-actin increased the pNPP-phosphatase activity of SSH1 by only \(2.2\)-fold (Fig. 5D). The marked difference between the extents of F-actin-mediated catalytic activation toward pNPP (\(2.2\)-fold) and P-cofilin (\(1,200\)-fold) suggests that F-actin predominantly affects the cofilin-directed phosphatase activity, but not the basal pNPP-phosphatase activity, of SSH1. We also examined the pNPP-phosphatase activity of SSH1(W458A), a mutant that is not activated by F-actin in cofilin-phosphatase assays (13). The pNPP-phosphatase activity of SSH1(W458A) was not significantly increased by F-actin (Fig. 5, C and D), which indicates that Trp-458 is involved in the F-actin-mediated activation of SSH1 in both cofilin-phosphatase and pNPP-phosphatase activities.

The A Domain Is Required for F-actin-induced SSH1 Cofilin-phosphatase Activation—We next examined the roles of the A and B domains in the regulation of the cofilin-phosphatase activity of SSH1, using the deletion mutants of GST-N461. In accordance with the results of the P-cofilin binding studies (Fig. 2C), N461ΔA, but not N461ΔB, exhibited cofilin-phosphatase activity (Fig. 6A). N461ΔA dose-dependently dephosphorylated P-cofilin, while N461ΔB failed to dephosphorylate it even at the highest dose (supplemental Fig. S4). These results indicate that the B domain, but not the A domain, is required for the cofilin-phosphatase activity of SSH1. To determine if the A domain is involved in the F-actin-mediated activation of SSH1, we analyzed the cofilin-phosphatase activity of GST-tagged N461 and N461ΔA in the absence or presence of F-actin. As shown in Fig. 6B, F-actin enhanced the cofilin-phosphatase activity of GST-N461, but not of GST-N461ΔA. These results suggest that the A domain is required for the F-actin-mediated activation of SSH1, although it is not essential for the basal cofilin-phosphatase activity (activity in the absence of F-actin).

Phosphatase Activity of SSH1 toward Coronin 1B—Coronin 1B, an actin-regulating protein, was recently reported as a novel substrate for SSH1 (28). To elucidate the substrate specificity of SSH1, we compared the intensities of the phosphatase activity toward P-cofilin and phospho-coronin 1B (P-cor1B). (Myc+His)-tagged P-cor1B was prepared by co-expression with GST-PKCζ, as reported (22), and subjected to an in vitro phosphatase assay with purified SSH1-(Myc+His). Unexpectedly, SSH1(WT) did not induce detectable dephosphorylation of P-cor1B, when measured by immunoblotting with anti-pSerPKC antibody (28) or by phosphoprotein analysis with Pro-Q staining, under the conditions where SSH1 efficiently dephosphorylated P-cofilin in a dose-dependent manner (Fig. 7A). In contrast, calf intestinal alkaline phosphatase (CIP) almost completely dephosphorylated P-cor1B. We also examined if SSH1 could dephosphorylate P-cor1B in the presence of F-actin. However, even in the presence of F-actin, SSH1(WT) exhibited no phosphatase activity toward P-cor1B, whereas the A domain is not required for the basal cofilin-phosphatase activity, but is required for the F-actin-mediated activation of SSH1. A, GST-N461 and its mutants were subjected to cofilin-phosphatase assays in the absence of F-actin. P-cofilin dephosphorylation was evaluated by Pro-Q staining, as in Fig. 3A (left). Relative P-cofilin levels (%) are shown as the means \pm S.D. of three independent experiments (right). B, GST-N461 and GST-N461ΔA were subjected to cofilin-phosphatase assays in the absence or presence of \(4 \mu M\) F-actin. P-cofilin dephosphorylation was evaluated as in A. Relative P-cofilin levels (%) are shown in the graphs as the means \pm S.D. of four independent experiments.

FIGURE 6. The A domain is not required for the basal cofilin-phosphatase activity, but is required for the F-actin-mediated activation of SSH1. A, GST-N461 and its mutants were subjected to cofilin-phosphatase assays in the absence of F-actin. P-cofilin dephosphorylation was evaluated by Pro-Q staining, as in Fig. 3A (left). Relative P-cofilin levels (%) are shown as the means \pm S.D. of three independent experiments (right). B, GST-N461 and GST-N461ΔA were subjected to cofilin-phosphatase assays in the absence or presence of \(4 \mu M\) F-actin. P-cofilin dephosphorylation was evaluated as in A. Relative P-cofilin levels (%) are shown in the graphs as the means \pm S.D. of four independent experiments.
SSH1(CS) Induces Aberrant Accumulation of F-actin and Cofilin in C2C12 Cells—We previously reported that the expression of SSH1(CS) in mouse C2C12 myoblast cells induces aberrant accumulation of F-actin and that SSH1(CS) colocalizes with the accumulated F-actin (21). To examine whether P-cofilin associates with SSH1(CS) within the cells, CFP-tagged SSH1 (WT or CS) was expressed in C2C12 cells, and the subcellular localization of SSH1, F-actin, and P-cofilin was analyzed by fluorescence microscopy (Fig. 8). As reported (21), SSH1(CS)-CFP aberrantly accumulated with F-actin in the cell periphery (Fig. 8A), while SSH1(WT)-CFP was diffusely distributed in the cytoplasm. Immunostaining with anti-P-cofilin antibody revealed that P-cofilin also accumulated with SSH1(CS) in SSH1(CS)-expressing cells, although it was uniformly distributed in the cytoplasm in cells expressing control CFP or SSH1(WT)-CFP (Fig. 8B). Quantitative analysis showed that over 80% of cells expressing SSH1(CS) displayed aberrant accumulation of F-actin and P-cofilin that were colocalized with SSH1(CS) (bottom panels in Fig. 8, A and B). These observations suggest that SSH1(CS) forms a stable enzyme-substrate complex with P-cofilin on F-actin in cultured cells. SSH1(CS), which failed to bind P-cofilin in an in vitro binding assay (Fig. 2B), did not induce aberrant accumulation of F-actin or P-cofilin in C2C12 cells (Fig. 8, A and B), indicating that the SSH-N domain is involved in the SSH1(CS)-induced aberrant accumulation of P-cofilin and F-actin in C2C12 cells.

The SSH-N Domain Is Involved in the Localization of SSH1 in the Rear of the Lamellipodium—To further examine the role of the SSH-N domain in cells, we analyzed the subcellular localization of SSH1-(Myc+His) and its deleted mutants in N1E-115 neuroblastoma cells by immunostaining with anti-Myc antibody (Fig. 9). N1E-115 cells that were transfected with active RacV12 generated the lamellipodial extension around the cell (29). When full-length SSH1(WT)-(Myc+His) was coexpressed with RacV12 in N1E-115 cells, SSH1-(Myc+His) colocalized with F-actin in the lamellipodium, and, in particular, accumulated in the rear of the lamellipodium (Fig. 9A), as we previously reported in chemokine-stimulated Jurkat T cells (13). The N-terminal fragment N461-(Myc+His) also accumulated in the rear of the lamellipodium (Fig. 9A). In contrast, an N-terminally deleted mutant PC-(Myc+His) (corresponding to amino acid residues 284–1049) uniformly distributed throughout the lamellipodium (Fig. 9A). Fig. 9B shows the quantitative analysis of the percentages of the cells, in which SSH1 or its deleted mutants were concentrated in the rear of the lamellipodium. These observations suggest that the SSH-N domain is required for the localization of SSH1 in the rear of the lamellipodium.

DISCUSSION

In this study, we examined the molecular mechanisms underlying the substrate specificity and F-actin-mediated catalytic activation of SSH1. Fig. 10 summarizes the results obtained in this study and previous studies. Substrate-trapping and cofilin-phosphatase assays revealed that both the noncatalytic B domain and the catalytic P domain are indispensable for P-cofilin recognition and basal cofilin-phosphatase activity of SSH1 (Figs. 2 and 6). The conserved Cys residue (Cys-393) in the active site signature motif in the P domain of SSH1 probably attacks the Ser-3-phosphate group of P-cofilin, in a manner similar to the mechanism proposed for other PTPs (1, 2). Because both the SSH-N domain and the P-domain alone failed to bind P-cofilin in the GST pull-down assay (Fig. 2), it is likely that the B and P domains of SSH1 individually have relatively weak binding ability toward P-cofilin, and that they cooperatively function to interact with P-cofilin. This is in contrast to the case of MKP-3, in which the N-terminal MAPK-binding domain tightly binds to its target ERK1/2 by itself (18). Because N461AN (composed of the P domain alone) exhibited pNPP-phosphatase activity with a level similar to that of N461, the SSH-N domain does not significantly contribute to the basal pNPP-phosphatase activity of SSH1, and the P domain probably retains the conformation required for the basal phosphatase activity by itself. Considering that the B domain is required for cofilin-phosphatase activity, but not for pNPP-phosphatase activity, the B domain presumably functions to recognize P-cofilin and orientate its Ser-3 phosphate group to a position suit-
able for recognition by the active site of the P-domain for the dephosphorylation reaction. The B domain may also alter the conformation of the P-domain to one required for exhibiting cofilin-phosphatase activity. Furthermore, the relatively weak binding affinity of the B domain of SSH1 to P-cofilin allows rapid substrate release after the dephosphorylation reaction, which may be important for the effective and continuous supply of active, free cofilin, leading to the rapid turnover of actin filaments.

Upon binding to F-actin, SSH1 undergoes marked activation of the cofilin-phosphatase activity. We previously showed that Trp-458 is critical for both F-actin-binding and F-actin-mediated activation of SSH1 (13, 15). In this study, we showed that the A domain is also essential for the F-actin-dependent SSH1 activation, but not for the F-actin-independent, basal cofilin-phosphatase activity (Fig. 6). It remains unknown whether the A domain directly interacts with F-actin to enhance cofilin-phosphatase activity or indirectly affects F-actin sensitivity of the B and P domains. We showed that F-actin increased more than 1200-fold the cofilin-phosphatase activity of SSH1, whereas it increased only 2.2-fold its pNPP-phosphatase activity (Figs. 4 and 5). These findings suggest that F-actin does not dramatically change the configuration of the active site in the P domain that is required for pNPP hydrolysis. Decoration of F-actin with phalloidin inhibited the binding of deP-cofilin to F-actin, but had no apparent effect on the F-actin-induced SSH1 activation, indicating that the activation is not due to the sequestration of the reaction product by F-actin. Thus, the marked increase in the cofilin-phosphatase activity of SSH1 is probably caused by an F-actin-induced change in the conformation of the SSH-N and P domains to one suitable for efficient P-cofilin dephosphorylation. The A domain and Trp-458 probably contribute to the F-actin binding and these conformational changes. Although we have demonstrated that the SSH-N domain has the potential to bind to the P domain,3 it is unlikely that the A domain acts as an autoinhibitory domain, blocking the accessibility of P-cofilin to the catalytic P-domain, because the level of cofilin-phosphatase activity of N461A is similar to that of N461 (Fig. 6A).

MKP-3 (also called Pyst1), a member of the DSPs, is activated by binding of its substrate ERK2 (17). Based on the three-dimensional structural study, a model for ERK2-induced catalytic activation of MKP-3 is proposed (30). Briefly, PTPs and DSPs have an invariant aspartate residue (Asp-262 in human MKP-3) in a so-called general acid loop, and this Asp residue is thought to participate in the dephosphorylation reaction as a proton donor for the substrate (16). In the absence of ERK2, MKP-3 takes an ‘open’ conformation, in which Asp-262 is located 5.5 Å distal to the active site, and has low catalytic activity. It is proposed that upon binding to ERK2, Asp-262 and its associated loop rotate to the position in close proximity to the catalytic Cys residue in the active site, and MKP-3 takes on a closed conformation, making it ready for ERK2 dephosphorylation (30). In contrast, in a DSP family member vaccinia H1-related phosphatase (VHR), this Asp residue-containing loop is close to the active site in its unliganded

3 S. Kurita and K. Mizuno, unpublished results.
SSH1 is positioned in the closed conformation in the absence of F-actin, it is likely that F-actin activates SSH1 by a mechanism distinct from that of MKP-3 by ERK2 binding, i.e. by the closure of the acid loop over the active site. Furthermore, binding of ERK2 increased the pNPP-phosphatase activity of MKP-3 by up to 30-fold (17). In contrast, F-actin stimulates the pNPP-phosphatase activity of SSH1 only by 2.2-fold, whereas it stimulates cofilin-phosphatase activity by ~1200-fold. These observations further support the hypothesis that the mechanism for F-actin-mediated SSH1 activation is distinct from that for MKP-3 activation. SSH1 appears to have a unique activation mechanism, in which the association with F-actin induces a conformational change in the N-terminal region that results in the adoption of an optimal conformation for efficient P-cofilin dephosphorylation.

A recently published study showed that coronin 1B is a substrate for SSH1 (28). However, in our in vitro phosphatase assay, SSH1, in either the absence or presence of F-actin, failed to dephosphorylate coronin 1B, under conditions where P-cofilin was effectively dephosphorylated (Fig. 7). Thus, at least in our in vitro phosphatase assay conditions, SSH1 prefers cofilin to coronin 1B, as a substrate. Further studies are required to understand the extent to which SSH1-mediated coronin 1B dephosphorylation contributes to the regulation of actin filament dynamics in cells.

Fluorescence microscopic analysis revealed that the expression of SSH1(CS) in C2C12 cells induced aberrant accumulation of F-actin and P-cofilin that colocalized with SSH1(CS) (Fig. 8). Aberrant accumulation of F-actin and SSH1(CS) was also observed in HeLa cells overexpressing SSH1(CS) (9, 10, 33). Considering that SSH1(CS) traps P-cofilin in vitro, the accumulation of P-cofilin is probably caused by the formation of the SSH1(CS)-P-cofilin complex in cells, which induces depletion of active cofilin and, as a consequence, accumulation of actin filaments in cells. Consistent with the results of the in vitro binding assays, SSH1AN(CS), which failed to bind P-cofilin, did not induce aberrant accumulation of P-cofilin or F-actin.
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F-actin. These results suggest that SSH1(CS) has the potential to bind P-cofilin in the cell, and that the SSH-N domain is required for this interaction.

We also showed that SSH1(WT) and N461, but not a PC mutant (deleted of the SSH-N domain), accumulated in the rear of the lamellipodium in RacV12-expressing N1E-115 cells (Fig. 9), which indicates that the SSH-N domain plays a critical role in the localization of SSH1 in the rear of the lamellipodium. For cells to migrate, actin filaments polymerize in the front and depolymerize in the rear of the lamellipodium (34). Cofilin promotes actin filament disassembly in the rear of the lamellipodium and contributes to the lamellipodium extension by supplying actin monomers for polymerization at the front (34, 35). SSH1 accumulated at the rear of the lamellipodium probably contributes to the local activation of cofilin in this region. Thus, the SSH-N domain appears to play a crucial role for the spatially restricted activation of SSH1, which may be important for effectively extending and maintaining lamellipodia.

In summary, our data indicate that the SSH-N domain is critical for P-cofilin recognition, F-actin-mediated activation, and subcellular localization of SSH1. The A and B domains play differential roles in F-actin-mediated activation and P-cofilin recognition, respectively. F-actin predominantly enhances the phosphatase activity of SSH1 toward its physiological substrate P-cofilin, probably by inducing a conformational change in the N-terminal region of SSH1. Future structural studies on the SSH-N and P domains of SSH1 will uncover the molecular mechanisms of regulation of SSH1 activity, including its F-actin-mediated activation.

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