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Sustained Activation of N-WASP through Phosphorylation Is Essential for Neurite Extension

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Summary

Neurite extension is a key process for constructing neuronal circuits during development and remodeling of the nervous system. Here we show that Src family tyrosine kinases and proteasome degradation signals synergistically regulate N-WASP in neurite extension. Src family kinases activate N-WASP through tyrosine phosphorylation, which induces Arp2/3 complex-mediated actin polymerization. Tyrosine phosphorylation of N-WASP also initiates its degradation through ubiquitination. When neurite growth is stimulated in culture, degradation of N-WASP is markedly inhibited, leading to accumulation of the phosphorylated N-WASP. On the other hand, under culture conditions that inhibit neurite extension, but favor proliferation, the phosphorylated N-WASP is degraded rapidly. Collectively, neurite extension is regulated by the balance of N-WASP phosphorylation (activation) and degradation (inactivation), which are induced by tyrosine phosphorylation.

Introduction

During neurite extension, actin polymerization occurs at the tip of the growing neurite, forming protrusive structures, such as filopodia and lamellipodia. Remodeling of the actin cytoskeleton is thought to be the basis of neurite formation. In this process, actin polymerization is regulated, at least in part, by neural Wiskott-Aldrich syndrome protein (N-WASP) because expression of mutant N-WASP that is defective in actin polymerization suppresses neurite extension (Banzai et al., 2000). N-WASP induces de novo polymerization of actin through activation of actin-related protein (Arp) 2/3 complex (Rohatgi et al., 1999; Yamaguchi et al., 2000).

Rho family small GTPases, such as Cdc42, Rac, and Rho, have fundamental roles in determining cell shape through regulation of the actin cytoskeleton (Hall, 1998). Among small GTPases, Cdc42 and Rac are important in neurite extension through formation of filopodia and lamellipodia (Kozma et al., 1997; Daniels et al., 1998). In particular, Cdc42 targets N-WASP and regulates actin assembly at the tip of growing neurite. Overexpression of the H208D mutant of N-WASP, in which a mutation in the CRIB region disrupts association with Cdc42, abolishes neurite extension both in neuroblastoma cells and in primary hippocampal neurons (Miki et al., 1998; Banzai et al., 2000).

In addition to the small GTPases, there are interactions between tyrosine kinases and WASP family proteins. Among tyrosine kinases, Btk associates with WASP through the SH3 domain and phosphorylates WASP (Oda et al., 1998; Baba et al., 1999). WASP is the homolog of N-WASP and is restricted in hematopoietic cells. However, the significance of this phosphorylation remains unclear. Src family proteins also have SH3 domains. WASP binds the SH3 domain of Fyn through the proline-rich region of WASP (Banin et al., 1996). We found that N-WASP also associates with Fyn through its SH3 domain (Miki et al., 1999; Fukuoka et al., 2001), although the significance of this interaction is not clear.

The Src and Fyn tyrosine kinases are expressed at high levels in the nervous system (Cotton and Brugge, 1983; Umemori et al., 1992). Src and Fyn are concentrated in neuronal growth cones (Maness et al., 1988; Bixby and Jhabvala, 1993), suggesting that they may play roles in axon growth and guidance. Indeed, Src/Fyn double-knockout mice exhibit defects in axon guidance and fasciculation (Morse et al., 1998). Further, cultured neurons lacking either Src or Fyn show defective neurite outgrowth in response to cell adhesion molecules (Beggs et al., 1994; Ignelzi et al., 1994).

Activities of Src family kinases are regulated through ubiquitin-dependent protein degradation by proteasomes (Hakak and Martin, 1999; Harris et al., 1999; Oda et al., 1999) as well as through phosphorylation and dephosphorylation (Brown and Cooper, 1996; Thomas and Brugge, 1997). Proteasomes are protease complexes that degrade proteins with ubiquitin tags. Activated Src is subject to ubiquitination and degradation, resulting in tight regulation of kinase activity.

Interestingly, inhibition of proteasome activity by specific inhibitors, such as lactacystin and MG-132, induces neurite formation, suggesting that protein degradation signals are also involved in neurite extension (Saito et al., 1992; Fenteany et al., 1994, 1995). Therefore, the overall activity of proteasomes is thought to be a negative regulator of neurite extension in PC12 cells (Obin et al., 1999). Further, chemotropic responses of retinal

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growth cones are mediated by proteasome-dependent protein degradation (Hu et al., 1997; Campbell and Holt, 2001). However, the mechanism by which the ubiquitin-dependent pathway is connected to reorganization of the actin cytoskeleton during development or remodeling of the nervous system is poorly understood.

In the present study, we show that signals from Src family kinases and ubiquitin proteasomes converge at N-WASP during neurite extension. Phosphorylation and degradation of N-WASP play an essential role in neurite extension.

Results

Phosphorylation of N-WASP by Fyn In Vitro

We first examined whether purified Fyn phosphorylates N-WASP, the domain structure of which is shown in Figure 1A. As shown in Figure 1B, purified Fyn phosphorylated full-length N-WASP after a 30 min reaction. To determine where in N-WASP phosphorylation occurred, N-WASP was divided into four domains (Figure 1A). Only the region between the IQ motif and the CRIB region was phosphorylated by Fyn. Approximately 0.7 mol of phosphate was incorporated into 1 mol of IQ-CRIB fragment. This fragment contains tyrosines at amino acids 172 and 253. Tyrosine 253 (Tyr253) was found to be the site of phosphorylation by Fyn because substitution of Tyr253 with phenylalanine eliminated phosphorylation both in vitro and in COS-7 cells (Figures 1B and 1C). In contrast, substitution of Tyr172 with phenylalanine did not affect phosphorylation of N-WASP (Figure 1C). To further characterize phosphorylation of N-WASP, an antibody specific for phosphorylated Tyr253 was raised. This antibody showed approximately 50,000-fold-greater affinity for peptides containing phosphorylated Tyr253 than for the nonphosphorylated form (data not shown). This antibody (anti-P-Tyr253 N-WASP) recognized purified N-WASP incubated with Fyn and ATP, but it did not recognize N-WASP incubated with Fyn alone (without ATP) (Figure 1D), confirming that Tyr253 is the site of phosphorylation in full-length N-WASP. Tyr253 is homologous to the residue in WASP that is phosphorylated by Btk (Baba et al., 1999), suggesting that this tyrosine is a conserved site for phosphorylation by various Src family and other tyrosine kinases.

When autophosphorylation of Fyn and phosphorylation of N-WASP were compared after a 10 min reaction, phosphorylation of N-WASP was much slower than autophosphorylation of Fyn (Figure 1E). Only 20% of N-WASP was phosphorylated, whereas approximately 90% of Fyn was phosphorylated. Thus, to assess the effects of molecules that bind N-WASP in Fyn-mediated phosphorylation, we examined phosphorylation of N-WASP in the presence of phosphatidylinositol 4,5-bisphosphate (PIP2), the active form of (GTPγS-loaded) Cdc42, and Ash/Grb2 (Takenawa and Miki, 2001). PIP2 dramatically enhanced phosphorylation of N-WASP by Fyn. With PIP2, approximately 80% of N-WASP molecules were phosphorylated, whereas, without PIP2, only approximately 20%–40% of N-WASP molecules were phosphorylated in the same amount of time (Figure 1E). Binding of PIP2 to the basic region of N-WASP adjacent to the phosphorylation site may enhance accessibility for Fyn. The effects of Cdc42 and Ash/Grb2 were not statistically significant (Figure 1E).

Phosphorylation of N-WASP Leads to Activation of the Arp2/3 Complex

N-WASP itself is inactive in actin polymerization because the C-terminal active region (VCA region; see Figure 1A) is usually masked by an interaction between itself and other regions of N-WASP (autoinhibition). The region around the phosphorylation site is thought to interact with the VCA region (Miki et al., 1998; Rohatgi et al., 1999; Kim et al., 2000); therefore, introduction of a negative charge through phosphorylation may cause a conformational change that releases the VCA region and results in Arp2/3 activation. Thus, we examined the effect of N-WASP phosphorylation on Arp2/3 complex-mediated actin polymerization with a pyrene actin assay, in which an increase in fluorescence indicates actin filament formation. In these experiments, phosphorylation of N-WASP by Fyn was confirmed by parallel experiments with radioisotope. We used N-WASP of which 50% was phosphorylated after a 60 min reaction with Fyn alone. Incubation at 37°C without Fyn did not release the autoinhibition of N-WASP (N-WASP WT), suggesting that the autoinhibited structure of N-WASP is stable under our experimental conditions. As shown in Figure 2A, phosphorylated N-WASP (N-WASP WT + Fyn + ATP) had higher actin polymerization activity than did unphosphorylated N-WASP complexed with an excess amount of the SH3 domain of Fyn (N-WASP + Fyn SH3) or full-length Fyn alone [N-WASP WT + Fyn (w/o ATP)]. Thus, the presence of Fyn alone at this concentration (17 nM) had little effect on Arp2/3 complex (60 nM) activation by N-WASP (100 nM), but the phosphorylation activated N-WASP (Figure 2A). This finding indicates that phosphorylation of N-WASP exposes the VCA region and activates Arp2/3-mediated actin polymerization. Therefore, the introduction of a negative charge by phosphorylation presumably releases the autoinhibited structure of N-WASP.

We next added PIP2 to phosphorylated N-WASP in an actin polymerization reaction (N-WASP WT + Fyn + ATP + PIP2) (Figure 2A). The addition of PIP2 enhanced actin polymerization induced by phosphorylated N-WASP, suggesting that PIP2 further increases phosphorylated N-WASP activity in nucleation of actin polymerization. We confirmed by Western blotting with anti-phosphotyrosine (4G10) antibody that, during polymerization reactions, further phosphorylation of N-WASP did not occur (data not shown). To confirm the synergistic activation by PIP2 and phosphorylation, we measured the number of generated barbed ends, which indicates the degree of Arp2/3 complex activation. The number of barbed ends generated by phosphorylated N-WASP together with PIP2 was larger than the total number of barbed ends generated by phosphorylated N-WASP alone and by unphosphorylated N-WASP with PIP2 (Figure 2B). Thus, PIP2 and phosphorylation act synergistically to activate N-WASP in Arp2/3 complex-mediated actin polymerization. The addition of Cdc42 to N-WASP activated by phosphorylation and PIP2 did not enhance actin polymerization (Figure 2A).

We then prepared Y253F N-WASP, in which Tyr253

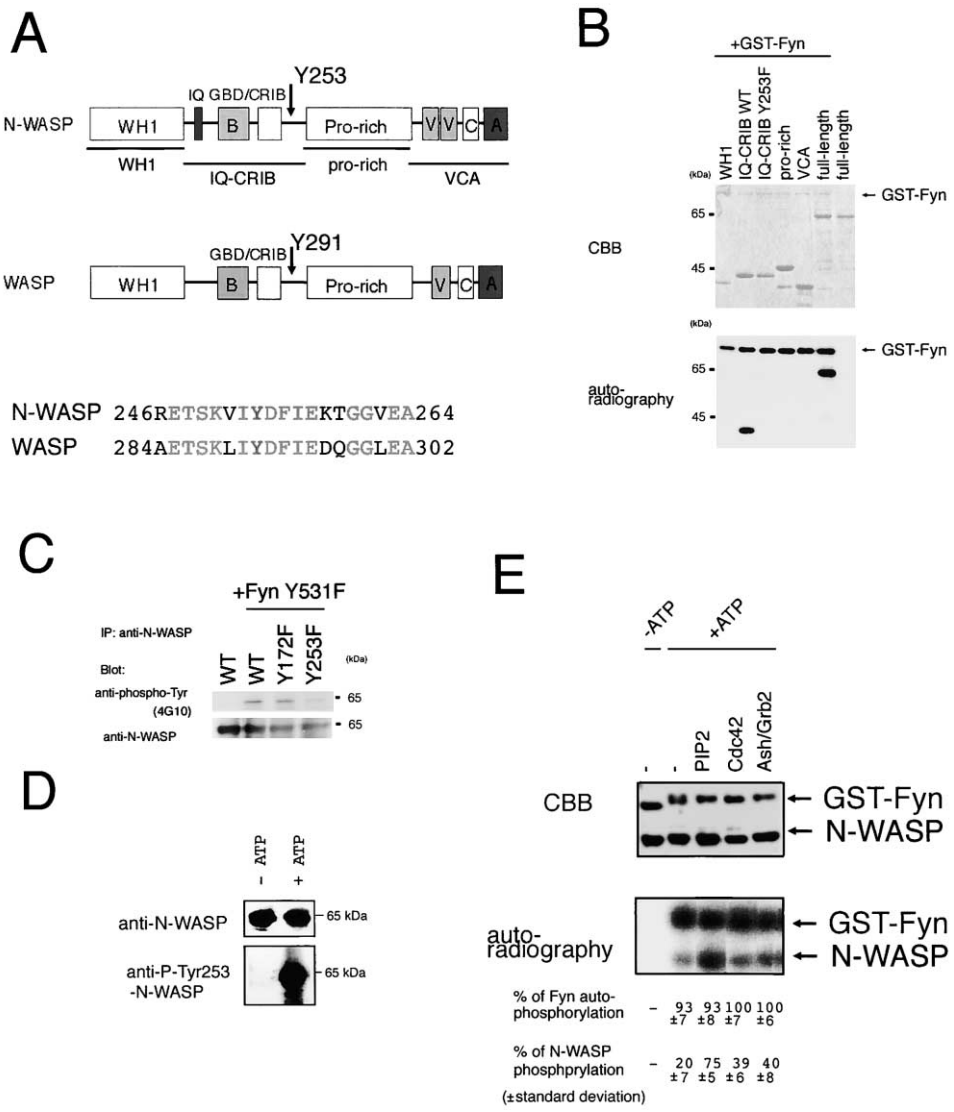


Figure 1. Phosphorylation of N-WASP by Fyn In Vitro

(A) Domain structures of N-WASP and WASP and amino acid alignments around the phosphorylation site. The alignment of amino acids around Tyr253 of N-WASP and Tyr291 of WASP is shown.

(B) In vitro phosphorylation of N-WASP by Fyn. Various domains of full-length N-WASP were mixed with Fyn and incubated with γ-^{32}P ATP for 30 min at 37°C. Phosphorylation of each fragment was then examined by SDS-PAGE and then by Coomassie brilliant blue (CBB) staining and subjected to autoradiography.

(C) Phosphorylation of Tyr253 in COS-7 cells. N-WASP with phenylalanine substituted for Tyr172 or Tyr253 was expressed in COS-7 cells with constitutively active (CA) Fyn (Y531F Fyn). N-WASP was immunoprecipitated, and phosphorylation was examined by Western blotting with anti-phosphotyrosine (P-Tyr) (4G10) antibody. The weak band at Y253F in 4G10 blot is endogenous N-WASP phosphorylated by Fyn.

(D) Specific recognition of phosphorylated N-WASP by anti-P-Tyr253 N-WASP antibody. Purified N-WASP was mixed with purified Fyn with or without ATP and then incubated for 30 min at 30°C as in (B). The mixture was then subjected to Western blotting with anti-P-Tyr253 N-WASP antibody and anti-N-WASP antibody.

(E) Enhancement of N-WASP phosphorylation by PIP2, Cdc42, or Ash/Grb2. N-WASP was phosphorylated with Fyn in vitro for 10 min at 37°C with PIP2 (4 μM), GTPγS-loaded Cdc42 (10 μM), or Ash/Grb2 (10 μM).

was replaced with phenylalanine. This mutant was not phosphorylated by Fyn and, therefore, is not activated [see N-WASP Y253F + Fyn + ATP and N-WASP Y253F + Fyn (w/o ATP)] (Figure 2C). In contrast, Y253F N-WASP was activated by Cdc42, PIP2, and a combination of PIP2 and Cdc42 in a manner similar to that of wild-type N-WASP (Figure 2C).

Because phosphorylation at Tyr253 activated N-WASP, we examined whether substitution of glutamic acid for Tyr253 (Y253E), which introduces a negative charge similar to that induced by phosphorylation, activates N-WASP by releasing the autoinhibited structure. Y253E N-WASP induces actin polymerization independently of other regulators (Figure 2D). Therefore, the introduction of a negative charge activates N-WASP without binding of Cdc42 or proteins with SH3 domains

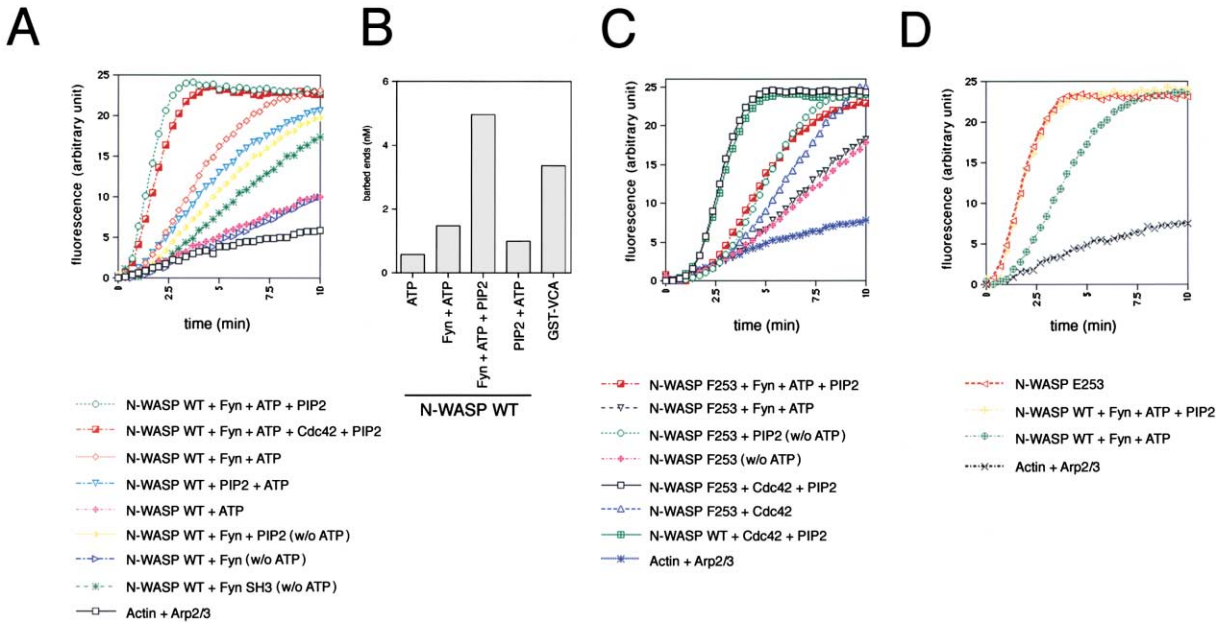


Figure 2. Activation of Arp2/3 Complex-Mediated Actin Polymerization by Phosphorylation of N-WASP

(A) Phosphorylated N-WASP was subjected to pyrene actin assay (N-WASP WT + Fyn + ATP), in which N-WASP was 100 nM (50% phosphorylated) with Fyn (17 nM). A phosphorylation reaction without ATP was performed as a negative control (N-WASP WT + Fyn w/o ATP). PIP2 and/or Cdc42 was added after the phosphorylation reaction, i.e., just before the addition of actin (N-WASP WT + Fyn + ATP + Cdc42 + PIP2 or N-WASP WT + Fyn + ATP + PIP2, respectively). N-WASP with Fyn SH3 domain at a higher concentration (1 μM) was assayed as a negative control (N-WASP + Fyn SH3). N-WASP alone was treated by the same procedure without Fyn as a negative control (N-WASP WT + ATP).

(B) The number of barbed ends, which indicates the degree of activation of actin polymerization, when Arp2/3 complex was activated by phosphorylated N-WASP alone, by nonphosphorylated N-WASP with PIP2, or by phosphorylated N-WASP with PIP2.

(C and D) The effect of amino acid substitution at Tyr253. N-WASP with substitution of Tyr253Phe (Y253F) (C) or Tyr253Glu (Y253E) (D) was subjected to pyrene actin assay. In (C), the effects of the phosphorylation reaction, Cdc42, and PIP2 were also examined.

(Takenawa and Miki, 2001). The strong activity of Y253E N-WASP in comparison with that of phosphorylated N-WASP (approximately 50% phosphorylated) is presumably due to a difference in the frequency of the introduced negative charge.

Phosphorylation of N-WASP in Neurite Extension

To address the physiological significance of phosphorylation of N-WASP, we investigated the phosphorylation status of endogenous N-WASP in rat pheochromocytoma PC12 cells and mouse neuroblastoma N1E 115 cells before and after neurite extension (differentiation). We also examined phosphorylation of N-WASP in primary cortical neurons. In primary neurons from the E14 cerebral cortex, phosphorylated N-WASP was present (Figure 3A). PC12 cells and N1E 115 cells proliferate, but do not extend neurites in the presence of serum (proliferating condition). On the other hand, they extend neurites when treated with nerve growth factor (NGF) or when cultured in the absence of serum (differentiating condition). When PC12 cells were treated with NGF, phosphorylation of N-WASP was increased (Figure 3B). Phosphorylation of N-WASP in N1E 115 cells was also increased under neurite growth conditions (Figure 3C). Phosphorylation of Tyr253 was confirmed with a phosphorylation-specific antibody against P-Tyr253 N-WASP (data not shown).

To confirm that phosphorylation of N-WASP is dependent on Src family kinases, we used pharmacological inhibition to reduce all activities of all nine Src family kinases. PC12 and N1E 115 cells as well as primary neurons were treated with PP2, a specific inhibitor of Src family kinases (Hanke et al., 1996). Phosphorylation of N-WASP was decreased significantly when cells were treated with PP2, but not with PP3, indicating that phosphorylation of Tyr253 is mediated by Src family kinases (Figures 3A–3C and data not shown).

To examine phosphorylation of N-WASP in response to stimuli other than NGF, we treated PC12 cells with EGF because PC12 cells respond to EGF, although it does not induce neurite extension (Boonstra et al., 1985; Lazarovici et al., 1987; Marshall, 1995). As shown in Figure 3D, phosphorylation of N-WASP was observed after EGF treatment, but this phosphorylation was not maintained, as it is with NGF.

Essential Role of N-WASP Phosphorylation in Neurite Extension

Because the expression of v-Src, which is a constitutively active mutant of Src, induces neurite extension in PC12 cells (Alema et al., 1985), Src family kinases are thought to be involved in neurite extension in PC12 and N1E 115 cells as well as in neurons. In PC12 and N1E 115 cells, the treatment with PP2 under culture conditions favoring neurite growth suppressed neurite outgrowth (Figures 4A and 4B). Expression of a dominant-negative (DN) Fyn, that is, a kinase-negative mutant (K299M Fyn), also suppressed neurite extension in both PC12 and N1E

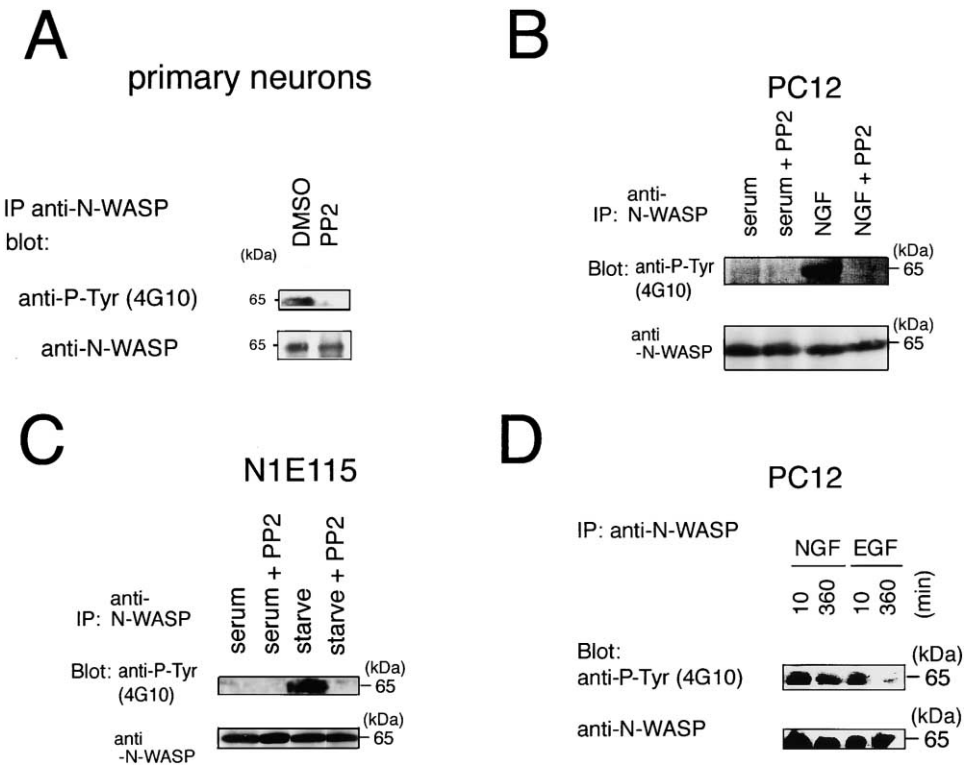


Figure 3. Phosphorylation of N-WASP in Neurite Extension

(A–C) Phosphorylation of N-WASP from (A) primary neurons, (B) PC12 cells, and (C) N1E 115 cells. Primary neurons were cultured with or without PP2 (10 μM) for 16 hr. PC12 cells were treated with NGF (100 ng/ml) with or without PP2 (10 μM) for 16 hr. N1E 115 cells were serum-starved for neurite induction with or without PP2 (10 μM) for 16 hr.

(D) Phosphorylation of N-WASP after treatment of PC12 cells with NGF or EGF. Cells were harvested after 10 or 360 min after NGF or EGF (100 ng/ml) treatment. N-WASP was immunoprecipitated and then subjected to Western blotting with anti-phosphotyrosine (P-Tyr) antibody (4G10) and anti-N-WASP antibody.

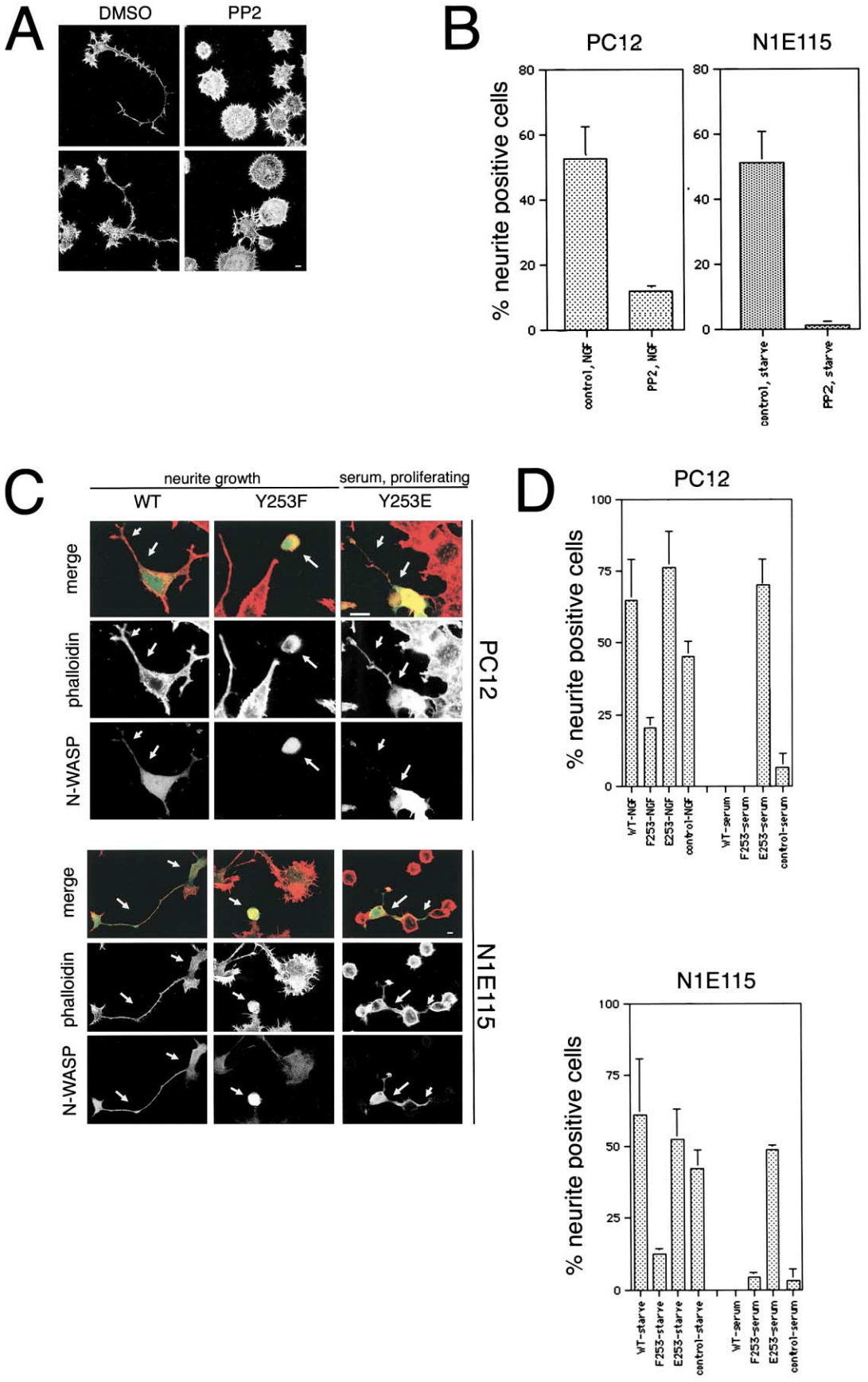
115 cells, whereas a constitutively active (CA) mutant of Fyn (Y531F Fyn) promoted neurite formation, showing the involvement of Src family kinases (Figure 5B and data not shown). Therefore, Src family kinases are essential for neurite extension in these cells as well as in neurons.

To document the importance of phosphorylation of N-WASP at Tyr253 in neurite extension, we examined whether cells expressing the Y253F mutant of N-WASP, which cannot be phosphorylated, can extend neurites. Expression of the Y253F mutant inhibited neurite extension induced under differentiating conditions (NGF or serum starvation). In contrast, cells expressing the Y253E mutant, which is active in vitro, formed neurites even when cultured under proliferating, nonneurite forming conditions, suggesting that activation of N-WASP by phosphorylation of Tyr253 is critical for inducing extension of neurites in these cells (Figures 4C and 4D). Further, ectopic expression of Y253E N-WASP enhanced neurite extension under differentiating conditions (Figures 4C and 4D). Expression of constitutively active Fyn also enhanced neurite extension under differentiating conditions, and this effect was blocked by Y253F N-WASP expression (Figures 5A and 5B). Further, the effect of PP2 or expression of DN Fyn on suppression of neurite extension in PC12 cells was canceled when Y253E N-WASP (active form of N-WASP) was expressed (Figures 5A and 5B). These results indicate that phosphorylation of N-WASP by Src family kinase is an essential event for inducing neurite.

We next examined whether phosphorylation of N-WASP is critical in neurite extension in primary neurons as in PC12 and N1E 115 cells. In primary cortical neurons, PP2, but not its inactive analog, PP3, inhibited neurite extension (Figure 6). These results demonstrate the essential role of Src family kinases in neurite extension in primary neurons. To document the importance of phosphorylation of Tyr253 in neurite extension by cortical neurons, we introduced plasmid expression for N-WASP or N-WASP mutants fused to GFP by in vivo electroporation (Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001). The expression of wild-type N-WASP had little effect on neurite extension. In contrast, expression of Y253F N-WASP inhibited neurite extension, whereas expression of Y253E N-WASP enhanced neurite extension in primary neuron cultures (Figure 6). Again, the effect of PP2 or expression of DN Fyn on suppression of neurite extension was canceled when Y253E N-WASP was expressed in primary neurons (Figure 6).

Src Family Kinases Regulate N-WASP

The suppressive effect of Y253F N-WASP expression is not due to its possible inability to receive Cdc42 signals


A
DMSO
PP2
B
PC12
N1E115
% neurite positive cells
0
20
40
60
80
control, NGF
PP2, NGF
control, starve
PP2, starve
C
neurite growth
serum, proliferating
WT
Y253F
Y253E
merge
phalloidin
N-WASP
PC12
N1E115
D
PC12
% neurite positive cells
0
25
50
75
100
WT-NGF
F253-NGF
E253-NGF
control-NGF
WT-serum
F253-serum
E253-serum
control-serum
N1E115
% neurite positive cells
0
25
50
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100
WT-starve
F253-starve
E253-starve
control-starve
WT-serum
F253-serum
E253-serum
control-serum

in vivo. In COS-7 cells, overexpression of N-WASP induces microspikes when cells are stimulated with epidermal growth factor (EGF) (Miki et al., 1996). This microspike formation is dependent on Cdc42 because the H208D mutant of N-WASP, which cannot bind with Cdc42, suppresses microspike formation (Fukuoka et al., 2001). In contrast, the Y253F mutant of N-WASP generated in the present study induced microspike formation in response to EGF in COS-7 cells (data not shown). Therefore, the Y253F mutant can respond through Cdc42 when expressed in cells, and this signaling pathway is predominant for N-WASP activation in COS-7 cells.

Cdc42 is shown to play a critical role during neurite extension and filopodium formation (Kozma et al., 1997; Daniels et al., 1998; Miki et al., 1998; Banzai et al., 2000). To assess the hierarchy of Cdc42 and Src family kinases in N-WASP regulation of neurite extension, we ectopically coexpressed DN Cdc42 and Y253E N-WASP. We also performed a series of expression studies by transfecting CA or DN Cdc42 and Fyn.

In PC12 cells, the activation of phosphorylation signals was effective for neurite extension. The expression of DN Cdc42 alone or DN Fyn alone suppressed neurite extension as reported previously (Figure 5B). Suppression of neurite extension by DN Cdc42 expression is restored, though not completely, by the expression of Y253E N-WASP or CA Fyn (Figures 5A and 5B). In addition, the expression of a double mutant H208D/Y253E N-WASP, which is defective in Cdc42 binding but that carries a negative charge as does phosphorylation, inhibited the suppressive effect of a single mutant H208D N-WASP for neurite extension. Similar results were obtained with N1E 115 cells.

In addition, to clarify whether there is a direct link between Cdc42 and Src family kinases, we examined the phosphorylation status of N-WASP in cells expressing CA or DN Cdc42 and Fyn. Phosphorylation of N-WASP was not changed by the expression of CA or DN Cdc42, whereas CA Fyn expression increased phosphorylation of N-WASP (Figure 5C). Therefore, Src family kinases are not directly downstream of Cdc42, because CA Cdc42 did not increase phosphorylation of N-WASP in the presence of NGF (Figure 5C) or serum (data not shown).

In primary cortical neurons, the phosphorylation signal is more effective than Cdc42 in forced expression experiments as well. Primary cortical neurons expressing Y253E N-WASP and DN Cdc42 had slightly shorter neurites than those expressing Y253E N-WASP alone. However, neurites of these cells overexpressing Y253E N-WASP and DN Cdc42 were longer than those expressing DN Cdc42 alone, suggesting that the phosphorylation signal acts overwhelmingly in neurite extension in cortical neurons from E14 mice in these conditions (Figure 6).

Inhibition of Proteasome Increases Phosphorylation of N-WASP and Neurite Extension

As shown in Figure 3, phosphorylated N-WASP is retained for a long time under differentiating (neurite growth) conditions. This may imply that the degradation process of phosphorylated N-WASP is suppressed in differentiating conditions because the inhibition of the proteasome pathway is involved in neurite extension (Saito et al., 1992; Fenteany et al., 1994, 1995).

To test this hypothesis, we first confirmed that treatment of PC12 and N1E 115 cells with proteasome inhibitors such as lactacystin and MG-132 induces neurite extension (Figure 7A). Second, we found that this effect of MG-132 was blocked by PP2, suggesting that neurite outgrowth induced by proteasome inhibition proceeds in an Src family kinase-dependent manner (Figures 7A and 7B). Third, we confirmed that, in parallel with neurite extension, phosphorylation of N-WASP increased after treatment with MG-132, even in the presence of serum, which inhibits neurite growth, by immunoprecipitation with anti-N-WASP antibody (Figure 7C). Phosphorylation at Tyr253 after MG-132 treatment was confirmed with anti-P-Tyr253 N-WASP antibody on the immunoprecipitates (data not shown). In primary neurons, phosphorylation of N-WASP is also increased in MG-132 or lactacystin treatment (Figure 7C). Therefore, protein degradation through the ubiquitin proteasome system appears to be involved in the regulation of N-WASP in neurite extension.

Regulation of N-WASP through Protein Degradation

Then, we investigated ubiquitination of N-WASP. To examine ubiquitination of endogenous N-WASP in PC12 and N1E 115 cells cultured under differentiating conditions (DC; NGF and serum starvation for neurite extension, respectively) or proliferating conditions (PC; serum addition), we immunoprecipitated N-WASP from these cells and examined the state of ubiquitination. Under PC, ubiquitinated N-WASP was detected rarely by Western blotting of anti-N-WASP immunoprecipitates with anti-multiubiquitin monoclonal antibody, suggesting that either phosphorylated N-WASP is degraded rapidly

Figure 4. Phosphorylation of N-WASP by Src Family Kinases Is Essential for Neurite Extension in PC12 and N1E 115 Cells

(A and B) Inhibition of neurite extension by PP2, a specific inhibitor of Src family kinases. N1E 115 cells were serum starved with or without PP2 (10 μM) for 32 hr to induce neurite formation. DMSO was added in negative controls. Phalloidin staining was performed to visualize actin filaments in (A). Bar, 10 μm. Percentages of neurite-bearing cells are shown in (B). Neurite-bearing cells were defined as cells with processes longer than the greatest diameter of the cell body. Bars represent standard deviations. Similar results were obtained with PC12 cells (data not shown).

(C and D) The effect of mutant N-WASP on neurite extension. PC12 cells and N1E 115 cells were transfected with construct expressing the indicated N-WASP mutant with a GFP tag. Cells were then treated with NGF for PC12 cells or serum starvation for N1E 115 cells to induce neurite extension (DC) or maintained in medium with serum to induce proliferation (PC). The representative cells transfected with wild-type, Y253F, and Y253E and stained with phalloidin are shown in (C). In the merged images, red indicates actin filaments, and green indicates ectopic expression of the N-WASP construct with a GFP tag. Arrows indicate cells with ectopic expression of the specific construct. Bar, 10 μm. The percentages of neurite-bearing cells are shown in (D).

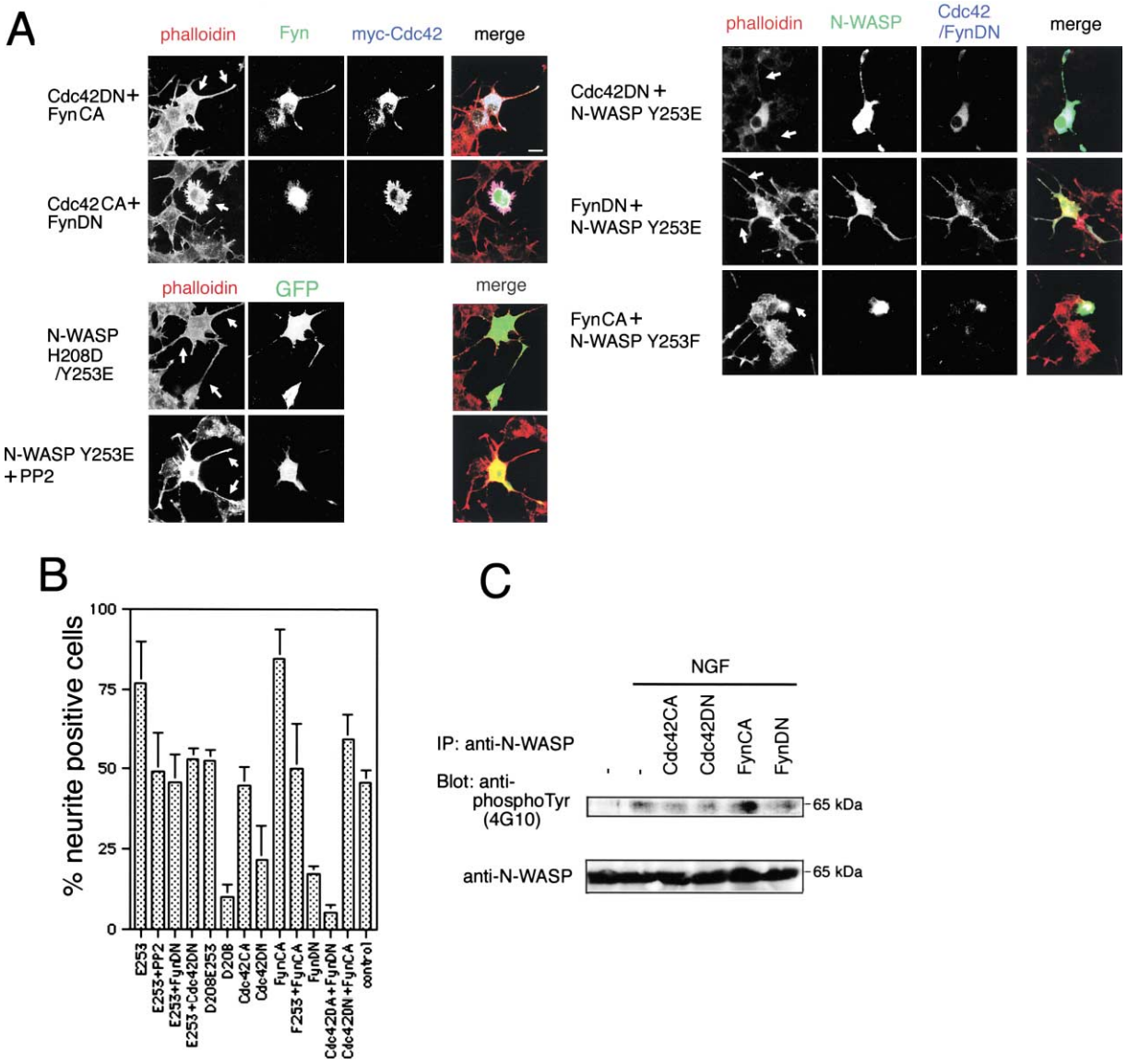


Figure 5. Relationship between Cdc42 and Src Family Kinases in Neurite Extension in PC12 and N1E 115 Cells

The effects of coexpression of constitutively active (CA) Cdc42 and dominant-negative (DN) Fyn, coexpression of DN Cdc42 and CA Fyn, coexpression of DN Cdc42 and Y253E N-WASP, and coexpression of DN Fyn and Y253E N-WASP on neurite extension under NGF. PC12 cells were cotransfected with mutant Cdc42, Fyn, and Y253E N-WASP as indicated. PC12 cells transfected with the N-WASP double mutant H208D/Y253E were also analyzed. Further, cells expressing Y253E N-WASP were treated with PP2, and neurite extension was analyzed. Representative transfected cells stimulated with NGF are shown in (A). In the merged images, the colors are as indicated in the panel labels. Bar, 10 μm. Arrows indicate cells with ectopic expression of specific constructs. Percentages of neurite-bearing cells are shown in (B). Similar results were obtained with N1E 115 cells.

(C) The effects of expression of CA or DN Cdc42 and CA or DN Fyn on phosphorylation of N-WASP of PC12 cells were analyzed by immunoprecipitation of N-WASP and then by Western blotting with anti-phosphotyrosine antibody (4G10). Because transfection efficiency was approximately 20%, transfection of DN Fyn did not suppress phosphorylation of N-WASP in immunoprecipitation experiments. Similar results were obtained with N1E 115 cells.

through proteasomes or that N-WASP is not ubiquitinated (Figure 7D). Ubiquitination of N-WASP was increased when proteasomes were inhibited with MG-132 in cells cultured under PC (Figure 7D). Further, in the pulse-chase experiments, degradation of N-WASP under PC is also inhibited by the proteasome inhibitor MG-132 (Figure 7E). Therefore, N-WASP appears to be phosphorylated by Src family kinases, even in the PC, but phosphorylated N-WASP is rapidly degraded.

When cells were cultured under DC, the ubiquitinated N-WASP was increased (Figure 7D). Ubiquitination of N-WASP under DC appeared to be dependent on phosphorylation of N-WASP because inhibition of Src family kinases by PP2 abolished ubiquitination of N-WASP (Figure 7D). The pulse-chase experiments demonstrated that degradation of N-WASP was downregulated in DC (Figure 7E), presumably explaining the increase in the ubiquitinated N-WASP. By either MG-132 or by DC, ubiquitinated N-WASP was prominently observed at a high molecular weight, above 140 kDa (Figure 7D), which

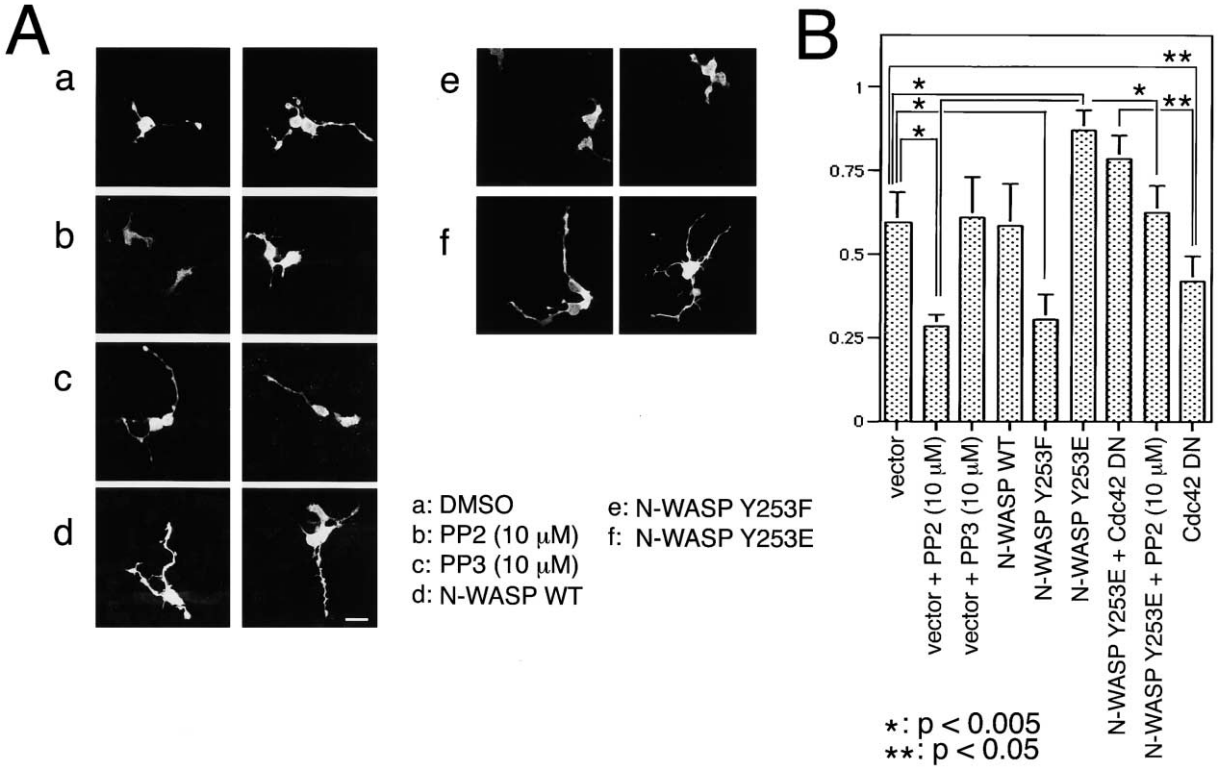


Figure 6. Essential Role of Phosphorylation of N-WASP by Src Family Kinases in Neurite Extension in Primary Neurons

(A) Neurite extensions of primary cortical neurons were analyzed with GFP fluorescence as a morphological marker. GFP expression (a) alone with DMSO (vehicle) control, (b) with PP2 (10 μM), and (c) with PP3 (10 μM); (d) wild-type N-WASP expression with bicistronic GFP expression; (e) Y253F N-WASP expression with bicistronic GFP expression; (f) GFP-tagged Y253E N-WASP expression. After culture for 18 hr, the neuron morphology was analyzed. Bar, 10 μm.

(B) The ratio of neurons with longer neurites (more than three times the length of the cell body) to neurons with shorter neurites. At least 100 cells were counted in every experiment. Data are the means of at least three independent experiments. Bars represent standard deviations. The significance of the difference between bars linked by lines was confirmed by Student's t test. Asterisk (*), $p < 0.005$; double asterisks (**); $p < 0.05$.

might indicate that N-WASP is ubiquitinated as a functional protein complex, for example, of a possible N-WASP dimer or of N-WASP and the other molecules.

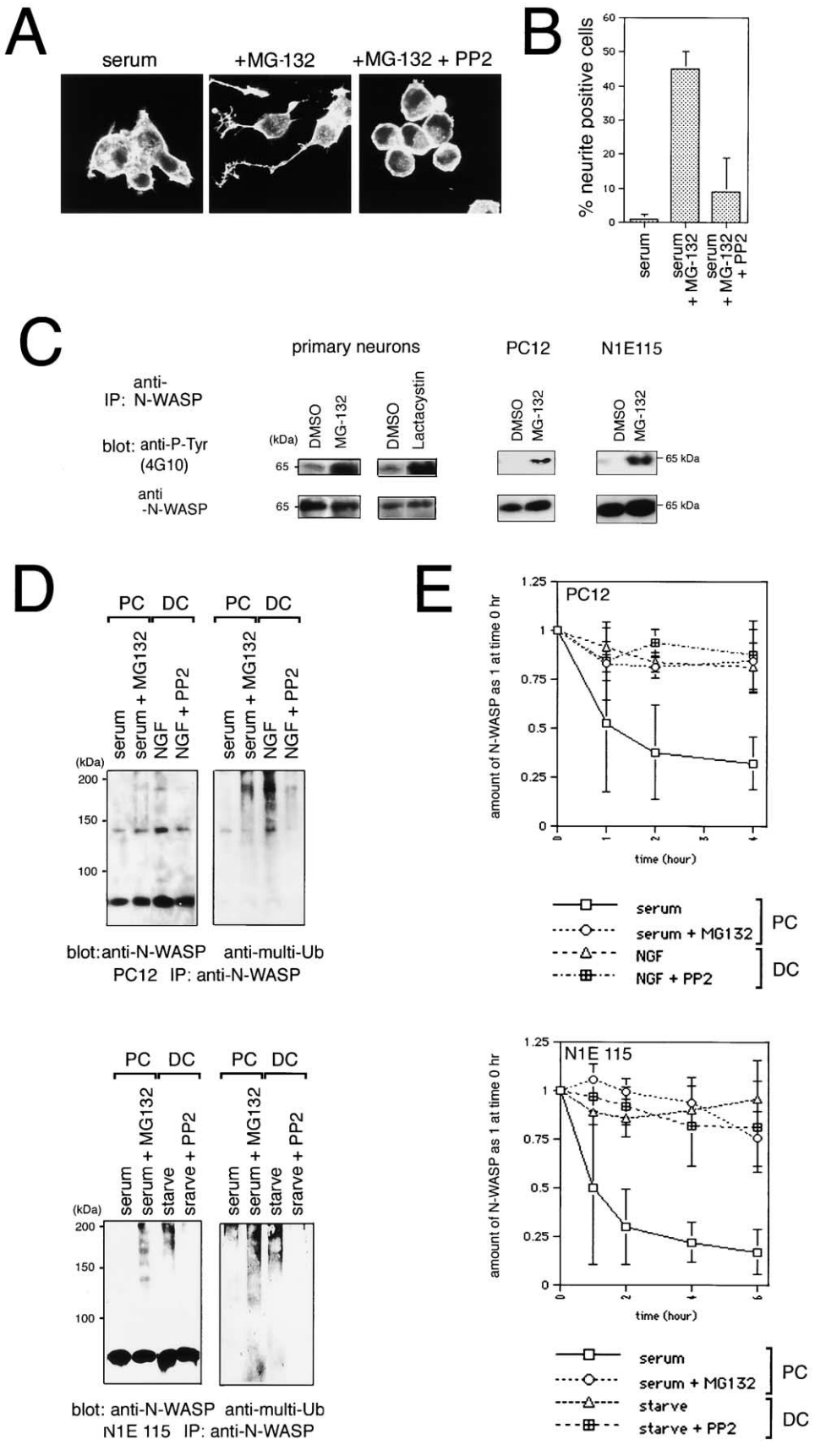
In primary cortical neurons, which are committed to neurite extension, ubiquitination of N-WASP was observed. Similar to the results from the cell lines, ubiquitination of N-WASP appears to be decreased by PP2 treatment and increased by MG-132 or lactacystin treatment (data not shown). Collectively, the inhibition of ubiquitin-dependent proteolysis of N-WASP occurs during conditions that favor neurite growth, resulting in the increase of phosphorylated N-WASP.

Discussion

In the present study, we showed that levels of active N-WASP, which are important for controlling neurite extension, are regulated through a balance of phosphorylation by Src family kinases and degradation by proteasomes. N-WASP is activated by Src family kinases through phosphorylation. When cells are cultured in conditions that favor proliferation, phosphorylated N-WASP is rapidly degraded through a ubiquitin proteasome pathway. Under neurite extension conditions, proteasomes are inhibited, leading to the accumulation of active N-WASP and neurite extension.

Possible Mechanism of Activation of N-WASP by Phosphorylation

We identified a novel mechanism for N-WASP activation. Phosphorylation of N-WASP at Tyr253 as well as the substitution of Tyr253 with glutamic acid activates N-WASP's ability in Arp2/3 complex-mediated actin polymerization. Tyr253 is located close to the GBD/CRIB motif of N-WASP. This region interacts with the VCA region, which is the Arp2/3 complex activating domain. Interactions between the basic GBD/CRIB motif region and VCA region block VCA binding with the Arp2/3 complex (autoinhibition) (Miki et al., 1998; Rohatgi et al., 1999; Higgs and Pollard, 2000; Kim et al., 2000). When Cdc42 activates N-WASP, Cdc42 induces a conformational change in GBD/CRIB, releasing association with VCA. In the case of phosphorylation, Tyr253 of N-WASP is likely to be located in one of the hydrophobic cores of the GBD/CRIB motif from structural analyses of WASP (Kim et al., 2000). Therefore, the introduction of a negative charge into this hydrophobic core through phosphorylation will cause a conformational change that disrupts autoinhibition. In addition to this mechanism, it is also possible that binding of the Fyn SH2 domain with the phosphorylation site released the autoinhibited structure. However, this is unlikely because the introduction of a negative charge by amino acid substitution


A
serum
+MG-132
+MG-132 + PP2
B
% neurite positive cells
serum
serum + MG-132
serum + MG-132 + PP2
C
primary neurons
PC12
N1E115
anti-
IP: N-WASP
blot: anti-P-Tyr
(4G10)
anti
-N-WASP
(kDa)
DMSO
MG-132
Lactacystin
65
65 kDa
D
PC
DC
serum
serum + MG132
NGF
NGF + PP2
(kDa)
200
150
100
blot:anti-N-WASP anti-multi-Ub
PC12 IP: anti-N-WASP
starve
srarve + PP2
N1E 115 IP: anti-N-WASP
E
PC12
N1E 115
amount of N-WASP as 1 at time 0 hr
time (hour)
serum
serum + MG132
NGF
NGF + PP2
starve
starve + PP2

(Y253E) activates N-WASP both in vivo and in vitro. In addition, the amino acid sequence of the N-WASP phosphorylation site (pYDFI) is not an Src family SH2 binding consensus sequence (pYEEI) (Songyang et al., 1993).

Phosphorylation of N-WASP and Neurite Extension

Phosphorylation of N-WASP by Src family tyrosine kinases is a critical event for neurite extension. In vitro, phosphorylation of N-WASP by Fyn, a member of Src family kinase, is enhanced slightly or significantly by several N-WASP binding molecules, including Cdc42, Ash/Grb2, and PIP2. These molecules alone can enhance N-WASP-induced actin polymerization (Rohatgi et al., 1999; Carlier et al., 2000); however, they only provide weak activation of N-WASP, indicating that a combination of these molecules or other activation mechanisms is necessary for full activation of N-WASP. Therefore, enhancement of phosphorylation by these molecules may be important for integrating several signaling pathways for neurite extension.

Interestingly, PIP2 provides the most effective enhancement of phosphorylation of N-WASP, suggesting that PIP2 works synergistically with Src family kinases in neurite extension. PIP2 activates N-WASP by itself and by enhancing the efficiency of N-WASP phosphorylation. PIP2 is thought to be involved in the regulation of the actin cytoskeleton through a variety of actin binding proteins (Laux et al., 2000; Sechi and Wehland, 2000). Therefore, PIP2 is likely to function as a positive regulator of both phosphorylation and activation of N-WASP in neurite induction.

Negative Regulation of N-WASP through Ubiquitination and Degradation

PC12 cells stimulated with NGF have elevated levels of ubiquitinated proteins, suggesting that the ubiquitin proteasome pathway is inhibited during neurite growth (Obin et al., 1999). For example, MAP kinase, a critical regulator of neurite extension, is upregulated by the treatment of cells with proteasome inhibitor (Hashimoto et al., 2000).

In the present study, we observed that N-WASP is a target of negative regulation by proteasomes. Phosphorylation of N-WASP, though it is an activating process, proceeded to ubiquitination and degradation through unknown mechanisms when cells were cultured under conditions favoring proliferation. This degradation appears to be suppressed when cells are cultured under neurite growth conditions, leading to accumulation of active N-WASP and neurite extension.

When cells are stimulated with NGF, the activity of Src is increased; however, this activation of Src only lasts about 3 hr in PC12 cells (Wooten et al., 2001), whereas neurite extension occurs over 1–2 days. We do not know how the activities of the other eight known Src family kinases are regulated. But, interestingly, in our present study, N-WASP phosphorylation persisted for a relatively long period during neurite extension. Therefore, other mechanisms must be necessary for maintenance of activated, phosphorylated N-WASP under NGF treatment. Reduced degradation of N-WASP is the most plausible mechanism underlying the maintenance of phosphorylated N-WASP levels. These findings suggest that NGF or neurite growth (differentiation) stimuli generate signals that negatively regulate degradation of N-WASP or other molecules.

Mechanism for Ubiquitination and Degradation of N-WASP

It is very likely that phosphorylation is tightly coupled with degradation of N-WASP because phosphorylated N-WASP accumulated and degradation of N-WASP decreased in the presence of proteasome inhibitors. This evidence suggests that phosphorylated N-WASP is ubiquitinated and then degraded to terminate neurite extension. Alternatively, it is still possible that ubiquitinated N-WASP with phosphorylation is more stable than ubiquitinated N-WASP without phosphorylation. In addition, it is notable that ubiquitinated N-WASP was observed at a much higher molecular weight than was N-WASP (Figure 7D). N-WASP might function in a huge protein complex, such as a possible N-WASP dimer or complex with molecules such as cortactin, as in podosome (Mizutani et al., 2002; Weaver et al., 2002). The function of these complexes might be regulated through ubiquitination. Further studies are needed to clarify the precise mechanisms of inhibition of degradation of N-WASP under differentiation/neurite outgrowth.

Figure 7. Effect of Proteasome Inhibitors on Phosphorylation of N-WASP

(A and B) The induction of neurite extension by proteasome inhibitors is dependent on the activities of Src family kinases. PC12 cells proliferating in serum were treated with MG-132 (20 μM) alone or in combination with PP2 (10 μM) for 16 hr. DMSO (vehicle) was added as a negative control. Phalloidin staining was then performed to visualize actin filaments (A). Bar, 10 μm. In (B), the percentages of neurite-bearing cells are shown. Neurite-bearing cells were defined as cells with processes longer than the greatest diameter of the cell body. Similar results were obtained with N1E 115 cells (data not shown).

(C) Primary neurons were cultured with or without MG-132 (5 μM). PC12 cells and N1E 115 cells cultured in medium with serum were treated with MG-132 (20 μM). N-WASP phosphorylation was analyzed by immunoprecipitation and then by Western blotting.

(D) Ubiquitination of N-WASP in cells cultured in the presence or absence of PP2 or MG-132 under proliferating and neurite growth conditions. PC12 cells or N1E 115 cells were induced, their neurite extension (differentiating condition [DC]) with or without PP2 or cultured in medium with serum (proliferating condition [PC]) with or without MG-132. Cell lysates were immunoprecipitated with anti-N-WASP antibody, and then subjected to Western blotting with anti-multiubiquitin antibody.

(E) Turnover of N-WASP by PC12 cells or N1E 115 cells cultured in the same conditions as those in (D). Cells were pulse labeled with ^{35}S-labeled methionine/cysteine and chased. N-WASP was then immunoprecipitated, analyzed by Western blotting, and subjected to autoradiography. Amounts of N-WASP immunoprecipitated, and radioactivity levels were measured by densitometry. The level of radioactivity per amount of N-WASP was considered to be 1 at time 0. The data shown are means of at least three independent experiments. Bars represent standard deviations.

Regulation of N-WASP for Neurite Extension

In the present study, we found that phosphorylation of N-WASP by Src family kinases is an essential step in neurite induction. One possible mechanism of neurite extension through N-WASP activation is as follows. Upon tyrosine phosphorylation of N-WASP by Src family kinases, the release of autoinhibition of N-WASP for actin polymerization and ubiquitination that targets N-WASP for degradation by proteasomes is induced. However, NGF or other differentiation stimuli simultaneously inhibit degradation of N-WASP. As a result, activated N-WASP accumulates without sustained activation by Src kinases, leading to neurite extension. In proliferating condition under serum or EGF, N-WASP is degraded rapidly, though it is activated through phosphorylation, resulting in the suppression of neurite formation.

In conclusion, neurite extension occurs when levels of phosphorylated N-WASP are increased by either activation of Src family kinases or inhibition of proteasomes. Thus, a balance between phosphorylation and degradation of N-WASP regulates neurite extension.

Experimental Procedures

Proteins

Various mutant and wild-type N-WASP proteins were expressed in Sf9 cells with the Bac-to-Bac baculovirus expression system (Gibco BRL) with N-terminal His_6-tag. Recombinant virus-infected Sf9 cells were lysed in lysis buffer (40 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% Triton X-100, 1 mM PMSF, and 1 μg/ml each of aprotinin and leupeptin). Lysates were then clarified by ultracentrifugation and affinity purified with Ni-NTA beads (Qiagen). GST-WH1 (amino acids 1–129), GST-IQ-CRIB (amino acids 127–277), GST-Pro-rich (amino acids 268–395), and GST-VCA (amino acids 392–505) were purified as described previously (Miki et al., 1996). Recombinant Cdc42 (isoform 1) was purified and loaded with GTPγS as described previously (Suetsugu et al., 1998). Arp2/3 complex was purified as described previously (Suetsugu et al., 2001). GST-Fyn was isolated as described previously (Nakazawa et al., 2001).

Phosphorylation of N-WASP In Vitro

Phosphorylation of N-WASP was performed as follows. Three micromolar N-WASP or its fragments, such as WH1, IQ-CRIB, pro-rich, VCA, and 0.5 μM of GST-Fyn, were mixed in solution containing 20 mM Hepes (pH 7.2), 10 mM $MgCl_2$, 3 mM $MnCl_2$, 150 mM KCl, and 1 mM ATP with or without 20 μCi/ml γ-^{32}P ATP. The mixture was incubated at 37°C for 10 min, analyzed by SDS-PAGE, and subjected to autoradiography.

Phosphorylated N-WASP for pyrene actin assay was prepared as follows. After the 60 min reaction, as described above, mixtures of Fyn and full-length N-WASP reacted with ATP were subjected directly to pyrene actin assay with 30-fold dilution to give 100 nM of N-WASP and 17 nM of Fyn. Approximately 50% of the N-WASP molecules were phosphorylated. Mixtures without Fyn, ATP, or both were included as negative controls and subjected to pyrene actin assays.

For the examination of molecules that enhance phosphorylation of full-length N-WASP, Cdc42 loaded with GTPγS (Suetsugu et al., 1998) and PIP2 was prepared (Rohatgi et al., 1999) as described previously. N-WASP (final 3 μM), GST-Fyn (2 μM), and molecules such as GTPγS-loaded Cdc42 (10 μM), PIP2 (4 μM), or Ash/Grb2 (10 μM) were mixed and incubated for 10 min at 37°C in solution, described above, and then analyzed by SDS-PAGE with 10% acrylamide gels. Gels were stained with Coomassie brilliant blue (CBB) and subjected to autoradiography. Bands corresponding to N-WASP and Fyn were excised from the gel, and the level of radioactivity was measured by scintillation counter. The percentages of phosphorylation of N-WASP and Fyn were then calculated.

Pyrene Actin Assay

Pyrene actin assay was performed as described previously (Rohatgi et al., 1999). Final concentrations of Arp2/3 complex, G-actin, and pyrenyl-actin were 60 nM, 2 μM, and 0.2 μM, respectively. The concentration of wild-type or mutant N-WASP was 100 nM, unless indicated otherwise. GTPγS-loaded Cdc42 and PIP2-containing vesicles were added to a final concentration of 500 nM and 1 μM, respectively. The concentration of full-length Fyn from the phosphorylation reaction mixture or SH3 domain of Fyn in the reaction mixture was 17 nM or 1 μM, respectively. Actin was added just before fluorescence and monitored with a fluorometer. The number of barbed ends was calculated at the point at which ~80% of G-actin was polymerized. The concentration of generated barbed ends was calculated with the equation k_+[actin monomers][barbed ends] = elongation rate. We assumed that pointed-end elongation was minimal; k_+ is a rate constant for the association of G-actin with filament ends.

Antibodies and Immunoprecipitation Analysis

Anti-N-WASP polyclonal antibody was produced as described previously (Miki et al., 1998). Anti-Fyn polyclonal antibody (sc-16) and anti-*myc* monoclonal antibody (9E10) were from Santa Cruz Biotech. Anti-phosphotyrosine monoclonal antibody (4G10) was from Upstate Biotech. Anti-ubiquitin monoclonal antibody (1B3) and anti-multiubiquitin antibody were from MBL. In some experiments, to detect ubiquitination of N-WASP, we conjugated anti-N-WASP and anti-multiubiquitin antibodies with biotin and detected them by peroxidase-conjugated avidin to reduce backgrounds on Western blots. Phosphorylation-specific antibody against N-WASP (anti-P-Tyr253 N-WASP antibody) was generated by immunization of rabbits with phosphorylated peptide (CTSKVS IpYDFI EK) conjugated with KLH, and then affinity purification and the removal of the fraction that reacted with nonphosphorylated peptide (CTSKVS IYDFI EK) were performed, as described previously (Nishizawa et al., 1991). Specificities for the phosphorylated and unphosphorylated peptides were examined by ELISA. The affinity for phosphorylated N-WASP peptide was more than 50,000-fold greater than the affinity for nonphosphorylated N-WASP. To detect phosphorylation of N-WASP, we harvested PC12, N1E 115, and COS-7 cells into lysis buffer containing pervanadate (Baba et al., 1999). N-WASP was immunoprecipitated with anti-N-WASP antibody as described previously (Fukuoka et al., 2001), and immunoprecipitates were subjected to Western blotting with anti-phosphotyrosine antibody (4G10) or anti-P-Tyr253 N-WASP antibody and with anti-N-WASP antibody.

Electroporation into Mouse Brain and Primary Cortical Neuron Culture

All surgery was performed according to the guidelines on animal experiments of the Japan Neuroscience Society. All efforts were made to minimize both the number of animals used and their suffering. Electroporation into mouse brain was performed as described previously (Tabata and Nakajima, 2001). Briefly, at embryonic day 14, pregnant ICR mice were anesthetized deeply, and the embryos were extracted. Approximately 2 μl of plasmid solution (7.5 mg/ml) was injected into the lateral ventricle of each embryo with a glass micropipette. The brain was placed in a tweezers-type electrode (CUY650-5; Tokiwa Science, Fukuoka, Japan), and an electronic pulse (35 V, 50 ms) was discharged five times at intervals of 950 ms with an electroporator (CUY21E; Tokiwa Science). After sitting on ice for approximately 1 hr, the cerebral cortices were dissected out and dispersed by trypsin and DNase I in Hanks' balanced salt solution. Cells were suspended in culture medium (Neurobasal medium [Invitrogen] supplemented with B27 [Invitrogen], glutamine, and antibiotics) and transferred to coverslips coated with poly-L-lysine at a density of 2.0×10^6 cells/cm^2. Expression of the ectopic protein was detected as early as 2 hr after plating (data not shown). Cells were incubated at 37°C for 18 hr, fixed, and observed under a fluorescent microscope. In some experiments, immunostaining with anti-GFP antibody was performed to obtain better resolution.

Pulse-Chase Experiments for Monitoring Turnover of N-WASP

PC12 and N1E 115 cells (2×10^6) were plated in a 6 cm dish and cultured overnight under neurite growth conditions (DC; NGF

treatment for PC12 cells and serum starvation for N1E 115 cells) or proliferating conditions (PC; serum addition) with or without 10 μM PP2 or 20 μM MG-132. Before labeling, cells were washed twice in DMEM without methionine and cysteine and then incubated in DMEM lacking methionine and cysteine for 1 hr at 37°C. Each 6 cm dish was labeled with 50 μCi of ^{35}S-labeled methionine/cysteine (Redivue Pro-Mix L-^{35}S In Vitro Cell Labeling Mix; Amersham) for a 3 hr pulse under DC or PC (serum without methionine and cysteine was used) with or without PP2 or MG-132, washed three times with phosphate-buffered saline (PBS), and chased with normal media with or without PP2 and MG-132. At each time point, cells were harvested, and N-WASP was immunoprecipitated with anti-N-WASP antibody. The level of radioactivity was assessed by autoradiography and then by densitometry with NIH image software. The radioactivity at time 0 was set as 1, and the relative radioactivity at each time point is shown with standard deviation as an error bar.

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