LIM Kinase and Slingshot Are Critical for Neurite Extension

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Cofilin and its closely related protein, actin-depolymerizing factor (ADF), are key regulators of actin cytoskeleton dynamics that have been implicated in growth cone motility and neurite extension. Cofilin/ADF are inactivated by LIM kinase (LIMK)-catalyzed phosphorylation and reactivated by Slingshot (SSH)-catalyzed dephosphorylation. Here we examined the roles of cofilin/ADF, LIMKs (LIMK1 and LIMK2), and SSHs (SSH1 and SSH2) in nerve growth factor (NGF)-induced neurite extension. Knockdown of cofilin/ADF by RNA interference almost completely inhibited NGF-induced neurite extension from PC12 cells, and double knockdown of SSH1/SSH2 significantly suppressed both NGF-induced cofilin/ADF dephosphorylation and neurite extension from PC12 cells, thus indicating that cofilin/ADF and their activating phosphatases SSH1/SSH2 are critical for neurite extension. Interestingly, NGF stimulated the activities of both LIMK1 and LIMK2 in PC12 cells, and suppression of LIMK1/LIMK2 expression or activity significantly reduced NGF-induced neurite extension from PC12 cells or chick dorsal root ganglion (DRG) neurons. Inhibition of LIMK1/LIMK2 activity reduced actin filament assembly in the peripheral region of the growth cone of chick DRG neurons. These results suggest that proper regulation of cofilin/ADF activities through control of phosphorylation by LIMKs and SSHs is critical for neurite extension and that LIMKs regulate actin filament assembly at the tip of the growth cone.

The regulation of actin cytoskeleton dynamics plays a fundamental role in cell shape change, motility, and migration in response to stimuli. In neurons, actin filaments accumulate at the distal tip of the growth cone in the growing neurite, and actin filament dynamics and reorganization are essential for controlling growth cone motility and morphology and determining the direction and speed of neurite extension (1–4). Cofilin and its closely related protein, actin depolymerizing factor (ADF), are key mediators of actin filament dynamics that act by stimulating the depolymerization and severing of actin filaments. The activities of cofilin and ADF are inhibited by phosphorylation at Ser-3 by LIM kinases (LIMKs, composed of LIMK1 and LIMK2) (6, 7) and TES kinases (TESKs, composed of TESK1 and TESK2) (8); the inactive Ser-3-phosphorylated cofilin and ADF (P-cofilin/P-ADF) are reactivated by dephosphorylation by Slingshot (SSH) family protein phosphatases (SSH1, SSH2, and SSH3) (9, 10) and chronophin (a haloacid dehalogenase) (11). Because cofilin/ADF and their upstream regulators, LIMKs and SSHs, are abundant in neuronal growth cones (12–15), these proteins have been implicated in the control of neurite extension and guidance through regulating actin filament dynamics.

Neurotrophins are known to regulate neurite outgrowth and guidance (2). Rat pheochromocytoma PC12 cells have been often used as a model system to investigate nerve growth factor (NGF)-induced neurite outgrowth. NGF induces cofilin/ADF dephosphorylation in PC12 cells (16), and overexpression of cofilin/ADF or SSH1 enhances neurite extension from PC12 cells and primary cultured neurons, such as chick dorsal root ganglion (DRG) or rat cortical neurons (13, 17). In contrast, overexpression of LIMK1 in neurons suppresses growth cone motility and extension (13). In addition, the growth cone collapse induced by semaphorin-3A (a repulsive guidance molecule) or Nogo-66 (a myelin-associated inhibitor of axon regeneration) requires transient activation of LIMK1 and cofilin phosphorylation in chick DRG neurons (18, 19). These results suggest that LIMK1 acts as a negative regulator of neurite outgrowth by inhibiting cofilin/ADF activity. However, recent studies have suggested that LIMK1 has a seemingly opposite function on neurite outgrowth: neurite extension from hippocampal neurons was enhanced by LIMK1 expression and suppressed by blockade of LIMK1 activation (14, 20–22). Thus, further studies are required to understand the role of LIMK1 in neurite extension.

In this study, we examined the roles of cofilin/ADF and its phosphorylation in neurite extension by knocking down the expression of cofilin/ADF, LIMK1/LIMK2, and SSH1/SSH2 using small interfering RNAs (siRNAs). Knockdown of cofilin/ADF markedly blocked NGF-induced neurite extension of PC12 cells, and knockdown of SSH1/SSH2 suppressed NGF-induced cofilin/ADF dephosphorylation and neurite extension, indicating that SSH1/SSH2-mediated cofilin/ADF dephosphorylation is crucial for neurite extension. LIMK1 and LIMK2 were also activated after NGF stimulation of PC12 cells, and knockdown of LIMK1/LIMK2 significantly suppressed NGF-induced neurite extension from PC12 cells and chick DRG neurons. Our results indicate that both LIMK1/LIMK2-mediated...
phosphorylation and SSH1/SSH2-mediated dephosphorylation of cofillin/ADF are important for neurite extension.

**EXPERIMENTAL PROCEDURES**

Materials—K252a, U73122, and wortmannin were purchased from Calbiochem (La Jolla, CA). Latrunculin A was from Molecular Probes (Eugene, OR). Rabbit polyclonal antibodies against rat/mouse ADF and SSH2 were generated against the C-terminal peptide of rat/mouse ADF and SSH2, respectively. Rabbit polyclonal antibodies against cofillin, P-cofilin, LIMK1, C10, and SSH1 were prepared as described previously (8, 23, 24). Rabbit polyclonal antibody against LIMK2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). S3 and RV peptides were designed and synthesized as described previously (18, 25).

Plasmids—The siRNA-targeting constructs were generated using pSUPER or pSUPER.retro.puro vectors (OligoEngine, Seattle, WA), as described previously (26). The 19-base targeting sequences were as follows: 5’-GACCTGGCTAGCCTTAGA-3’ (rat LIMK1), 5’-GGACAAAGAGCTGAATCTG-3’ (rat LIMK2), 5’-GAGAGCTGCCGATGAC-3’ (rat SSH1), 5’-TGCGTCAACTTAGAGGAC-3’ (rat SSH2), 5’-GCAGAATTTACAAGCTAC-3’ (rat cofillin), 5’-GAGCGAGTATCGAAATA-3’ (rat ADF), 5’-GGAGCTGTCCGCTTGTAG-3’ (chick LIMK1), and 5’-CTGCTTAATCATGTGGAT-3’ (chick LIMK2). We also used the second siRNA sequences (termed siRNA2) targeting rat and chick LIMKs as follows: 5’-GAAGGACTACTGGGCCCGC-3’ (rat LIMK1), 5’-GTGGAGAGTTGCAACCAGA-3’ (rat LIMK2), 5’-GATTC-ATCGGACTGCTTCTAC-3’ (chick LIMK1), and 5’-GGACAAGAGCTCAGCAACCT-3’ (chick LIMK2). As a control, we used a non-targeting sequence, 5’-TCTTCCCCCAAGAAGATA-3’, which does not exist in the rat or chick genome. Expression plasmids coding for N-terminally Myc-tagged chick LIMK1 (chLIMK1) and chLIMK2 and C-terminally Myc-tagged chick cofillin and ADF were constructed by inserting their full-length cDNAs into pMyc-C1 or pcDNA3.1/Myc-His(-) mammalian expression vector containing the Myc epitope tag (13, 27). Expression plasmid (pEFYP-C1) coding for yellow fluorescent protein (YFP) was purchased from Clontech.

Cell Culture and Electroporation—PC12 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal bovine serum. Cells were plated in poly-L-lysine-coated dishes. For the RNA interference experiments, cells were plated in poly-L-lysine- and laminin-coated glass-bottom culture dishes, and further incubated for 12 h before time-lapse observation. CHO-K1 cells were maintained in minimum essential medium–α supplemented with 9% fetal bovine serum and transfected using Lipofectamine-2000 (Invitrogen), according to the manufacturer’s instructions.

Generation of PC12 Cell Lines—Retrovirus stocks encoding human SSH1 or mouse SSH2 were obtained by transfection of pLNCX plasmids (Clontech) encoding human SSH1 or mouse SSH2 cDNAs with packaging vectors (retrovirus packaging kit Amphi, Takara Bio, Otsu, Japan) into 293T cells and by harvesting conditioned growth medium containing secreted retrovirus. Virus stocks were filtered through a 0.45-μm filter, supplemented with 8 μg/ml polybrene, and incubated with PC12 cells for 48 h. PC12 cells expressing retrovirus-encoding cDNAs were selected for growth in medium containing 800 μg/ml G418.

Recombinant Herpes Simplex Virus Preparation and Infection—The recombinant herpes simplex virus stocks were prepared as described previously (13). For infection, freshly dissociated DRG neurons were allowed to adhere to dishes for 30 min and then incubated with recombinant viral stocks for 12 h before analysis.

Immunoprecipitation and Immunoblot Analyses—Immunoprecipitation and immunoblot analyses were performed as described previously (28).

In Vitro Kinase Assay—Serum-starved PC12 cells were stimulated with 100 ng/ml NGF and lysed in kinase buffer (50 mM Heps (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 5% glycerol, 1 mM MgCl2, 1 mM MnCl2, 10 mM NaF, 1 mM Na2VO4, 1 mM dithiothreitol, 1 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin). LIMK1 or LIMK2 was immunoprecipitated with anti-LIMK1 or anti-LIMK2 antibodies and subjected to an in vitro kinase assay using His6-tagged cofillin as a substrate, as described (13). Reaction mixtures were separated on SDS-PAGE and analyzed by autoradiography, Amido Black staining, and immunoblotting with anti-LIMK1 and anti-LIMK2 antibodies.

Neurite Outgrowth Assay—PC12 cells were cotransfected with pSUPER and YFP plasmids (4:1) by electroporation, cultured for 32 h and further serum-starved for 16 h. Then, neurite outgrowth was stimulated with 50 ng/ml NGF and allowed to proceed for 48 h in Dulbecco’s modified Eagle’s medium containing 0.1% bovine serum albumin. The cells with neurites longer than two cell body lengths of YFP-positive cells were scored as the neurite-bearing cells. The mean lengths of the longest neurites were measured for YFP-positive cells with neurites longer than one cell body length.
FIGURE 1. SSH1 and SSH2 are involved in NGF-induced cofilin/ADF dephosphorylation in PC12 cells. A, NGF induces cofilin/ADF dephosphorylation. PC12 cells were stimulated with NGF, and cell lysates were analyzed by immunoblotting with anti-P-cofilin and anti-cofilin antibodies. B, suppression of SSH1/SSH2 expression by siRNA. PC12 cells transfected with siRNA plasmids were selected by culturing for 44 h with puromycin, then serum-starved for 16 h. Expression of endogenous SSH1, SSH2, and β-actin was analyzed by immunoblotting with the indicated antibodies. C, effects of SSH1/SSH2 siRNA on NGF-induced cofilin/ADF dephosphorylation. PC12 cells transfected with siRNA plasmids were cultured for 44 h with puromycin, serum-starved for 16 h, then stimulated with NGF for 10 min. Cell lysates were analyzed by immunoblotting as described for A. Relative P-cofilin/P-ADF levels are shown as the means ± S.D. of three independent experiments, with the values in untreated cells taken as 100% (bottom panel). D, expression of siRNA-resistant human SSH1 or mouse SSH2 blocks the inhibitory effect of the SSH1/SSH2 double knockdown on NGF-induced cofilin/ADF dephosphorylation. PC12 cells stably expressing human SSH1-Myc (hSSH1-Myc/PC12 cells) or mouse SSH2-Myc (mSSH2-Myc/PC12 cells) were transfected with siRNA plasmids targeting rat SSH1/SSH2, cultured for 44 h with puromycin, serum-starved for 16 h, then stimulated with NGF for 10 min. Cell lysates were analyzed by immunoblotting with anti-P-cofilin or anti-cofilin antibodies. Expression of endogenous (Endo.) SSH1 and SSH2 was analyzed by immunoblotting with anti-SSH1 and anti-SSH2 antibodies, and expression of hSSH1-Myc and mSSH2-Myc was analyzed by immunoblotting with an anti-Myc antibody. The anti-SSH2 antibody recognized both endogenous rat SSH2 and mSSH2-Myc, whereas the anti-SSH1 antibody recognized endogenous rat SSH1 but not hSSH1-Myc. Relative P-cofilin/P-ADF levels are shown as the means ± S.D. of three independent experiments, with the values in untreated cells taken as 100% (bottom panel). E, effects of various inhibitors on NGF-induced cofilin/ADF dephosphorylation. PC12 cells were pretreated with control Me2SO (Vehicle), 0.2 µM K252a, 5 µM U73122, or 0.5 µM latrunculin-A for 30 min and then stimulated with NGF for 10 min. Cell lysates were analyzed as in A. Experiments were repeated twice, and similar results were obtained.
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quantification of areas of protrusion, a series of images was digitized. Growth cones were outlined at 30-s intervals, and the outlines were used to calculate areas of new protrusion of the growth cone perimeter by binary segmentation using IPLab image analysis software (Scanalytics, Fairfax, VA). The index of growth cone motility was calculated by dividing the average area of new protrusions of each growth cone measured at 30-s intervals over a total recording time of 3 min by the average growth cone perimeter during 3 min of recording. The rate of neurite extension was defined as the mean distance that the center of the growth cone migrated during 10 min of recording. Statistical analyses were performed using Student’s t test.

Immunofluorescence and Quantification of Actin Assembly—DRG explants were fixed with 4% paraformaldehyde containing 10% sucrose for 20 min followed by −20 °C methanol for 5 min. The explants were blocked with 2% fetal bovine serum in phosphate-buffered saline for 1 h and then incubated with rabbit polyclonal anti-P-cofilin and P-ADF antibodies that recognize both P-cofilin and P-ADF (8) and a monoclonal anti-β-actin antibody (AC-15, Sigma) overnight at 4 °C, washed three times with phosphate-buffered saline, and incubated with fluorescein- and rhodamine-conjugated secondary antibodies for 1 h at room temperature. To quantify the actin assembly at the leading edge of the growth cone, DRG neurons were stained with anti-β-actin antibody, and noncollapsed growth cones were randomly selected and fluorescence images were acquired using a Coollsnap HQ-cooled CCD camera driven by Q550FW Imaging Software. Growth cones were outlined on the fluorescence image by automatic outline tool and the average fluorescence intensity (mean pixel density) in a region of 5 μm width inside the front edge of the growth cone was measured using NIH image (Version 1.63). The fluorescence intensities were collected from 20–25 different growth cones per each sample group. Statistical analysis was performed using Student’s t test.

Time-lapse Video Fluorescence Image Analysis and Quantification—Live growth cones of chick DRG neurons were observed using an inverted fluorescence microscope (model DMI1RE, Leica) equipped with a 40× phase-contrast objective lens, as described (13). Time-lapse fluorescence images were captured every 30 s for 15 min with 50–100-ms exposures, using a Coollsnap HQ-cooled CCD camera (Roper Scientific) driven by Q550FW Imaging Software (Leica).
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Both SSH1 and SSH2 Are Involved in NGF-induced Cofilin/ADF Dephosphorylation in PC12 Cells—To investigate the effect of cofilin/ADF phosphoregulation on NGF-induced neurite extension, we first analyzed changes in P-cofilin/P-ADF levels in PC12 cells after NGF stimulation. Immunoblot analysis with an anti-P-cofilin antibody, which recognizes both P-cofilin and P-ADF (8), revealed that NGF induced cofilin/ADF dephosphorylation in PC12 cells (Fig. 1A), as previously reported (16). To examine whether SSHs are responsible for the NGF-induced cofilin/ADF dephosphorylation, we suppressed SSH1 and SSH2 expression with siRNAs, using a pSUPER.retro.puro vector containing the puromycin-resistance gene to allow selection of siRNA-resistant cells. A transfection of cofilin/ADF siRNAs had no apparent effect on the viability of PC12 cells (supplemental Fig. S1). To quantify the effect of cofilin/ADF siRNA on neurite extension, we scored the percentage of neurite-bearing cells with neurites longer than two cell body lengths in YFP-positive cells (Fig. 2C). We also compared the mean lengths of the longest neurites of YFP-positive cells with neurites longer than two cell body lengths in YFP-positive cells (Fig. 2C).

RESULTS

NGF-induced neurite outgrowth from PC12 cells, we suppressed the expression of endogenous cofilin and ADF in PC12 cells by transfecting cofilin and ADF siRNA plasmids (Fig. 2A). The PC12 cells were cotransfected with YFP plasmids to permit the transfected cells to be visualized. The transfected cells were cultured for 32 h, serum-starved for 16 h, and then stimulated with NGF for 48 h (Fig. 2B). Transfection of cofilin/ADF siRNAs had no apparent effect on the viability of PC12 cells (supplemental Fig. S1). To quantify the effects of cofilin/ADF siRNA on neurite extension, we scored the percentage of neurite-bearing cells with neurites longer than two cell body lengths in YFP-positive cells (Fig. 2C). We also compared the mean lengths of the longest neurites of YFP-positive cells with neurites longer than one cell body (Fig. 2D). Individual knockdown of cofilin or ADF significantly reduced both the number of neurite-bearing cells and the mean neurite length, compared with cells treated with a control siRNA. ADF knockdown suppressed neurite outgrowth more prominently than cofilin knockdown. When both cofilin and ADF were knocked down simultaneously, the percentage of neurite-bearing cells drastically decreased (Fig. 2B, 2C). Cotransfection of chick cofilin or ADF, whose expression was not affected by rat cofilin or ADF siRNA, significantly blocked the inhibitory effects of cofilin/ADF knockdown on NGF-induced neurite extension (Fig. 2D).

NGF-induced cofilin/ADF dephosphorylation requires Trk, phospholipase C, and F-actin. To examine whether cofilin and ADF affect NGF-induced neurite outgrowth from PC12 cells, we suppressed the expression of endogenous cofilin and ADF in PC12 cells by transfecting cofilin and ADF siRNA plasmids (Fig. 2A). The PC12 cells were cotransfected with YFP plasmids to permit the transfected cells to be visualized. The transfected cells were cultured for 32 h, serum-starved for 16 h, and then stimulated with NGF for 48 h (Fig. 2B). Transfection of cofilin/ADF siRNAs had no apparent effect on the viability of PC12 cells (supplemental Fig. S1). To quantify the effects of cofilin/ADF siRNA on neurite extension, we scored the percentage of neurite-bearing cells with neurites longer than two cell body lengths in YFP-positive cells (Fig. 2C). We also compared the mean lengths of the longest neurites of YFP-positive cells with neurites longer than one cell body (Fig. 2D). Individual knockdown of cofilin or ADF significantly reduced both the number of neurite-bearing cells and the mean neurite length, compared with cells treated with a control siRNA. ADF knockdown suppressed neurite outgrowth more prominently than cofilin knockdown. When both cofilin and ADF were knocked down simultaneously, the percentage of neurite-bearing cells drastically decreased (Fig. 2B, 2C). Cotransfection of chick cofilin or ADF, whose expression was not affected by rat cofilin or ADF siRNA, significantly blocked the inhibitory effects of cofilin/ADF knockdown on NGF-induced neurite extension (Fig. 2D).

 Knockdown of SSH1/SSH2 suppresses NGF-induced neurite extension of PC12 cells. A, PC12 cells were cotransfected with YFP and the indicated siRNA plasmids, cultured for 32 h, and serum-starved for 16 h. Cells were then stimulated with NGF and cultured for 48 h. Cells were visualized by their YFP fluorescence. Scale bar, 50 μm. B, quantitative analysis of the number of neurite-bearing cells. The number of neurite-bearing cells was measured as in Fig. 2C. * , p < 0.05 and ** , p < 0.01, compared with control siRNA cells. C, quantitative analysis of the mean neurite length of PC12 cells transfected with siRNA plasmids. The mean length of the longest neurites of YFP-positive cells with at least one neurite exceeding one cell body length was measured as in Fig. 2D. * , p < 0.01 and ** , p < 0.001, compared with control siRNA cells. D, expression of siRNA-resistant hSSH1 or mSSH2 blocks the inhibitory effect of SSH1/SSH2 knockdown on NGF-induced neurite extension. PC12 cells stably expressing hSSH1-Myc (hSSH1-Myc/PC12 cells) or mSSH2-Myc (mSSH2-Myc/PC12 cells) were cotransfected with YFP and siRNA plasmids, cultured, and stimulated with NGF, as in A. Cells were visualized by YFP fluorescence, and the number of neurite-bearing cells was measured as in B.

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NGF induces LIMK1/LIMK2 Activation in PC12 Cells—NGF induces cofilin/ADF dephosphorylation, raising the possibility that the kinase activity of LIMKs may be negatively regulated by NGF stimulation. However, when we examined the changes in the kinase activities of LIMK1 and LIMK2 in PC12 cells after NGF stimulation, both LIMK1 and LIMK2 were activated rather than repressed. LIMK1 activity increased 1.7-fold at 2 min after NGF treatment and then reverted to the basal level by 30 min (Fig. 4A). In contrast, LIMK2 activity gradually increased, reaching 1.5-fold by 30 min after NGF treatment (Fig. 4B). Pretreatment of PC12 cells with wortmannin, an inhibitor of phosphoinositide 3-kinase, blocked the NGF-induced activation of LIMK1 but not of LIMK2 (Fig. 4C, 4D). In contrast, Y-27632, a specific inhibitor of ROCK, had no apparent effect on NGF-induced LIMK1 or LIMK2 activation (Fig. 4, C and D).

LIMK1 and LIMK2 Are Critical for NGF-induced Neurite Extension from PC12 Cells—To examine whether LIMK1 and LIMK2 contribute to NGF-induced neurite extension from PC12 cells, we introduced siRNA plasmids targeting rat LIMK1 and LIMK2 into the cells, which suppressed the expression of endogenous LIMK1 and LIMK2, respectively (Fig. 5A). The kinase activity of LIMK1 and LIMK2 in cells expressing the corresponding siRNA also decreased, according to the decrease in their expression levels (supplemental Fig. S2). Knockdown of LIMK1, LIMK2, or both significantly reduced the number of neurite-bearing cells and the mean neurite length, compared with control cells (Fig. 5, B–D). LIMK2 knockdown suppressed neurite extension more prominently than LIMK1 knockdown. Similar results were obtained by using another set of siRNAs for rat LIMK1 and LIMK2 (supplemental Fig. S3). These results suggest that both LIMK1 and LIMK2 are critical for NGF-induced neurite extension of PC12 cells. We also analyzed the P-cofilin/P-ADF levels in unstimulated PC12 cells expressing LIMK1/LIMK2 siRNAs. The P-cofilin/P-ADF levels reduced in cells expressing LIMK2 or LIMK1/LIMK2 double knockdown cells but not LIMK1 knockdown cells (supplemental Fig. S4), which suggests that effects of SSH1/SSH2 siRNA on neurite extension are attributable to the suppression of SSH1/SSH2 expression and both SSH1 and SSH2 act to promote NGF-induced neurite extension from PC12 cells.

SSH1 and SSH2 Are Critical for NGF-induced Neurite Extension from PC12 Cells—We next examined whether SSH1 and SSH2 are involved in NGF-induced neurite extension from PC12 cells by knocking down SSH1 and SSH2 with siRNAs (Fig. 3A, see also Fig. 1B). The percentage of neurite-bearing cells was significantly reduced by SSH1 or SSH2 single knockdown or SSH1/SSH2 double knockdown, compared with control siRNA (Fig. 3B). The mean neurite length was also reduced in single and double SSH knockdown cells (Fig. 3C). In PC12 cells stably expressing human SSH1 or mouse SSH2, the number of neurite-bearing cells (Fig. 3D) and the mean neurite length (not shown) were not affected by either single or double knockdown of endogenous rat SSH1/SSH2, which indicates that the

neurite extension (Fig. 2E), which indicates that the effects of cofilin/ADF siRNAs are due to the suppression of cofilin/ADF expression. These results suggest that cofilin/ADF are essential for NGF-induced neurite outgrowth from PC12 cells.

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LIMK2, but not LIMK1, is mainly involved in maintenance of the P-cofilin/P-ADF levels in unstimulated PC12 cells.

LIMK1 and LIMK2 Are Critical for the Growth Cone Extension and Motility of Chick DRG Neurons—To assess the roles of LIMK1 and LIMK2 in the growth cone extension and motility of neurons in primary culture, we used time-lapse fluorescence microscopy to analyze the effects of LIMK1 and LIMK2 knockdown on the growth cone movement of chick DRG neurons. When cotransfected into CHO cells, the siRNA plasmids targeting chLIMK1 and chLIMK2 strongly silenced the expression of Myc-chLIMK1 and Myc-chLIMK2, respectively (Fig. 6A). To analyze the growth cone motility, chick DRG neurons were cotransfected with YFP plasmids and siRNA plasmids for chLIMK1 or chLIMK2 using Nucleofector II, cultured for 48 h, replated the neurons in laminin-coated dishes, and further cultured them for 12 h before time-lapse observation. NGF was always included in the culture medium. Representative live images of growth cones are shown in Fig. 6B. Time-lapse observations revealed that single knockdown of LIMK1 or LIMK2, or double knockdown of LIMK1/LIMK2, markedly decreased the rate of growth cone extension, compared with the rate in DRG neurons transfected with a control siRNA (Fig. 6B). To quantify the data, we measured the distance that the center of the growth cone migrated during 10 min of recording. The average rate of neurite extension of the neurons expressing LIMK1, LIMK2, and LIMK1/LIMK2 siRNA were 13.8 ± 1.6 μm/10 min, 16.1 ± 1.6 μm/10 min, and 10.2 ± 1.4 μm/10 min, respectively, corresponding to 50%, 59%, and 37% of the rate of the control siRNA neurons (27.4 ± 1.5 μm/10 min) (Fig. 6C). Similar results were obtained by using another set of siRNAs for chick LIMK1 and LIMK2 (supplemental Fig. S5). We also quantified the motility of the growth cones. The motility index of the growth cone was calculated by dividing the area of new protrusions at 30-s intervals by the average growth cone perimeter. The average value was calculated from recording of each growth cone (26–28 different growth cones) over a total of 3 h. The motility indices of growth cones of LIMK1, LIMK2, and LIMK1/LIMK2 siRNA neurons were 0.24 ± 0.02 μm/30 s, 0.26 ± 0.02 μm/30 s, and 0.23 ± 0.02 μm/30 s, respectively, corresponding to 59%, 65%, and 56% of the index of control siRNA neurons (0.40 ± 0.02 μm/30 s) (Fig. 6D). These results suggest that both LIMK1 and LIMK2 are critical for neurite extension and growth cone motility of chick DRG neurons. Immunostaining with anti-P-cofilin antibody revealed that the P-cofilin level in DRG neurons expressing chLIMK1/chLIMK2 siRNAs was significantly low, compared with the surrounding siRNA-non-expressing neurons or control siRNA-expressing neurons (supplemental Fig. S6), which indicates that LIMK1/LIMK2 are involved in growth cone extension and motility through cofilin/ADF phosphorylation.

S3 Peptide Suppresses the Growth Cone Extension and Motility of Chick DRG Neurons—To further examine the role of LIMKs in growth cone extension and motility, we used a cell-permeable peptide inhibitor of LIMKs (S3 peptide), which contains the N-terminal 16-amino acid sequence of cofilin and the cell-permeable sequence motif of penetratin (18, 25). As a control, the reverse (RV) peptide containing the reverse sequence of cofilin and the penetratin sequence was also tested. Chick DRG explants were cultured overnight, and then the movement of the growth cones was monitored by time-lapse microscopy after treatment with the S3 or RV peptide for 1 h. Treatment with S3 peptide, but not RV peptide, reduced the neurite extension rate and the growth cone motility (Fig. 7A). To quantify the rate of neurite extension and the motility of growth cones, DRG neurons were infected with herpes simplex virus coding for YFP...
and treated with S3 or RV peptide for 1 h, after which the motility of the growth cones was monitored by time-lapse fluorescence microscopy. S3 peptide, but not RV peptide, markedly reduced the rate of neurite extension (S3, 6.7 ± 1.9 μm/10 min; RV, 19.8 ± 1.7 μm/10 min) (Fig. 7B) and the motility of growth cones (S3, 0.3 ± 0.02 μm/30 s; RV, 0.44 ± 0.02 μm/30 s) (Fig. 7C), compared with non-treated growth cones (18.2 ± 1.9 μm/10 min and 0.43 ± 0.02 μm/30 s). Thus, S3 peptide produced a phenotype similar to that of neurons transfected with LIMK1/LIMK2 siRNA, supporting the importance of LIMK1/LIMK2-catalyzed cofilin phosphorylation for neurite extension from and growth cone motility of chick DRG neurons.

Effects of S3 Peptide on P-cofilin Levels and Actin Filament Assembly in Growth Cones of Chick DRG Neurons—We examined the effects of S3 peptide treatment on the P-cofilin levels and actin filament assembly in growth cones of chick DRG neurons. Chick DRG explants that had been cultured overnight were treated for 1 h with S3 or RV peptide and then fixed and stained with anti-P-cofilin and anti-β-actin antibodies. The anti-P-cofilin antibody recognized P-cofilin even in the presence of S3 or RV peptide, indicating that S3 or RV peptide does not interfere with the ability of anti-P-cofilin antibody (supplemental Fig. S7). Treatment with S3 peptide significantly reduced the P-cofilin levels in DRG neurons, compared with those of untreated or RV peptide-treated neurons (Fig. 8A, top panels). Intensive F-actin assembly was observed in the leading edge of the growth cones in untreated and RV peptide-treated neurons, whereas the S3 peptide-treated neurons exhibited weaker F-actin staining in the periphery of the growth cones (Fig. 8A, bottom panels). Quantitative analysis of the intensity of anti-β-actin immunofluorescence revealed that actin assembly in the peripheral region of the growth cone was significantly reduced by treatment with S3 peptide (Fig. 8B). These observations suggest that S3 peptide treatment suppressed cofilin phosphorylation and actin filament assembly at the tip of the growth cone.

FIGURE 6. Knockdown of LIMK1/LIMK2 suppresses growth cone extension and motility of chick DRG neurons. A, suppression of chick LIMK1/LIMK2 expression by siRNA. CHO-K1 cells were cotransfected with the siRNA plasmids and the plasmids encoding Myc-chLIMK1 or Myc-chLIMK2. After 60 h in culture, the expression of Myc-chLIMK1, Myc-chLIMK2, and actin was analyzed by immunoblotting with anti-Myc and anti-β-actin antibodies. B, time-lapse images of the growth cones of chick DRG neurons cotransfected with YFP and siRNA plasmids. The dotted line in each frame is a fixed reference. Scale bar, 10 μm. C, quantitative analysis of the growth cone extension rate. The extension rate was calculated by measuring the migration distance of the growth cone center for 10 min. The data represent means ± S.E. from 25–30 different growth cones. *, p < 0.001, compared with control siRNA cells. D, quantitative analysis of the growth cone motility. Growth cone motility was determined as the mean values of the newly protruded areas of growth cones measured every 30 s during 3-min recording, divided by the mean length of the perimeter of the growth cone. The data represent means ± S.D. from 25–30 different growth cones. *, p < 0.001, compared with control siRNA cells.
DISCUSSION

In this study, we examined the roles of cofilin/ADF and their upstream regulators, LIMK1/LIMK2 and SSH1/SSH2, in NGF-induced neurite extension from PC12 cells or chick DRG neurons. Consistent with a previous report (16), NGF induced cofilin/ADF dephosphorylation in PC12 cells. This dephosphorylation was repressed by double knockdown of both SSH1 and SSH2 but not by single knockdowns of either SSH1 or SSH2, and expression of siRNA-resistant human SSH1 or mouse SSH2 restored NGF-induced cofilin/ADF dephosphorylation in SSH1/SSH2 double-knockdown cells. These results suggest that both SSH1 and SSH2 are responsible for NGF-induced cofilin/ADF dephosphorylation and that they function redundantly in this process. Knockdown of SSH1/SSH2 also significantly suppressed NGF-induced neurite extension from PC12 cells, which suggests that SSH1/SSH2 acts through cofilin/ADF dephosphorylation to promote neurite extension. However, double knockdown of SSH1/SSH2 only partially suppressed NGF-induced neurite extension, whereas double knockdown of cofilin/ADF almost completely inhibited it. These results suggest that other cofilin/ADF phosphatases, such as SSH3 or chronophin, may be involved in the maintenance of basal cofilin/ADF activity (9–11). K252a blocked NGF-induced dephosphorylation of cofilin/ADF, which indicates that NGF induces cofilin/ADF dephosphorylation via the high-affinity NGF receptor TrkA. U73122, an inhibitor of phospholipase C, also blocked NGF-induced cofilin/ADF dephosphorylation. Phospholipase C acts upstream of calcium signaling, and SSH1 activation and cofilin/ADF dephosphorylation are induced by calcium and calcineurin signals in other types of cells (16, 29), making it possible that phospholipase C mediates NGF-induced SSH activation and cofilin/ADF dephosphorylation. Phospholipase C acts upstream of calcium signaling, and SSH1 activation and cofilin/ADF dephosphorylation are induced by calcium and calcineurin signals in other types of cells (16, 29), making it possible that phospholipase C mediates NGF-induced SSH activation and cofilin/ADF dephosphorylation.

We showed in this study that both LIMK1 and LIMK2 are activated after NGF stimulation in PC12 cells, although the time courses of their activation differ; LIMK1 is activated transiently, while LIMK2 activity increases gradually up to 30 min. LIMK1 activation was inhibited by wortmannin, but not by Y-27632, while LIMK2 activation was not affected by either wortmannin or Y-27632. These results suggest that LIMK1, but not LIMK2, is activated downstream of phosphoinositide 3-kinase and that the Rho-ROCK signaling pathway is not involved in NGF-induced LIMK1/LIMK2 activation. Thus, the signaling mechanisms that lead to NGF-induced LIMK1 and LIMK2 activation significantly differ, and
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LIMK1 and LIMK2 seem to play distinct roles in NGF-induced neurite extension. Interestingly, knockdown of LIMK1, LIMK2, or both significantly suppressed NGF-induced neurite extension from PC12 cells and chick DRG neurons. Similarly, the cell-permeable S3 peptide, which inhibits LIMK1/LIMK2 activity, reduced the P-cofilin/P-ADF levels in the growth cone.

We have provided evidence that both LIMK1/LIMK2 and SSH1/SSH2 are required for NGF-induced neurite extension. LIMKs are therefore activated by factors that stimulate neurite extension, such as NGF (shown in this study), as well as neurite retraction, such as semaphorin-3A and Nogo (18, 19), and are essential for both attractive and repulsive guidance signaling. It is probable that the local and temporal regulation of LIMKs and SSHs and the balance of their activities are important for driving the attractive or repulsive response of growth cones. Further studies on the mechanisms of local and temporal regulation of LIMK and SSH activities within growth cones in response to various external cues will help to elucidate the mechanism of growth cone guidance.

REFERENCES


FIGURE 8. Effects of S3 peptide treatment on the P-cofilin level and F-actin assembly in chick DRG growth cones. A, immunofluorescence analyses. Chick DRG neurons were incubated with 20 μg/ml S3 or RV peptide for 1 h or left untreated (–), and the cells were fixed and stained with anti-P-cofilin (top panels) and anti-β-actin (bottom panels) antibodies. Scale bar, 10 μm. B, quantitative analysis of actin assembly in the periphery of the growth cone. Actin assembly was measured as the average fluorescence intensity in a region of 5 μm width inside the front edge of the growth cone. The left images show the region measured. Scale bar, 5 μm. The data represent means ± S.E. from 20–25 different growth cones, with the mean fluorescence intensity of untreated growth cones normalized to 100%. *, p < 0.001 compared with untreated neurons.
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