Calcium Signal-induced Cofilin Dephosphorylation Is Mediated by Slingshot via Calcineurin*

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Cofilin, an essential regulator of actin filament dynamics, is inactivated by phosphorylation at Ser-3 and reactivated by dephosphorylation. Although cofilin undergoes dephosphorylation in response to extracellular stimuli that elevate intracellular Ca2+ concentrations, signaling mechanisms mediating Ca2+-induced cofilin dephosphorylation have remained unknown. We investigated the role of Slingshot (SSH) 1L, a member of a SSH family of protein phosphatases, in mediating Ca2+-induced cofilin dephosphorylation. The Ca2+ ionophore A23187 and Ca2+-mobilizing agonists, ATP and histamine, induced SSH1L activation and cofilin dephosphorylation in cultured cells. A23187- or histamine-induced SSH1L activation and cofilin dephosphorylation were blocked by calcineurin inhibitors or a dominant-negative form of calcineurin, indicating that calcineurin mediates Ca2+-induced SSH1L activation and cofilin dephosphorylation. Importantly, knockdown of SSH1L expression by RNA interference abolished A23187- or calcineurin-induced cofilin dephosphorylation. Furthermore, calcineurin dephosphorylated SSH1L and increased the cofilin-phosphatase activity of SSH1L in cell-free assays. Based on these findings, we suggest that Ca2+-induced cofilin dephosphorylation is mediated by calcineurin-dependent activation of SSH1L.

Actin cytoskeletal reorganization is essential for numerous cell activities, including migration, morphological change, and vesicle transport. The actin-depolymerizing factor/cofilin family proteins are key regulators for actin filament dynamics and reorganization, with potential to bind to actin monomers and filaments and stimulate depolymerization and severance of actin filaments (1–4). These activities of actin-depolymerizing factor/cofilin (henceforth referred to as cofilin) are negatively regulated by phosphorylation at Ser-3 and reactivated by dephosphorylation (5, 6). Cofilin undergoes phosphorylation and dephosphorylation in response to various extracellular stimuli that trigger changes in the actin cytoskeleton (1–4). Given the essential role of cofilin in actin filament dynamics, it is important to elucidate signal transduction mechanisms that regulate cofilin phosphorylation and dephosphorylation in order to better understand stimulus-induced actin cytoskeletal remodeling and the cell activities related to it.

LIM kinases (LIM kinases 1 and 2) and testicular protein kinases (testicular protein kinases 1 and 2), which specifically phosphorylate cofilin at Ser-3, have been identified as mediators of cofilin phosphorylation (7–11). LIM kinases are activated after stimulation of cells by extracellular cues, such as lysophosphatidic acid (12) and stromal cell-derived factor (13), through Rho-family GTPases, Rho, Rac, and Cdc42, and their downstream protein kinases, such as ROCK and PAK (7–9, 12–15). In contrast, testicular protein kinase 1 is activated downstream of the integrin-mediated signaling pathway (10). Thus, cofilin phosphorylation/inactivation is induced by multiple pathways, all of which are dependent on extracellular cues.

As for phosphatases responsible for cofilin dephosphorylation, genetic and biochemical analyses have led to identification of a Slingshot (SSH) family of protein phosphatases (SSH in Drosophila and SSH1L, SSH2L, and SSH3L in mammals), which can specifically dephosphorylate and reactivate an inactive Ser-3-phosphorylated cofilin (P-cofilin), both in vitro and in vivo (16–19). Cofilin dephosphorylation is induced in response to various extracellular stimuli in many different cell types (1–4, 20–30). Although multiple signaling pathways, including those involving Ca2+, cAMP, and phosphoinositide 3-kinase, have been proposed for stimulus-induced cofilin dephosphorylation (20–28), neither downstream signaling pathways nor the roles of SSHs in stimulus-induced cofilin dephosphorylation have been determined. We recently showed that SSH1L is activated downstream of phosphoinositide 3-kinase in insulin-stimulated cells, but the signaling pathway linking phosphoinositide 3-kinase and SSH1L has remained unknown (29). We also showed that the cofilin-phosphatase activity of SSH1L is increased by association with actin filaments and suppressed by 14-3-3 proteins (30). However, the mechanisms regulating SSH1L activity through F-actin and 14-3-3 proteins are not well understood.

In the present study, we investigated the signaling mechanism of Ca2+ signal-induced cofilin dephosphorylation. Previous studies reported that elevation of the intracellular Ca2+ concentration stimulates cofilin dephosphorylation in a variety of cell types (20–24), and this dephosphorylation is blocked by inhibitors of calcineurin, a Ca2+/calmodulin-dependent protein phosphatase (also called protein phosphatase 2B), thus indicating that calcineurin is involved in Ca2+-induced cofilin dephosphorylation (23, 31). However, it remained to be determined whether or not calcineurin directly dephosphorylates cofilin or merely mediates cofilin dephosphorylation in cells through ac-

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1 The abbreviations used are: SSH, Slingshot; CnA, calcineurin A β-isotype; HA, hemagglutinin; P-cofilin, Ser-3-phosphorylated cofilin; siRNA, small interfering RNA; WT, wild-type.
tivation of a cofilin-specific phosphatase, such as SSH1L. Here we now provide evidence that Ca\textsuperscript{2+}-induced cofilin dephosphorylation is mediated by SSH1L via calcineurin.

**EXPERIMENTAL PROCEDURES**

**Materials**—A23187, histamine, cyclosporin A, FK506, cypermethrin, calmodulin, and recombinant active calcineurin Aα containing calcineurin B subunit were purchased from Calbiochem, and okadaic acid was purchased from Wako (Osaka, Japan). Mouse monoclonal antibody against Myc epitope (9E10) and rat monoclonal antibody against hemagglutinin (HA) epitope (3F10) were purchased from Roche Diagnostics. Rabbit polyclonal antibodies to P-cofilin, cofilin, and SSH1L were prepared as described previously (10, 19). An anti-β-actin monoclonal antibody (AC-15) was purchased from Sigma. Fluorescein isothiocyanate-conjugated anti-rat IgG and rhodamine-conjugated anti-rabbit IgG antibody were purchased from Chemicon (Temecula, CA).

**Plasmid Construction**—Expression plasmids coding for Myc epitope-tagged wild-type SSH1L (SSH1L(WT)) and phosphatase-dead SSH1L (SSH1L(CS)) were constructed as described previously (16, 17). Plasmids for HA epitope-tagged calcineurin A β-isotype (ChA), its constitutively active (ΔCaH) and dominant-negative (ΔCaH160Q) mutants, and calcineurin B were constructed as described previously (31, 32). pSSH1L plasmid, which allows coexpression of SSH1L and RNAi (SSH1LsiRNA) was provided by Dr. R. Agami (The Netherlands Cancer Institute, Amsterdam, The Netherlands) (33). An SSH1L siRNA plasmid (pSUPER-SSH1L) that targets the human SSH1L mRNA sequence (TCGTCACCCAAGAAAGATA) was constructed as described previously (29). A mutated SSH1L siRNA plasmid, pSUPER-SSH1L(mt), was constructed by substituting two bases in the target sequence (TCGTCACCAAGAAAGATA).

**Cell Culture and Transfection**—293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. 293T and HeLa cells were transfected with expression plasmids using FuGENE 6 transfection reagent (Roche Applied Science) and Lipofectamine (Invitrogen), respectively. Cells were used for assays after being in culture for 30–44 h.

**Detection of the Level of P-cofilin**—Serum-starved 293T or HeLa cells were cultured twice in phosphate-buffered saline, harvested, and lysed with lysis buffer (50 mM HEPES, pH 7.4, 1% Nonidet P-40, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, and 10 μg/ml leupeptin). After incubation on ice for 30 min, lysates were incubated in Laemmli's sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 1 mM dithiothreitol, 1% SDS, and 0.02% bromphenol blue) for 5 min at 95°C, and aliquots were separated by SDS-PAGE. Proteins were transferred onto polyvinylidene difluoro membranes. The membranes were analyzed by immunoblotting using an anti-P-cofilin and anti-cofilin antibodies. Immunoblot analysis was performed as described previously (15).

**In Vitro Phosphatase Assay of SSH1L**—Cofilin-His\textsubscript{6} expressed in Vero cells was purified and used as a substrate for in vitro phosphatase reactions. 293T or HeLa cells were lysed in phosphate buffer (50 mM HEPES, 1 mM NaCl, 1% Nonidet P-40, 5% glycerol, 1 mM dithiothreitol, and 10 μg/ml leupeptin). 293T and HeLa cells were transfected with expression plasmids using FuGENE 6 transfection reagent (Roche Applied Science) and Lipofectamine (Invitrogen), respectively. Cells were used for assays after being in culture for 30–44 h.

**Dephosphorylation of SSH1L by Calcineurin in Cell-free Assays**—Lysates of 293T cells transfected with plasmids for Myc-SSH1L were immunoprecipitated with anti-Myc antibody or control IgG. The precipitates were incubated for 1 h at 30°C with 7.5 units/μl recombinant active calcineurin Aα containing calcineurin B subunit, 14 μg/ml calmodulin, and 6.3 μg/ml cofilin-His\textsubscript{6} in 20 μl of reaction buffer (50 mM HEPES, pH 7.4, 0.1 mM CaCl\textsubscript{2}, 0.5 mM EDTA, 3 mM MgCl\textsubscript{2}, 100 mM NaCl, 1 mM dithiothreitol, 0.5 mg/ml bovine serum albumin, and 1 μg/ml leupeptin) and then further incubated for 30 min at 30°C in reaction buffer containing 0.25 mg/ml P-actin. Reaction mixtures were separated by SDS-PAGE and analyzed by immunoblotting using antibodies to cofilin, P-cofilin, and SSH1L.

**Ca\textsuperscript{2+} and Calcineurin-dependent Activation of Slingshot**

**RESULTS**

**Ca\textsuperscript{2+} Ionophore Stimulates Cofilin Dephosphorylation and SSH1L Activation**—It was reported that cofilin undergoes dephosphorylation in response to extracellular stimuli that increase the intracellular Ca\textsuperscript{2+} concentration (20–24). To explore signaling pathways of Ca\textsuperscript{2+}-induced cofilin dephosphorylation, we first examined the effects of the Ca\textsuperscript{2+} ionophore A23187 on levels of cofilin phosphorylation in cultured cells. Serum-starved 293T or HeLa cells were exposed to 5 μM A23187 for different time periods, and cell lysates were analyzed by immunoblotting with an anti-P-cofilin antibody that specifically recognizes P-cofilin and an anti-cofilin antibody that recognizes both phosphorylated and non-phosphorylated forms of cofilin (10). Stimulation of 293T or HeLa cells with A23187 significantly decreased the level of P-cofilin in a time-dependent manner, without affecting the total cofilin level (Fig. 1A). A23187-induced cofilin dephosphorylation was also observed in WI-38 human lung fibroblasts (data not shown). These findings suggest that the increase in intracellular Ca\textsuperscript{2+} concentrations induces cofilin dephosphorylation in various types of cells.

We next analyzed changes in cofilin-phosphatase activity of endogenous SSH1L after stimulating the cells with A23187. Serum-starved 293T or HeLa cells were incubated with or without 5 μM A23187 for 10 min, cell lysates were prepared, and SSH1L was immunoprecipitated with an anti-SSH1L antibody. The immunoprecipitates were subjected to an in vitro phosphatase assay, using a recombinant phosphorylated cofilin-His\textsubscript{6} substrate. Cofilin-phosphatase activity was measured by the decrease in P-cofilin immunoreactivity. In both 293T and HeLa cells, the cofilin-phosphatase activity of SSH1L significantly increased after A23187 stimulation (Fig. 1B). These results suggest that Ca\textsuperscript{2+} signaling induces both SSH1L activation and cofilin dephosphorylation.

**Ca\textsuperscript{2+}-mobilizing Agents Induce Cofilin Dephosphorylation and SSH1L Activation**—Extracellular ATP and histamine are known to elevate the intracellular Ca\textsuperscript{2+} concentration by mobilizing Ca\textsuperscript{2+} from internal stores in 293T and HeLa cells, respectively (24). We asked whether these Ca\textsuperscript{2+}-mobilizing agents would induce cofilin dephosphorylation and SSH1L activation in these cells. The level of P-cofilin significantly decreased after exposure of 293T cells to 50 μM ATP (Fig. 2A, top panel). In vitro phosphatase assay showed that the cofilin-phosphatase activity of SSH1L in 293T cells increased after ATP stimulation (Fig. 2A, bottom panel). In a similar manner, stimulation of HeLa cells with 10 mM histamine induced both cofilin dephosphorylation and SSH1L activation (Fig. 2B). Therefore extracellular Ca\textsuperscript{2+}-mobilizing agents do have the potential to induce cofilin dephosphorylation and SSH1L activation.

**Calcineurin Inhibitors Block Ca\textsuperscript{2+}-induced Cofilin Dephosphorylation and SSH1L Activation**—A23187-induced cofilin dephosphorylation was reported to be blocked by calcineurin inhibitors, which suggested that calcineurin is involved in the Ca\textsuperscript{2+}-induced cofilin dephosphorylation (23). However, it is unclear whether or not calcineurin directly dephosphorylates cofilin. We examined the effects of calcineurin inhibitors on A23187- or histamine-induced cofilin dephosphorylation and SSH1L activation in HeLa cells. When HeLa cells were preincubated with calcineurin inhibitors (10 μM cyclosporin A, 50 nM FK506, or 100 nM cypermethrin) and then treated with 5 μM A23187 for 10 min, A23187-induced cofilin dephosphorylation
Ca^2+ /Calcineurin-dependent Activation of Slingshot

Fig. 1. Calcium ionophore A23187 induces cofilin dephosphorylation and SSH1L activation. A, calcium ionophore-induced cofilin dephosphorylation. Serum-starved 293T (left panels) or HeLa (right panels) cells were stimulated with 5 μM A23187 for the indicated times. Cell lysates were immunoprecipitated with anti-P-cofilin and anti-cofilin antibodies. Bottom panels show the relative P-cofilin levels, with the value in non-treated cells taken as 100%. B, calcium ionophore-induced SSH1L activation. Serum-starved 293T or HeLa cells were stimulated with 5 μM A23187 for 10 min. Cell lysates were immunoprecipitated (IP) with anti-SSH1L antibody or control IgG and subjected to an in vitro phosphatase assay, using cofilin-His6 as a substrate. Reaction mixtures were analyzed by immunoblotting with anti-P-cofilin, anti-cofilin, and anti-SSH1L antibodies. Bottom panels show the relative P-cofilin levels after the in vitro cofilin-phosphatase assay, with the value obtained from control IgG precipitates taken as 100%.

Fig. 2. Cofilin dephosphorylation and SSH1L activation in ATP-stimulated 293T cells and histamine-stimulated HeLa cells. A, ATP-induced cofilin dephosphorylation and SSH1L activation in 293T cells. Serum-starved 293T cells were stimulated with 50 μM ATP for the indicated times. Cell lysates were analyzed by immunoblotting with antibodies to P-cofilin and cofilin (top panel). SSH1L was immunoprecipitated and subjected to in vitro phosphatase assay, as described in Fig. 1B (bottom panel). B, histamine-induced cofilin dephosphorylation and SSH1L activation in HeLa cells. Serum-starved HeLa cells were stimulated with 10 μM histamine for the indicated times. Changes in the levels of P-cofilin (top panel) and SSH1L activity (bottom panel) were measured in cells as described in A.

was almost completely blocked in the presence of any one of these calcineurin inhibitors (Fig. 3A, top panel). Interestingly, A23187-induced SSH1L activation was also suppressed by calcineurin inhibitors (Fig. 3A, bottom panel), which indicates that calcineurin is required for the Ca^{2+}-induced SSH1L activation. Both histamine-induced cofilin dephosphorylation and SSH1L activation in HeLa cells were inhibited by pretreatment of the cells with FK506 (Fig. 3B). These findings suggest that Ca^{2+}-induced cofilin dephosphorylation and SSH1L activation are dependent on calcineurin activity and that calcineurin functions upstream of SSH1L. In addition, pretreatment of cells with 1 μM okadaic acid (an inhibitor of protein phosphatases type 1 and 2A) had no apparent effect on histamine-induced cofilin dephosphorylation and SSH1L activation (Fig. 3C), which suggests that neither protein phosphatase type 1 nor 2A is involved in Ca^{2+}-induced cofilin dephosphorylation and SSH1L activation.

Expression of Dominant-negative Calcineurin Suppresses A23187-induced Cofilin Dephosphorylation and SSH1L Activation—To further examine the role of calcineurin in Ca^{2+}-induced cofilin dephosphorylation and SSH1L activation, we overexpressed either HA-tagged wild-type calcineurin A (CnA(WT)) or its dominant-negative form (∆CnA(H160Q)) in HeLa cells and analyzed changes in the levels of P-cofilin and SSH1L activity before and after A23187 treatment. Expression of CnA(WT) alone had no apparent effect, unless cells had been exposed to agents that increased the intracellular Ca^{2+} concentration (32). A23187-induced cofilin dephosphorylation and SSH1L activation were suppressed in cells expressing ∆CnA(H160Q), but not in cells expressing CnA(WT) (Fig. 4). These data further indicate that calcineurin activity is required for Ca^{2+}-induced SSH1L activation and cofilin dephosphorylation.

Phosphatase-inactive SSH1L Suppresses A23187-induced Cofilin Dephosphorylation—To examine the role of SSH1L in Ca^{2+}-induced cofilin dephosphorylation, we overexpressed either SSH1L(WT) or SSH1L(CS) in HeLa cells and analyzed changes in P-cofilin levels before and after A23187 stimulation. In cells expressing SSH1L(WT), the level of P-cofilin decreased significantly even before A23187 stimulation and declined further after stimulation (Fig. 5A). In contrast, in cells expressing SSH1L(CS), a phosphatase-inactive Slingshot-1L mutant in which catalytic Cys is replaced by Ser, A23187-induced cofilin dephosphorylation was significantly suppressed (Fig. 5A). Similar results were obtained when CnA(WT) was co-expressed with either SSH1L(WT) or SSH1L(CS) in HeLa cells. A23187-induced cofilin dephosphorylation in CnA(WT)-expressing cells was stimulated by co-expression of SSH1L(WT) and inhibited by SSH1L(CS) (Fig. 5B, compare lanes 3, 6, and 9). These findings suggest that the phosphatase activity of SSH1L is
critical for Ca$^{2+}$/calcineurin-induced cofilin dephosphorylation and that SSH1L(CS) acts as a dominant-negative mutant.

Suppression of SSH1L Expression by siRNA Inhibits A23187-induced Cofilin Dephosphorylation—To confirm that SSH1L mediates Ca$^{2+}$/calcineurin-induced cofilin dephosphorylation, expression of endogenous SSH1L in HeLa cells was suppressed by transfection of a pSUPER siRNA expression plasmid, pSUPER-SSH1L, which directs the synthesis of an siRNA targeting human SSH1L (29). An empty pSUPER vector and siRNA plasmid mutated in the SSH1L target sequence, pSUPER-SSH1L(mt), were transfected as control experiments. Immunoblot analysis revealed that transfection with pSUPER-SSH1L significantly reduced the level of endogenous SSH1L expression, whereas control vector or mutated siRNA plasmid did not suppress it (Fig. 6A). HeLa cells were co-transfected with CnA(WT) and siRNA plasmids, and changes in P-cofilin levels in cells were analyzed before and after A23187 stimulation. In cells transfected with pSUPER vector and pSUPER-SSH1L(mt), A23187 stimulation induced cofilin dephosphorylation (Fig. 6B, lanes 3 and 9). In contrast, in cells transfected with pSUPER-SSH1L, A23187-induced cofilin dephosphorylation was significantly inhibited (Fig. 6B, lane 6). These observations further suggest that SSH1L plays a critical role in Ca$^{2+}$/calcineurin-induced cofilin dephosphorylation.
induced cofilin dephosphorylation by staining cells with an anti-P-cofilin antibody. HeLa cells transfected with plasmids coding for HA-tagged constitutively active calcineurin A and calcineurin B (ΔCnA/B) with or without pSUPER-SSH1L siRNA plasmids were cultured for 44 h and then co-stained with anti-HA and anti-P-cofilin antibodies. As shown in the left panels of Fig. 7, the P-cofilin level significantly decreased in cells expressing ΔCnA/B (indicated by arrowheads), compared with the levels in neighboring non-expressing cells. In contrast, the P-cofilin levels in cells co-transfected with ΔCnA/B and pSUPER-SSH1L were similar to those in surrounding cells (Fig. 7, right panels), which indicates that SSH1L is required for ΔCnA/B-induced cofilin dephosphorylation. These observations strongly suggest that calcineurin induces cofilin dephosphorylation via SSH1L activation.

Calcineurin Dephosphorylates and Activates SSH1L in Cell-free Assays—We next examined whether calcineurin has the potential to dephosphorylate and activate SSH1L in cell-free assays. Lysates of 293T cells transfected with plasmids for Myc-SSH1L were immunoprecipitated with anti-Myc antibody or control IgG, the precipitates were incubated in the presence or absence of recombinant active calcineurin, and the levels of SSH1L phosphorylation were analyzed (Fig. 8A). The amounts of phosphorylated SSH1L (P-SSH1L) and total SSH1L were measured by Pro-Q Diamond phosphoprotein staining and Coomassie Brilliant Blue staining, respectively. Pro-Q Diamond staining revealed that Myc-SSH1L was phosphorylated in 293T cells (Fig. 8A, lane 3) and dephosphorylated in vitro by treatment with active calcineurin (Fig. 8A, lane 5). Calcineurin-catalyzed dephosphorylation was blocked by pretreatment of calcineurin with pyrophosphate, an inhibitor of phosphatases including calcineurin (Fig. 8A, lane 6). These results suggest that SSH1L is a phosphoprotein in cultured cells and that calcineurin has the potential to dephosphorylate SSH1L in cell-free assays. The slight increase in the P-SSH1L level by pyrophosphate may be due to the inhibitory effect on SSH1L autodephosphorylation (Fig. 8A, lane 4). We also examined the effect of active calcineurin on the cofilin-phosphatase activity of SSH1L in cell-free assays. SSH1L purified from 293T cells by immunoprecipitation was incubated with or without recombinant active calcineurin and subjected to the in vitro cofilin-phosphatase assay (Fig. 8B). Incubation of SSH1L with calcineurin significantly increased the cofilin-phosphatase activity of SSH1L (Fig. 8B, lanes 3 and 4). In control experiments, calcineurin alone had no apparent effect on the P-cofilin level (Fig. 8B, lane 2). These results suggest that calcineurin has the potential to stimulate the cofilin-phosphatase activity of SSH1L, but not to catalyze cofilin dephosphorylation itself, in cell-free assays. These data, taken together with the data of Fig. 8A, indicate that calcineurin appears to activate SSH1L by dephosphorylation.

**DISCUSSION**

Cofilin plays an essential role in regulating actin filament dynamics. Its actin-depolymerizing and -severing activities are inhibited by phosphorylation at Ser-3 by LIM kinases and...
Calcineurin dephosphorylates and activates SSH1L in vitro. A, dephosphorylation of SSH1L by calcineurin. Lysates of 293T cells expressing Myc-SSH1L were immunoprecipitated with anti-Myc antibody or control IgG. The precipitates were incubated with recombinant active calcineurin (active Cn) and/or pyrophosphate (Cn inhibitor), as indicated. Reaction mixtures were run on SDS-PAGE, and phospho-SSH1L (P-SSH1L) and total SSH1L were measured by Pro-Q Diamond and Coomassie Brilliant Blue (CBB) staining, respectively. Bottom panel shows the relative phospho-SSH1L levels after the in vitro cofilin-phosphatase assay, with the value obtained from control IgG precipitates in the absence of calcineurin taken as 100%. Results are shown as the means ± S.D. of three independent experiments. B, activation of SSH1L by calcineurin. Lysates of 293T cells were immunoprecipitated with anti-SSH1L antibody or control IgG. The precipitates were incubated with calcineurin and/or pyrophosphate, as indicated. Reaction mixtures were analyzed by immunoblotting with anti-P-cofilin, anti-cofilin, and anti-SSH1L antibodies. Bottom panel shows the relative P-cofilin levels after the in vitro cofilin-phosphatase assay, with the value obtained from control IgG precipitates in the absence of calcineurin taken as 100%. Results are shown as the means ± S.D. of three independent experiments.

Fig. 8. Calcineurin dephosphorylates and activates SSH1L in vitro. A, dephosphorylation of SSH1L by calcineurin. Lysates of 293T cells expressing Myc-SSH1L were immunoprecipitated with anti-Myc antibody or control IgG. The precipitates were incubated with recombinant active calcineurin (active Cn) and/or pyrophosphate (Cn inhibitor), as indicated. Reaction mixtures were run on SDS-PAGE, and phospho-SSH1L (P-SSH1L) and total SSH1L were measured by Pro-Q Diamond and Coomassie Brilliant Blue (CBB) staining, respectively. Bottom panel shows the relative phospho-SSH1L levels after the in vitro cofilin-phosphatase assay, with the value obtained from control IgG precipitates in the absence of calcineurin taken as 100%. Results are shown as the means ± S.D. of three independent experiments. B, activation of SSH1L by calcineurin. Lysates of 293T cells were immunoprecipitated with anti-SSH1L antibody or control IgG. The precipitates were incubated with calcineurin and/or pyrophosphate, as indicated. Reaction mixtures were analyzed by immunoblotting with anti-P-cofilin, anti-cofilin, and anti-SSH1L antibodies. Bottom panel shows the relative P-cofilin levels after the in vitro cofilin-phosphatase assay, with the value obtained from control IgG precipitates in the absence of calcineurin taken as 100%. Results are shown as the means ± S.D. of three independent experiments.

testicular protein kinases and reactivated by dephosphorylation by SSH family phosphatases (7–17). Although a variety of extracellular stimuli induce cofilin dephosphorylation, the signaling pathways that lead to cofilin dephosphorylation have not been well understood. In the present study, we provided evidence that Ca2⁺ signal-induced cofilin dephosphorylation is mediated by consecutive activation of two protein phosphatases, calcineurin and SSH1L. We showed that Ca2⁺ ionophore and Ca2⁺-mobilizing agents induced SSH1L activation and cofilin dephosphorylation, both of which were suppressed by inhibition of calcineurin. These data indicate that Ca2⁺-induced SSH1L activation and cofilin dephosphorylation are mediated by calcineurin. Importantly, knockdown of endogenous SSH1L expression by siRNA abolished Ca2⁺- or calcineurin-induced cofilin dephosphorylation. Furthermore, calcineurin dephosphorylated SSH1L and stimulated cofilin-phosphatase activity of SSH1L in cell-free assays, but it had no apparent activity to catalyze cofilin dephosphorylation directly. Taken together, these findings suggest that SSH1L is essential for Ca2⁺-induced cofilin dephosphorylation as a downstream effector of calcineurin and that calcineurin stimulates cofilin dephosphorylation apparently only through SSH1L activation. Thus, we propose a novel signaling cascade of Ca2⁺-calcineurin-SSH1L-cofilin for Ca2⁺ signal-induced cofilin dephosphorylation and activation.

The precise mechanism of calcineurin-mediated SSH1L activation is still unclear. It is important to identify the residue(s) of SSH1L that is phosphorylated in resting cells but becomes dephosphorylated by calcineurin in response to stimulation of the Ca2⁺-induced signaling pathway. SSH1L activity is inhibited by association with 14-3-3 proteins, in a manner dependent on the phosphorylation of SSH1L serine residues (30), and it is possible that calcineurin stimulates SSH1L activity by dephosphorylating the serine residues that are involved in 14-3-3 binding. In the case of Ca2⁺-induced apoptosis, calcineurin-mediated dephosphorylation of BAD causes its dissociation from 14-3-3 and translocation to mitochondria to inhibit Bcl-xL (35). Additional studies are necessary to determine whether calcineurin induces SSH1L activation through a similar mechanism.

Ca2⁺ is a versatile intracellular signal mediator that can regulate many different cellular processes, including those related to actin cytoskeletal rearrangement. Ca2⁺-mediated cofilin dephosphorylation has been observed in various cell types and is thought to contribute to various cell responses, such as thrombin-induced platelet aggregation (20), chemotactic peptide-induced neutrophil migration (28), and nicotine-induced noradrenaline secretion by adrenal chromaffin cells (24). Because cofilin appears to play an essential role in actin filament dynamics and remodeling, Ca2⁺-induced SSH1L activation and cofilin dephosphorylation/activation must represent an important signaling pathway supporting stimulus-induced actin cytoskeletal changes and related cell responses. In neurons, intracellular Ca2⁺ signals play important roles in regulating growth cone motility and guidance (36). Local Ca2⁺ signals can cause growth cone steering responses, with higher Ca2⁺ signals mediating attraction and lower Ca2⁺ signals mediating repulsion (37). Based on findings that both cofilin and SSH1L stimulate growth cone motility and extension (18, 38), local activation of the Ca2⁺-SSH1L-cofilin signaling pathway appears to stimulate the directional steering of growth cones. In addition, Ca2⁺ and calcineurin are known to regulate neuronal plasticity and memory formation in the central nervous system (39). Modulation of actin cytoskeletal dynamics in dendritic spines by cofilin phosphorylation and dephosphorylation contributes to spine structure, persistence of long-term potentiation, and synaptic plasticity (40, 41). Thus, Ca2⁺/calcineurin-induced SSH1L activation may be one of the mechanisms by which Ca2⁺/calcineurin regulates neuronal actin cytoskeletal dynamics and hence modulates synaptic function and neuronal plasticity.

Various signaling pathways are involved in stimulus-induced cofilin dephosphorylation. In insulin-stimulated cells, phosphoinositide 3-kinase and its product, phosphatidylinositol-3,4,5-trisphosphate, are involved in SSH1L activation and cofilin dephosphorylation (29). In other types of cells, eAMP and protein kinase C signals stimulate cofilin dephosphorylation (1, 21–23). In contrast to the Ca2⁺ signal, cAMP- or protein kinase C signal-induced cofilin dephosphorylation is insensitive to calcineurin or calmodulin inhibitors (23, 28), which indicates the existence of calcineurin-independent signaling pathways for cofilin dephosphorylation. Additional studies are required to determine whether SSH1L, other members of a SSH family (e.g., SSH2L and SSH3L), or other more general...
classes of protein phosphatases, such as protein phosphatase type 1 and 2A (27), are involved in cAMP- and protein kinase C-induced coflin dephosphorylation. Indeed, coflin dephosphorylation/activation appears to be one of the important and convergent points in a cell signaling network through which a variety of extracellular stimuli regulate actin cytoskeletal dynamics and organization.

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REFERENCES

1. Moon, A., and Drubin, D. G. (1995) Mol. Biol. Cell 6, 1423–1431
2. Bamburg, J. R. (1999) Annu. Rev. Cell Dev. Biol. 15, 185–230
3. Carlier, M.-F., Ressad, F., and Pantaloni, D. (1999) J. Biol. Chem. 274, 33827–33830
4. Condeelis, J. (2001) Trends Cell Biol. 11, 288–293
5. Agnew, B. J., Minamide, L. S., and Bamburg, J. R. (1995) J. Biol. Chem. 270, 17582–17587
6. Morigyama, K., Iida, K., and Yahara, I. (1996) Genes Cells 1, 73–86
7. Yang, N., Higuchi, O., Ohashi, K., Nagata, K., Wada, A., Kangawa, K., Nishida, E., and Mizuno, K. (1998) Nature 393, 809–812
8. Arber, S., Barbayannis, F. A., Hanser, H., Schneider, C., Stanyon, C. A., Bernard, O., and Caronni, P. (1999) Nature 393, 805–809
9. Amano, T., Tanabe, K., Eto, T., Narumiya, S., and Mizuno, K. (2001) Biochem. J. 354, 149–159
10. Toshima, J., Toshima, J. Y., Takeuchi, K., Mori, R., and Mizuno, K. (2001) J. Biol. Chem. 276, 31449–31458
11. Maekawa, M., Ishizaki, T., Boku, S., Watanabe, N., Fujita, A., Iwamatsu, A., Nishida, E., and Mizuno, K. (1998) Nature 393, 809–812
12. Nakamura, T., Muto, Y., and Kainosho, M. (2000) Biochem. Biophys. Res. Commun. 279, 1269–1275
13. Ishida, K., Matsumoto, T., and Nishida, E. (2002) J. Biol. Chem. 277, 13317–13324
14. Watanabe, N., Fujita, A., Iwamatsu, A., and Nishida, E. (1998) J. Biol. Chem. 273, 11673–11677
15. Kashiwagi, A., Nakamura, T., and Nishida, E. (1999) Biochem. Biophys. Res. Commun. 264, 641–646
16. Endo, M., Ohashi, K., Sasaki, Y., Goshima, Y., Niwa, R., Uemura, T., and Mizuno, K. (2003) J. Neurosci. 23, 2527–2537
17. Nishida, E., and Mizuno, K. (1998) J. Biol. Chem. 273, 33450–33455
18. Groth, R. D., Fukushima, H., Nishida, E., and Mizuno, K. (2000) J. Neurosci. 20, 2459–2469
19. Kaji, N., Ohashi, K., Shuin, M., Niwa, R., Uemura, T., and Mizuno, K. (2003) J. Biol. Chem. 278, 33450–33455
20. Davidson, M. M., and Haslam, R. J. (1994) Biochem. J. 301, 41–47
21. Okada, K., Takano-Omuro, H., Obinata, S., and Abe, H. (1996) Exp. Cell Res. 227, 116–122
22. Takuma, T., Ichida, T., Yokoyama, N., Tamura, S., and Obinata, T. (1996) J. Biochem. (Tokyo) 120, 35–41
23. Meberg, P. J., Ono, S., Minamide, L. S., Takahashi, M., and Bamburg, J. R. (1998) Cell Motil. Cytoskeleton 34, 172–190
24. Birkenfeld, J., Klotzmann, B., Betz, H., and Roth, D. (2001) Biochem. Biophys. Res. Commun. 286, 493–498
25. Suzuki, K., Yamaguchi, T., Tanaka, T., Kawanishi, T., Nishimaki-Mogami, T., Yamamoto, K., Tsuji, T., Irimura, T., Hayakawa, T., and Takahashi, A. (1995) J. Biol. Chem. 270, 19551–19556
26. Lee, K. H., Meuer, S. C., and Samstag, Y. (2000) Eur. J. Immunol. 30, 892–899
27. Ambach, A., Saunus, J., Konstandin, M., Wesselborg, S., Meuer, S. C., and Samstag, Y. (2000) Eur. J. Immunol. 30, 3422–3431
28. Zhan, Q., Bamburg, J. R., and Badway, J. A. (2003) Cell Motil. Cytoskeleton 54, 1–15
29. Nishita, M., Wang, Y., Tomizawa, C., Suzuki, A., Niwa, R., Uemura, T., and Mizuno, K. (2004) J. Cell Biol. 165, 465–471
30. Obinata, T., Ohashi, K., Mizuno, K., and Narumiya, S. (1999) Biochem. Biophys. Res. Commun. 261, 657–664
31. Obinata, T., Ohashi, K., Mizuno, K., and Narumiya, S. (1999) Biochem. Biophys. Res. Commun. 261, 657–664
32. Wang, H.-G., Pathan, N., Ethis, I. M., Krajewski, S., Yamaguchi, Y., Shibasaki, F., Price, E. R., Milan, D., and McKeon, F. (1996) Nature 382, 370–373
33. Aguilar, G., Campanella, M., Manzotti, E., Pinton, P., Sanzi, M., Moretti, S., Piva, R., Rizzuto, R., and del Ser, L. (2005) Biochem. Biophys. Res. Commun. 330, 657–664
34. Shibasaki, F., Price, E. R., Milan, D., and McKeon, F. (1996) Nature 382, 370–373
35. Meberg, P. J., Takeuchi, K., Mori, R., and Mizuno, K. (2001) Mol. Biol. Cell 12, 1131–1145
36. Meberg, P. J., and Bamburg, J. R. (2000) J. Biol. Chem. 275, 3577–3582
37. Meberg, P. J., Ono, S., Minamide, L. S., Takahashi, M., and Bamburg, J. R. (1998) J. Biol. Chem. 273, 17582–17587
38. Nakamura, T., Muto, Y., and Kainosho, M. (2000) Biochem. Biophys. Res. Commun. 279, 1269–1275