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Identification of multiple actin-binding sites in cofilin-phosphatase Slingshot-1L

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Abstract **Slingshot-1L (SSH1L) is a phosphatase that specifically dephosphorylates and activates cofilin, an actin-severing and -depolymerizing protein. SSH1L binds to and is activated by F-actin in vitro, and co-localizes with F-actin in cultured cells. We examined the F-actin-binding activity, F-actin-mediated phosphatase activation, and subcellular distribution of various mutants of SSH1L. We identified three sites involved in F-actin binding of SSH1L: Trp-458 close to the C-terminus of the phosphatase domain, an LHK motif in the N-terminal region, and an LKR motif in the C-terminal region. These sites play unique roles in the control of subcellular localization and F-actin-mediated activation of SSH1L.**

Keywords: Slingshot; Cofilin; Actin-binding; Phosphatase

1. Introduction

Actin filament dynamics play an essential role in various cell activities, including migration, morphological change and cytokinesis. Cofilin is an actin-binding protein that severs actin filaments and stimulates depolymerization, thereby playing a key role in actin filament dynamics and reorganization [1,2]. Cofilin is inactivated through phosphorylation of Ser-3 by LIM-kinases or related TES-kinases [3–5], and the inactive Ser-3-phosphorylated cofilin (P-cofilin) is reactivated through dephosphorylation by Slingshot (SSH) family protein phosphatases, composed of SSH1L, -2L and -3L in mammals [6,7].

We previously showed that a long form of Slingshot-1 (SSH1L) binds to F-actin in cell-free assays and co-localizes with F-actin in cultured cells [6,7]. We also found that the cofilin-phosphatase activity of SSH1L is markedly enhanced by its association with F-actin [8]. After cell stimulation with growth factors or chemokines, SSH1L is translocated into F-actin-rich lamellipodia where it is activated by F-actin, and consequently dephosphorylates and activates cofilin locally in the lamellipodium [8–10]. The knockdown of SSH1L by RNA interference has been shown to impair directional cell migration [10]. These results suggest that F-actin-binding and F-actin-mediated activation of SSH1L are crucial to directional cell migration through local stimulation of cofilin activity and changes in actin filament dynamics in the lamellipodium [10].

In the present study, we examined the F-actin-binding activity, F-actin-mediated phosphatase activation, and subcellular localization of various deletion and amino acid substitution mutants of SSH1L. Our results suggest that there are at least three F-actin-binding sites in SSH1L and that these sites are differentially involved in the subcellular localization and F-actin-mediated activation of SSH1L.

2. Materials and methods

2.1. Plasmid construction

Expression plasmids encoding C-terminally (Myc+His)-tagged (consisting of a Myc epitope peptide and six His residues) human SSH1L were constructed using the pcDNA3.1/Myc-His(+) vector (Invitrogen), as described [6]. The plasmids encoding deletion mutants of human SSH1L were generated by PCR amplification and subcloning into the pcDNA3.1/Myc-His(+) vector. The plasmids for substitution mutants were constructed using a site-directed mutagenesis kit (Stratagene).

2.2. F-actin co-sedimentation assay

The F-actin co-sedimentation assay was performed as described previously [6,7]. Rabbit skeletal muscle actin (Sigma) was polymerized into F-actin by incubating at 20 °C for 1 h in F-buffer (50 mM Tris–HCl, pH 6.8, 100 mM NaCl, 0.2 mM ATP, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 1 mM dithiothreitol). (Myc+His)-tagged SSH1L mutants, expressed in 293T cells, were purified on Ni-NTA-agarose, eluted with 15 μl of 0.5 M imidazole/50 mM HEPES (pH 7.4), and diluted with 55 μl of F-buffer. The molar concentration of each SSH1L mutant was adjusted, based on the calculated molecular weight and the densitometric intensity of the immunoblot, compared with the amounts of standard SSH1L purified from baculovirus expression system. Co-sedimentation assay was done by adding 30 μl of solution containing about 0.36 μM SSH1L mutants and 0.1 mg/ml BSA to 30 μl of F-buffer containing 20 μM polymerized actin. After incubation for 1 h at 20 °C, the reaction mixture was centrifuged at 100 000 × g for 30 min, and equal portions of the supernatant and pellet were separated by SDS–PAGE, and analyzed by immunoblotting using an anti-Myc monoclonal antibody (9E10; Roche Applied Science) and Amido Black staining.

2.3. Cofilin-phosphatase assay

SSH1L-(Myc+His) and its mutants expressed in 293T cells were immunoprecipitated with anti-Myc antibody and incubated for 1 h at 30 °C with 100 ng cofilin-$(His)_6$ in the presence or absence of 5 μg

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Abbreviations: FL, full-length; P-cofilin, Ser-3-phosphorylated cofilin; SSH, Slingshot; SSH1L, a long form of Slingshot-1; WT, wild-type

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F-actin in 20 μl of F-buffer [8]. Reaction mixtures were separated by SDS–PAGE, and P-cofilin was analyzed by staining with the Pro-Q Diamond phosphoprotein gel stain kit (Invitrogen).

2.4. Cell culture and staining

HeLa and 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. HeLa and 293T cells were transfected, using Lipofectamine (Invitrogen) and FuGene6 (Roche Applied Science), respectively. For cell staining, HeLa cells were fixed and co-stained with anti-Myc antibody (9E10) and rhodamine-phalloidin (Molecular Probes), as described [7].

3. Results

3.1. Identification of F-actin-binding sites of SSH1L

SSH1L has the domain structure, composed of the N-terminal A, B, P (phosphatase) domains, conserved between an SSH family, and the *S* (serine-rich) domain near the C-terminus, conserved between SSH1L and 2L (Fig. 1A). To determine the F-actin-binding site(s) of SSH1L, we constructed expression plasmids coding for a set of deletion mutants of SSH1L (Fig. 1A). The C-terminally (Myc+His)-tagged SSH1L and its mutants were expressed in 293T cells. Immunoblot analyses of cell lysates using an anti-Myc antibody confirmed the expression of SSH1L mutant proteins of the predicted molecular sizes (Fig. 1B). Since the wild-type phosphatase domain fragment [P(WT)] was insoluble, but its phosphatase-dead form [P(CS)] (catalytic Cys-393 replaced by Ser) was soluble (Fig. 1B), we used P(CS) for the F-actin-binding assays. The almost equimolar concentrations of SSH1L-(Myc+His) mutants purified from cell lysates were subjected to an in vitro F-actin co-sedimentation assay (Fig. 1C). The assays revealed that the full-length (FL) SSH1L almost completely co-sedimented with F-actin. In

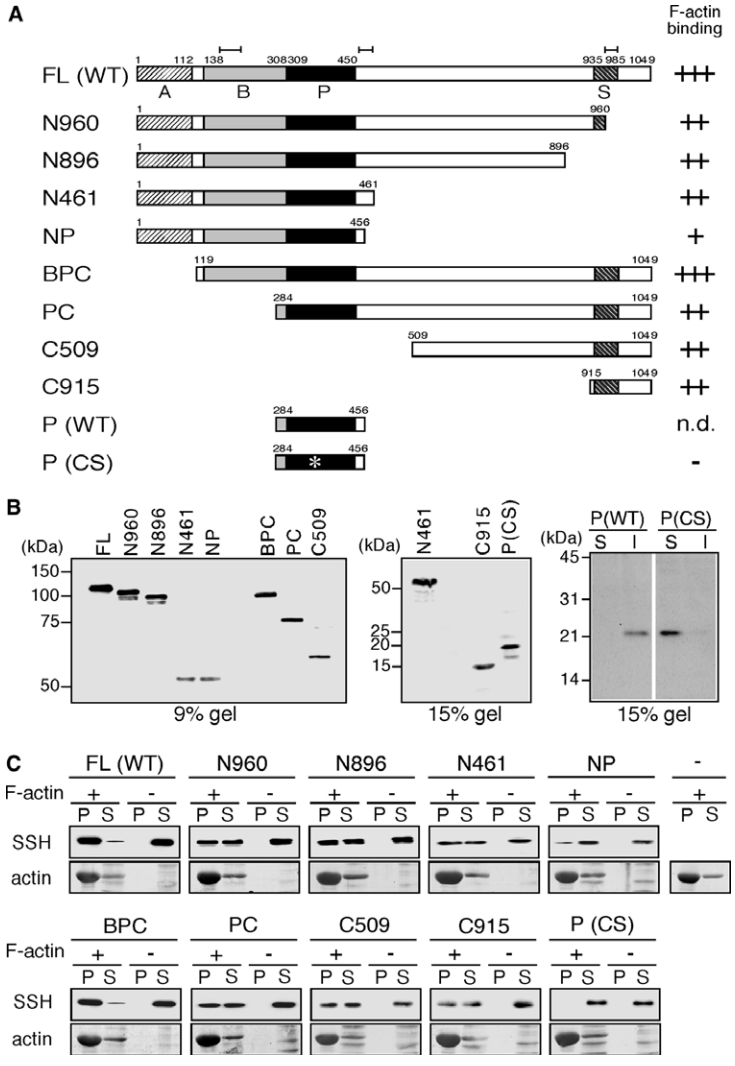

Fig. 1. Identification of F-actin-binding regions of SSH1L. (A) Schematic diagrams of SSH1L and its deletion mutants. The highly conserved regions of SSH family proteins are denoted as A, B, P (phosphatase) and S (serine-rich) domains [7]. Numbers on the bars indicate amino acid residues. An asterisk indicates the site where Cys-393 was replaced by Ser. The relative amounts of SSH1L mutants co-sedimenting with F-actin (estimated from Fig. 1C) are noted at the right of the diagram. n.d., not determined. (B) Expression of SSH1L and its mutants. (Myc+His)-tagged SSH1L and its mutants expressed in 293T cells were separated on 9% and 15% SDS–PAGE and analyzed by immunoblotting with anti-Myc antibody. For cells expressing P(WT) or P(CS), equal portions of soluble (S) and insoluble (I) fractions of the cell lysates were analyzed by immunoblotting (right panel). (C) F-actin-binding assays of SSH1L mutants. SSH1L-(Myc+His) and its mutants expressed in 293T cells were purified and the almost equimolar concentrations of SSH1L mutants were incubated with (+) or without (−) F-actin. After centrifugation, equal amounts of pellet (P) and supernatant (S) fractions were analyzed by immunoblotting with anti-Myc antibody. Actin was detected by Amido black staining.

contrast, F-actin binding of the C-terminal deletion mutants, N960, N896 and N461, was weaker and only about half of these deletion mutant proteins co-sedimented with F-actin. These data suggest that the C-terminal region (amino acids 961–1049) is involved in the F-actin-binding activity of SSH1L. The N-terminal deletion mutant BPC almost completely co-sedimented with F-actin, but only about half of PC and C509 mutants did, which indicates the involvement of the B domain (amino acids 119–283) in F-actin binding of SSH1L. The phosphatase domain fragment [P(CS)] did not bind to F-actin. Interestingly, the F-actin-binding activity of NP was significantly lower than that of N461, which suggests that the short sequence of amino acids 457–461 is involved in F-actin binding of SSH1L. Thus, the B domain (amino acids 119–283), the short sequence near the C-terminus of the phosphatase domain (amino acids 457–461), and the C-terminal region (amino acid residues 961–1049) each appear to be involved in F-actin-binding activity of SSH1L.

To further define the F-actin-binding sites of SSH1L, we constructed various site-directed mutants of SSH1L fragments. To determine the residue(s) essential for F-actin binding of SSH1L in the region of amino acids 456–461, we constructed a set of substitution mutants of N461 and examined their F-actin-binding activity (Fig. 2A). Co-sedimentation assays revealed that only faint amounts of the W458A (Trp-458 replaced by Ala) and 3A (Leu-457, Trp-458 and Arg-459 replaced by Ala) mutants of N461 co-sedimented with F-actin, whereas other substitution mutants bound to F-actin at levels similar to that of N461(WT). This suggests that the Trp-458 residue is critical for F-actin binding of SSH1L.

Previous studies have proposed that motifs homologous to LKHAET in actobindin (Fig. 2B) form a short actin-binding site [11–14]. The LKHAET-like motif is found in many actin-binding proteins, including villin, thymosin-β4, and verprolin [11–14]. On the basis of similarity to this motif, we assumed that LHKACE (amino acids 185–190) in the B domain and LKRSHS (amino acids 973–978) in the S domain of SSH1L might be involved in F-actin binding. Co-sedimentation assays revealed that the F-actin-binding activities of NP(3A) (Leu-185, His-186, and Lys-187 replaced by Ala) and C915(3A) (Leu-973, Lys-974, and Arg-975 replaced by Ala) were significantly decreased, compared to the parental NP and C915 fragments (Fig. 2C). In contrast, the C915(5A) mutant, in which five basic amino acids (Arg-1023, Lys-1030, Lys-1034, Lys-1041, and Lys-1048) near the extremely C-terminus were replaced by Ala, bound to F-actin at a level similar to that of C915(WT), indicating that these basic residues are not required for F-actin binding of C915 (Fig. 2C). Taken together these results suggest that there are at least three sites involved in F-actin binding of SSH1L: Trp-458 in the C-terminal region flanking the P domain, an LHK motif in the B-domain, and an LKR motif in the S domain.

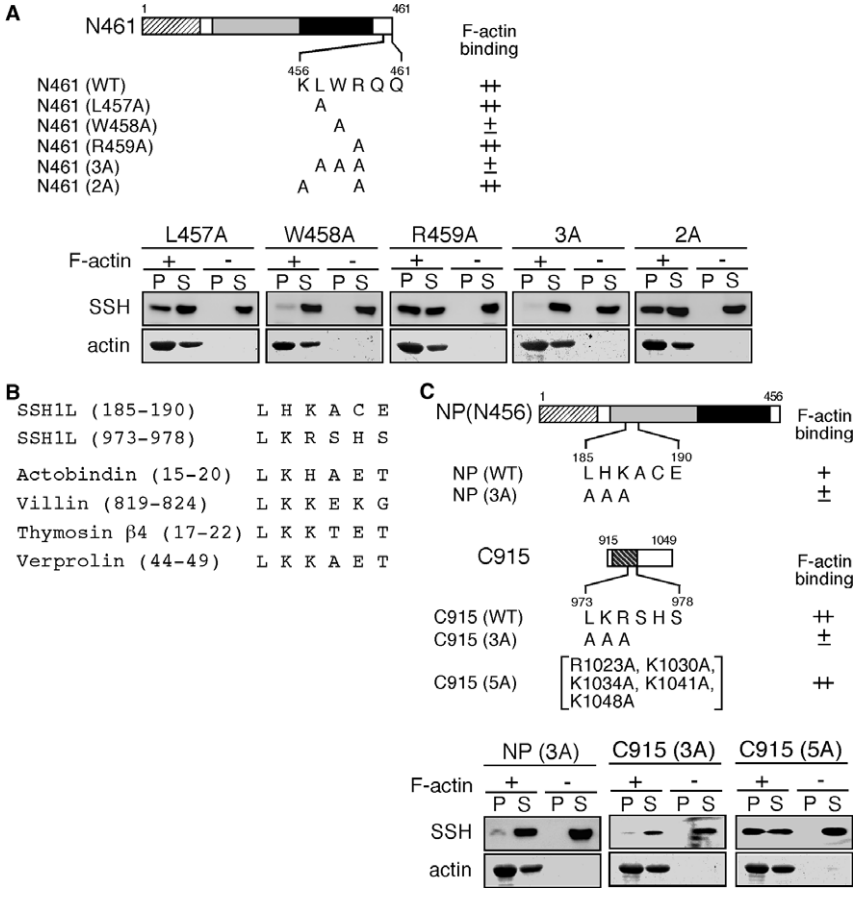

Fig. 2. Identification of amino acid residues of SSH1L involved in F-actin binding. (A) F-actin-binding assays of point mutants of N461. (Myc+His)-tagged N461 point mutants were expressed in 293T cells and analyzed as in Fig. 1C. (B) Alignment of the sequences of the putative actin-binding sites in SSH1L with the LKHAET-like actin-binding motifs in various actin-binding proteins. (C) F-actin binding assays of point mutants of NP and C915. (Myc+His)-tagged NP and C915 point mutants were expressed in 293T cells and analyzed as in Fig. 1C.

3.2. F-actin-mediated activation of SSH1L

We previously showed that SSH1L phosphatase activity is highly stimulated by F-actin [8]. Our data also demonstrated that the phosphatase activity of N960, N698 and N461 were activated by F-actin, but the activity of NP and N461(W458A) was not, indicating that Trp-458 is crucial for F-actin-mediated activation of SSH1L [10]. In this study, we examined whether or not the N-terminally truncated BPC mutant is activated by F-actin. (Myc+His)-tagged SSH1L mutants were subjected to an in vitro cofilin-phosphatase assay in the presence or absence of F-actin, using P-cofilin as a substrate (Fig. 3). As previously reported [8,10], SSH1L(FL) was activated by F-actin, but NP or phosphatase-dead C393S mutant was not. Unexpectedly, BPC was not activated by F-actin, although it retained almost complete F-actin-binding activity. This suggests that the A domain is involved in the process of F-actin-mediated activation.

3.3. Subcellular localization of SSH1L mutants

We next examined the subcellular distribution of SSH1L mutants. (Myc+His)-tagged SSH1L and its mutants were expressed in HeLa cells and the cells were co-stained with an anti-Myc antibody to detect SSH1L mutants and rhodamine-phalloidin to detect F-actin (Fig. 4). As reported [6,7], SSH1L(FL) co-localized well with F-actin and accumulated on the cortical actin bundles at the cell periphery and on stress fibers. In contrast, the C-terminal truncation mutants, N896 (data not shown), and N960 and N461 (Fig. 4), only partially co-localized with actin filaments at the cell periphery. In contrast to N461, N461(W458A) was distributed diffusely in the cytoplasm. This correlates well with the findings of the in vitro F-actin-binding assay (Fig. 2A) and further suggests that Trp-458 plays a critical role in F-actin-binding. NP partially co-localized with F-actin near the plasma membrane at the cell periphery, but NP(3A) diffusely distributed in the cytoplasm, which indicates that the LHK motif in the B-domain plays a role in SSH1L co-localization with F-actin near the plasma membrane. BPC co-localized well with F-actin and accumulated both on the cortical actin bundles and stress fibers, but PC and C509 only partially co-localized with F-actin at the cell periphery, which further implicates the B domain in F-actin association. In contrast to C509, C915 co-localized well with stress fibers, which suggests that the region of amino acids 509–914 may have some inhibitory effect on the localization of C915 on stress fibers. P(CS) was diffusely distributed in the cytoplasm, whereas P(WT) was detected as speckles in the cytoplasm. Since P(WT) was not solubilized by cell lysis (Fig. 1B), the punctate distribution is likely to represent insoluble aggregates within the cell.

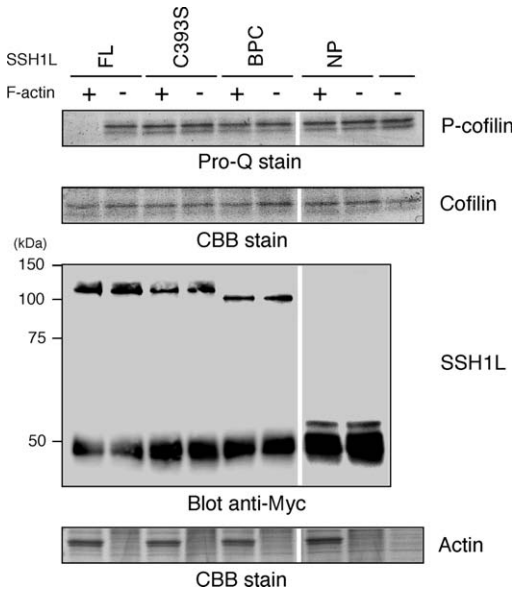

Fig. 3. F-actin-mediated activation of SSH1L and its mutants. (Myc+His)-tagged SSH1L mutants were precipitated with anti-Myc antibody and subjected to an in vitro phosphatase assay, using $(His)_6$-cofilin as a substrate, in the presence or absence of F-actin. Cofilin-phosphatase activity was assessed by Pro-Q and Coomassie brilliant blue (CBB) staining of *P*-cofilin and cofilin, respectively. SSH1L mutants and actin in the reaction mixtures were analyzed with anti-Myc and CBB staining.

4. Discussion

Our data demonstrate that SSH1L has at least three F-actin-binding sites in different domains of the protein. Characterization of various SSH1L mutants indicates that these actin-binding sites play different roles in regulating the subcellular localization and F-actin-mediated activation of SSH1L. The C-terminal deletion mutants (N960, N896, N698 and N461) had reduced actin-binding activity and only partially co-localized with F-actin, but were fully activated by F-actin [10 and in this study]. These data indicate that the LKR actin-binding motif in the C-terminal region is involved in the localization of SSH1L to actin filaments in cells but does not contribute to the process of F-actin-induced phosphatase activation. In contrast, Trp-458 near the C-terminus of the phosphatase domain is indispensable for F-actin-mediated activation of SSH1L [10]. Because NP, but not NP(3A), has weak F-actin-binding activity and co-localized with F-actin near the plasma membrane, the LHK motif in the B domain is likely involved in the localization of SSH1L to F-actin near the plasma membrane. Interestingly, BPC was not activated by F-actin, even though it displayed almost complete F-actin-binding activity. Since BPC retained the basal cofilin-phosphatase activity (activity in the absence of F-actin), the A domain is required for F-actin-mediated activation, but not for basal activity. The A domain may be involved in F-actin-mediated activation through co-operative interactions with Trp-458.

We previously showed that 14-3-3 proteins bind to SSH1L, and this binding required the phosphorylation of Ser-937 and Ser-978 of SSH1L [8]. In unstimulated cells, 14-3-3 proteins negatively regulate SSH1L activity by sequestering it in the cytoplasm and preventing F-actin-mediated activation [8]. Since the LKR actin-binding motif in the S domain is very close to one of the 14-3-3 binding sites (Ser-978), it is possible that 14-3-3 proteins sterically hinder SSH1L binding to F-actin.

Overexpression of the phosphatase-dead (CS) form of full-length SSH1L frequently evoked robust assembly of actin filaments within cells [6,7]. Expression of WT SSH1L can induce aberrantly thick actin bundles, as well, although this effect was noted infrequently [7,15]. These data suggest that the full-length SSH1L has F-actin-bundling activity, irrespective of

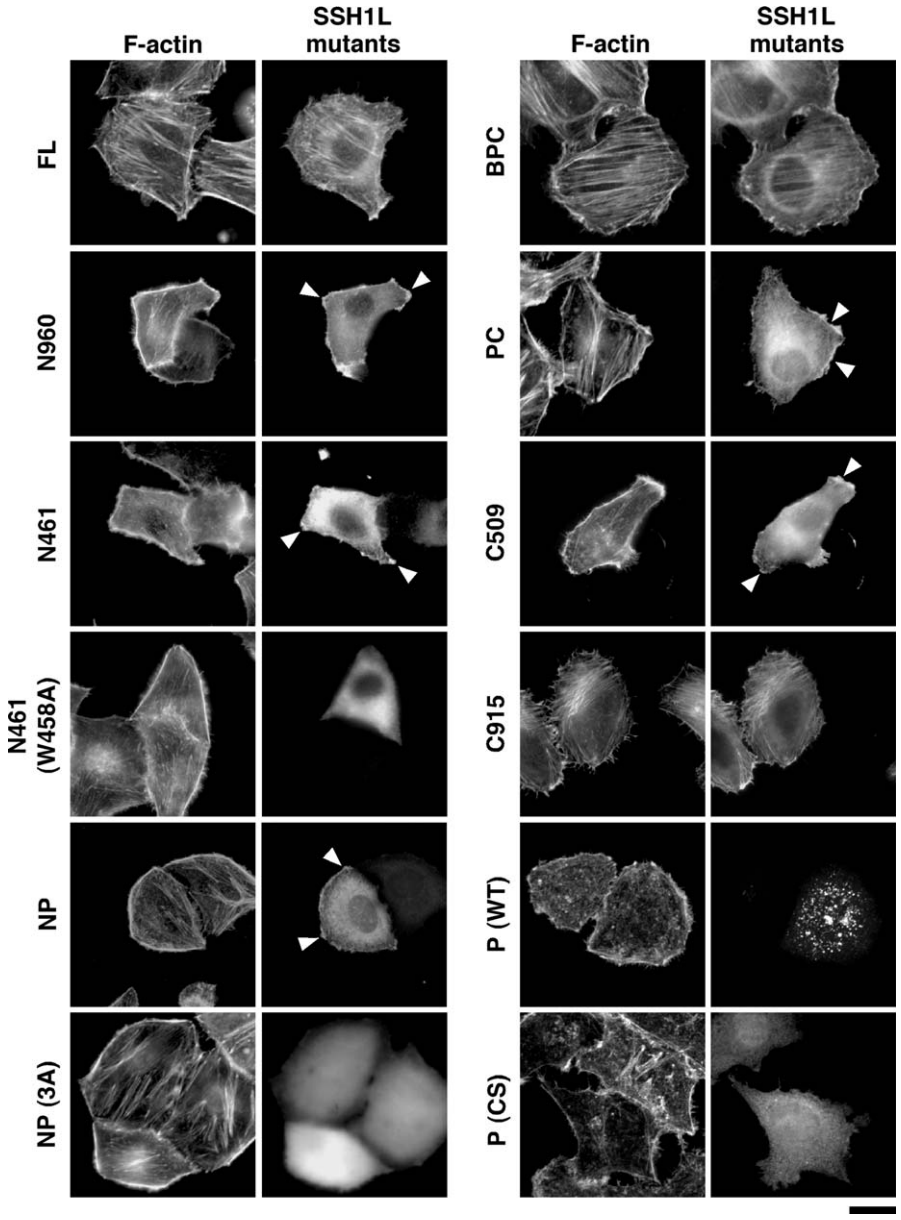

Fig. 4. Subcellular localization of SSH1L and its mutants. (Myc+His)-tagged SSH1L mutants were expressed in HeLa cells. Cells were co-stained with anti-Myc for SSH1L mutants (right panels) and rhodamine-phalloidin for F-actin (left panels). Arrowheads in panels for N960, N461, NP, PC and C509 indicate the positions where SSH1L mutants co-localize with F-actin. Scale bar, 10 μm.

cofilin-phosphatase activity. Since the SSH1L deletion mutants, such as N461, NP, and C508, did not induce such phenotypes, multiple F-actin-binding sites in SSH1L may be related to the F-actin-bundling activity of SSH1L.

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References

[1] Bamburg, J.R., McGough, A. and Ono, S. (1999) Putting a new twist on actin: ADF/cofilins modulate actin dynamics. Trends Cell Biol. 9, 364–370.

[2] Pollard, T.D. and Borisy, G.G. (2003) Cellular motility driven by assembly and disassembly of actin filaments. Cell 112, 453–465.

[3] Yang, N., Higuchi, O., Ohashi, K., Nagata, K., Wada, A., Kangawa, K., Nishida, E. and Mizuno, K. (1998) Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. Nature 393, 809–812.

[4] Arber, S., Barbayannis, F.A., Hanser, H., Schneider, C., Stanyon, C.A., Bernard, O. and Caroni, P. (1998) Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. Nature 393, 805–809.

[5] Toshima, J., Toshima, J.Y., Amano, T., Yang, N., Narumiya, S. and Mizuno, K. (2001) Cofilin phosphorylation by testicular protein kinase 1 and its role in integrin-mediated actin reorganization and focal adhesion formation. Mol. Biol. Cell 12, 1131–1145.

[6] Niwa, R., Nagata-Ohashi, K., Takeichi, M., Mizuno, K. and Uemura, T. (2002) Control of actin reorganization by Slingshot, a family of phosphatases that dephosphorylate ADF/cofilin. Cell 108, 233–246.

[7] Ohta, Y., Kousaka, K., Nagata-Ohashi, K., Ohashi, K., Muramoto, A., Shima, Y., Niwa, R., Uemura, T. and Mizuno, K. (2003) Differential activities, subcellular distribution and tissue expression patterns of three members of Slingshot family phosphatases that dephosphorylate cofilin. Genes Cells 8, 811–824.
[8] Nagata-Ohashi, K., Ohta, Y., Goto, K., Chiba, S., Mori, R., Nishita, M., Ohashi, K., Kousaka, K., Iwamatsu, A., Niwa, R., Uemura, T. and Mizuno, K. (2004) A pathway of neuregulin-induced activation of cofilin-phosphatase Slingshot and cofilin in lamellipodia. J. Cell Biol. 165, 465–471.
[9] Nishita, M., Wang, Y., Tomizawa, C., Suzuki, A., Niwa, R., Uemura, T. and Mizuno, K. (2004) Phosphoinositide 3-kinase-mediated activation of cofilin phosphatase Slingshot and its role for insulin-induced membrane protrusion. J. Biol. Chem. 279, 7193–7198.
[10] Nishita, M., Tomizawa, C., Yamamoto, M., Horita, Y., Ohashi, K. and Mizuno, K. (2005) Spatial and temporal regulation of cofilin activity by LIM kinase and Slingshot is critical for directional cell migration. J. Cell Biol. 171, 349–359.
[11] Vancompernolle, K., Vandekerckhove, J., Bubb, M.R. and Korn, E.D. (1991) The interfaces of actin and *Acanthamoeba* actobindin. Identification of a new actin-binding motif. J. Biol. Chem. 266, 15427–15431.
[12] Friederich, E., Vancompernolle, K., Huet, C., Goethals, M., Finidori, J., Vandekerckhove, J. and Louvard, D. (1992) An actin-binding site containing a conserved motif of charged amino acid residues is essential for the morphogenic effect of villin. Cell 70, 81–92.
[13] Van Troys, M., Dewitte, D., Goethals, M., Carlier, M.F., Vandekerckhove, J. and Ampe, C. (1996) The actin binding site of thymosin β4 mapped by mutational analysis. EMBO J. 15, 201–210.
[14] Vaduva, G., Martin, N.C. and Hopper, A.K. (1997) Actin-binding verprolin is a polarity development protein required for the morphogenesis and function of the yeast actin cytoskeleton. J. Cell Biol. 139, 1821–1833.
[15] Soosairajah, J., Maiti, S., Wiggan, O., Sarmiere, P., Moussi, N., Sarcevic, B., Sampath, R., Bamburg, J.R. and Bernard, O. (2005) Interplay between components of a novel LIM kinase-slingshot phosphatase complex regulates cofilin. EMBO J. 24, 473–486.