Essential Role of Neural Wiskott-Aldrich Syndrome Protein in Neurite Extension in PC12 Cells and Rat Hippocampal Primary Culture Cells

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Neural Wiskott-Aldrich syndrome protein (N-WASP) is an actin-regulating protein that induces filopodium formation downstream of Cdc42. It has been shown that filopodia actively extend from the growth cone, a guidance apparatus located at the tip of neurites, suggesting their role in neurite extension. Here we examined the possible involvement of N-WASP in the neurite extension process. Since verprolin, coflin homology and acidic region (VCA) of N-WASP is known to be required for the activation of Arp2/3 complex that induces actin polymerization, we prepared a mutant (Δcof) lacking four amino acid residues in the coflin homology region. The corresponding residues in WASP had been reported to be mutated in some Wiskott-Aldrich syndrome patients. Expression of Δcof N-WASP suppressed neurite extension of PC12 cells. In support of this, the VCA region of Δcof cannot activate Arp2/3 complex enough compared with wild-type VCA. Furthermore, H208D mutant, which has been shown unable to bind to Cdc42, also works as a dominant negative mutant in neurite extension assay. Interestingly, the expression of H208D-Δcof double mutant has no significant dominant negative effect. Finally, the expression of the Δcof mutant also severely inhibited the neurite extension of primary neurons from rat hippocampus. Thus, N-WASP is thought to be a general regulator of the actin cytoskeleton indispensable for neurite extension, which is probably caused through Cdc42 signaling and Arp2/3 complex-induced actin polymerization.

The actin cytoskeleton plays a critical role in the regulation of cellular morphological change in response to various external stimuli (1, 2). In the case of neural development, actin filaments have been shown to accumulate at the growth cone (3), a guidance apparatus located at the tip of growing neurites. It has long been suggested that the actin cytoskeletal reorganization at growth cones including filopodium and lamellipodium formation is the key determinant of the direction and/or speed of neurite extension. Thus, clarification of the regulatory mechanism behind the reorganization of the actin cytoskeleton in neurons will ultimately lead to a better understanding of neural development.

Evidence has been accumulating that Rho family small GTPases regulate the reorganization of the actin cytoskeleton (4). Indeed, two family members, Cdc42 and Rac, are shown to induce filopodium and lamellipodium formations, respectively (5–7). Furthermore, recent reports demonstrated that their function is essential for neurite extension in N1E-115 and PC12 cells (8, 9). However, the target proteins that function in neurite extension in neurons have yet to be identified.

We found N-WASP as a 65-kDa protein that binds to the SH3 domains of Ash/Grb2 adaptor protein (10). N-WASP possesses a GBD/CRIB motif through which N-WASP directly binds to activated Cdc42 (11). In addition, N-WASP can induce filopodium formation downstream of Cdc42 in COS 7 cells (11). We have also shown that verprolin homology domain (V), which is an actin-binding site, is essential for filopodium formation (12), and coflin homology domain (C) and the acidic (A) region in the C terminus are binding sites for Arp2/3 complex (13). Furthermore, it has been made clear that Arp2/3 complex-induced actin polymerization is markedly enhanced by N-WASP only in the presence of Cdc42 and phosphatidylinositol 4,5-bisphosphate (13).

Considering that N-WASP was originally found and shown to be most abundant in brain (10, 14), it is quite probable that N-WASP plays an important role in neurite extension.

To examine this possibility, we prepared a mutant N-WASP (Δcof) that has mutations in the coflin homology domain through which N-WASP regulates Arp2/3 complex-induced actin polymerization. By expressing wild-type and Δcof N-WASP in PC12 cells and primary culture neurons obtained from rat hippocampus, we show here that N-WASP is a general regulator of neurite extension.

EXPERIMENTAL PROCEDURES

Antibodies—The polyclonal anti-N-WASP antibody and the antibody specific for Arp3 were made, respectively, as described previously (10, 15). The secondary antibodies linked to alkaline phosphatase (used in Western blotting) and fluorescein (used in immunofluorescence microscopy) were from Promega and Cappel, respectively. The monoclonal antibody specific for neuronal class III β-tubulin and the polyclonal antibody specific for β-galactosidase were purchased from Berkeley Antibody Co. and Chemicon, respectively.

Construction of Mutants, Such as Δcof, H208D Mutant, and H208D-Δcof Double Mutant—Wild-type rat N-WASP cDNA has been isolated and reported previously (16). A mutant (H208D) in which a histidine residue (H) conserved in all the GBD/CRIB motifs is replaced by an

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aspartate (D) was prepared by site-directed mutagenesis. Another mutant with deletion of the cofilin homology domain (Δcof; substituting amino acids Lys-Arg-Ser-Lys (473–476) for Asp-Ile (GATATC; EcoRV site)) was made by polymerase chain reaction (PCR) using two sets of oligonucleotide primers (5′-CCGCTCGAGACCATGAGCTCGGAGCCAC-3′ and 5′-GGGGATATCCTGCATCACTTCCATCAGCGCA-3′ for the former half and 5′-CCGGATATCCTGCATCACTTCCATCAGCGCA-3′ and 5′-CGAATTCTCAGTCTTCCCACTCATCATCATC-3′ for the latter half). After the confirmation of the nucleotide sequences, the two PCR products were ligated at the EcoRV site. H208D mutant was made as described previously (11). The double-mutated N-WASP (H208D-Δcof) was prepared from mutants H208D and Δcof by truncation and ligation at XhoI sites. The nucleotide sequences of all mutants were confirmed.

GST Fusion Proteins—The GST fusion proteins of the C-terminal region of wild-type N-WASP and Δcof (GST-VCA and GST-Δcof-VCA) were constructed, expressed, and purified as described previously (10).

In Vitro Binding Assay Using GST Fusion Proteins—Each GST fusion protein was immobilized on glutathione-Sepharose beads and incubated with protein samples (Arp2/3 complex or actin purified from rabbit muscle) and nucleated actin polymerization. Fluorescence intensity that reflects actin polymerization (2.2 μM actin and 60 nM Arp2/3 complex) was followed in the absence (○, actin only; ■, actin and Arp2/3 complex) or presence of various amounts of GST-VCA (▲, 100 nM; □, 30 nM), and GST-Δcof-VCA (▲, 100 nM; ▼, 30 nM).

Fig. 1. Cofilin homology domain is important for N-WASP-activated Arp2/3 complex-induced actin polymerization. A, amino acid sequences at cofilin homology domain of rat wild-type and Δcof N-WASP and human WASP. Modified amino acid residues in Δcof are boxed in black. B, schematic structures of GST fusion proteins of VCA-region of N-WASP. Functional domains of N-WASP are indicated as boxes (abbreviations used are: verp, verprolin homology domain; cof, cofilin homology domain; acidic, acidic amino acid residue-rich region). C, binding of Arp2/3 complex (upper figure) and actin (lower figure) to GST-VCA and GST-Δcof-VCA. GST-VCA and GST-Δcof-VCA were incubated with Arp2/3 complex purified from bovine brain or actin purified from rabbit muscle and precipitated with glutathione-Sepharose. The bound proteins were analyzed by Western blotting with anti-Arp3 antibody or Coomassie staining. D, Δcof mutation reduces the activation activity of VCA region for Arp2/3-induced actin polymerization. Fluorescence intensity that reflects actin polymerization (2.2 μM actin and 60 nM Arp2/3 complex) was followed in the absence (○, actin only; ■, actin and Arp2/3 complex) or presence of various amounts of GST-VCA (▲, 100 nM; □, 30 nM), and GST-Δcof-VCA (▲, 100 nM; ▼, 30 nM).

Fig. 2. Construction of H208D, Δcof, and H208D-Δcof N-WASP. A, schematic structures of wild-type, H208D, Δcof, and H208D-Δcof N-WASP. Functional domains of N-WASP are indicated as boxes (abbreviations used are: WH1, WASP homology 1; basic, basic amino acid residue-rich region; GBD, GBD/CRIB motif domain; Pro-rich, proline-rich region; verp, verprolin homology domain; cof, cofilin homology domain; acidic, acidic amino acid residue-rich region). B, Western blot analysis of lysates from wild-type, Δcof, H208D, or H208D-Δcof N-WASP-expressing PC12 cells. Untransfected cells and GFP-transfected cells are also analyzed as controls. The bands of N-WASP are indicated by an arrow.
with the same stimulation. The percentage of neurite-bearing cells for each treatment were counted. Results are expressed as the mean of at least three independent experiments. Similar results were recorded. At least 150 cells body lengths were scored as neurite bearing. Three separate experiments were done, and similar results were recorded. At least 150 cells for each treatment were counted. Results are expressed as the mean percentage of neurite-bearing cells. Symbols indicate the result of a Student's t test; *, p < 0.01, compared with each column of GFP-expressing cells with the same stimulation.

Fig. 4. Quantitation of either NGF- or cAMP-induced neurite extension from PC12 cells. Cells with neurites of greater than two body lengths were scored as neurite bearing. Three separate experiments were done, and similar results were recorded. At least 150 cells for each treatment were counted. Results are expressed as the mean percentage of neurite-bearing cells. Symbols indicate the result of a Student's t test; *, p < 0.01, compared with each column of GFP-expressing cells with the same stimulation.

Fig. 3. Effect of the expression of wild-type and Δcof N-WASP on NGF- and cAMP-induced neurite extension from PC12 cells. Wild-type, Δcof, H208D, and H208D-Δcof N-WASP were transiently expressed in PC12 cells, and then the cells were stimulated with 100 ng/ml NGF or with 1 mM Bt,cAMP for 72 h. Cells were fixed and stained with phalloidin and anti-N-WASP antibody for immunofluorescence microscopy. GFP-transfected cells, either unstimulated or stimulated with NGF or cAMP, were also stained as a control. The left-hand panel of each row shows PC12 cells stained with phalloidin to visualize actin filaments. The right-hand panel shows the same cells stained with anti-N-WASP antibody or ob.
A

![Western blot of LacZ, wild-type, ∆cof N-WASP](image)

B

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![Graph showing percentage of neurite-bearing cells](image)

**Fig. 5.** ∆cof N-WASP inhibits neurite extension from primary culture hippocampal neural cells. Wild-type and ∆cof N-WASP were transiently expressed in rat hippocampal primary culture cells using recombinant adenovirus (m.o.i. 50). Cells were cultured for 72 h after infection, and then some cells were used for Western blotting. Others were fixed and stained with anti-β-tubulin antibody and anti-N-WASP antibody for immunofluorescence microscopy. Cells infected with LacZ-expressing recombinant adenovirus were also fixed and stained with anti-β-galactosidase antibody as a control. A, cell lysates were subjected to Western blotting with anti-N-WASP antibody. B, fluorescent images of typical fields are shown. The left-hand panel shows the cells stained with anti-β-tubulin antibody to visualize neural cells. The middle panel shows the same cells stained with anti-N-WASP or anti-β-galactosidase antibody. The right-hand panel shows the merged image of the left-hand and the middle panel of each row. Anti-β-tubulin signal and anti-N-WASP or anti-β-galactosidase signal are shown as red and green, respectively, and thus areas with overlapping anti-β-tubulin staining and anti-N-WASP or anti-β-galactosidase staining are yellow. After infection, the percentage of neurite-bearing cells. Cells with neurites of greater than five body lengths were scored as neurite-bearing, and cells aggregated into groups of more than five were not counted. Three separate experiments were done, and similar results were recorded. At least 40 cells for each treatment were counted. Results are expressed as the mean percentage of neurite-bearing cells. Symbols indicate the result of the chi-squared test; *, p < 0.01, compared with the control (LacZ).
expressed to Western blot analysis with anti-N-WASP antibody (Fig. 2B). All recombinant N-WASP proteins were found to be expressed at similar levels.

We next investigated the morphological changes of PC12 cells in both the presence and absence of NGF. Typical photomicrographs of cells are shown in Fig. 3. Cells transfected with GFP develop neurites in the presence but not in the absence of NGF. The GFP expression had no significant effect on neurite extension in PC12 cells (data not shown). In contrast, transient overexpression of H208D mutant, which cannot bind to Cdc42, strongly inhibits neurite outgrowth induced by NGF and makes the cells round like unstimulated cells. The expression of Δcof mutant also suppresses the long neurite extension, but compared with H208D mutant-expressing cells, Δcof-expressing cells bear short processes in response to NGF, and thus, the expression of Δcof is not as effective as H208D in blocking the morphologic change induced by NGF. These differences may be explained by the data that GST-Δcof-VCA still has a weak Arp2/3 complex-activating function (Fig. 1, C and D). On the other hand, the expression of H208D-Δcof mutant N-WASP does not inhibit neurite extension induced by NGF, suggesting that H208D-Δcof can bind neither Cdc42 nor Arp2/3 complex, and thus, Cdc42 signal is transmitted to Arp2/3 complex through endogenous N-WASP. Among GFP-transfected cells, the proportion of neurite-bearing cells was 50% in the presence of NGF (Fig. 4). However, among Δcof-expressing cells expressing wild-type N-WASP in PC12 cells (data not shown). In contrast, transient overexpression of H208D mutant-expressing cells, Δcof-expressing cells bear short processes in response to NGF, and thus, the expression of Δcof is not as effective as H208D in blocking the morphologic change induced by NGF. These differences may be explained by the data that GST-Δcof-VCA still has a weak Arp2/3 complex-activating function (Fig. 1, C and D). On the other hand, the expression of H208D-Δcof mutant N-WASP in PC12 cells has no significant effect on neurite extension. In contrast to the Δcof-expressing cells, the cells overexpressing wild-type N-WASP develop neurites in response to NGF (Fig. 3). The proportion of neurite-bearing cells in wild-type N-WASP-expressing cells was 53% in the presence of NGF. These results indicate that the expression of wild-type N-WASP in PC12 cells has no significant effect on neurite extension and that wild-type N-WASP expression alone is not sufficient to induce neurite extension in the absence of NGF. On the other hand, only 11% of H208D mutant-transfected cells showed neurite extension, indicating that Cdc42 is essential to this process. Interestingly, neurite extension by NGF was almost recovered to normal levels (48%) in H208D-Δcof double mutant-expressing cells.

N-WASP Functions in cAMP-induced Neurite Extension—It is known that neurite extension of PC12 cells is also induced by several other stimulators, such as KCl-induced depolarization and cAMP (29, 30). To examine whether N-WASP plays a critical role in neurite extension induced by stimulants other than NGF, the effects of the expression of N-WASP mutants on neurite extension induced by cAMP were investigated. After the transfection with either GFP, wild-type N-WASP, Δcof, H208D mutant, or H208D-Δcof double mutant, cells were stimulated with Bt2cAMP, an analogue of cAMP that can permeate the cell membrane. As shown in Fig. 3, the expression of Δcof also results in an inhibition of neurite extension, whereas cells expressing wild-type N-WASP normally develop neurites. H208D mutant also strongly inhibits neurite extension. On the other hand, H208D-Δcof double mutant does not. The proportion of neurite-bearing cells is 58% among GFP-transfected cells, 22% among Δcof-expressing cells, and 59% among wild-type N-WASP-expressing cells (Fig. 4). On the other hand, it is 17% among H208D mutant-expressing cells and 49% among H208D-Δcof double mutant-expressing cells stimulated with Bt2cAMP. Thus, as the case of NGF stimulation, the expression of H208D mutant inhibits neurite outgrowth most severely, and Δcof mutant also inhibits it significantly in response to Bt2cAMP, whereas the expression of H208D-Δcof double mutant does not have a significant effect on the neurite extension. Effects of N-WASP Expression on Neurite Extension from Rat Hippocampal Primary Culture Cells—The results presented above suggest N-WASP to be a generally required component in neurite extension in PC12 cells. However, the PC12 cell line, which is routinely used as an experimental model of neural cells, does not have all the properties of neural cells. To investigate the role of N-WASP in neurite extension in more native conditions, we carried out experiments with rat hippocampal primary culture cells. Primary culture cells were prepared from rat hippocampus, and adenosine-mediated gene transduction was done. To estimate N-WASP expression mediated by recombinant adenosivirus, the virus-infected cell lysates were subjected to Western blot analysis with anti-N-WASP antibody. As shown in Fig. 5A, in cells infected with wild-type and Δcof recombinant N-WASP adenoviruses (m.o.i. 50), 15-fold or more N-WASP was detected than in the control cells infected with LacZ-expressing recombinant adenoviruses (m.o.i. 50). Under this condition, the Δcof expression inhibits neurite extension (Fig. 5B). In contrast, LacZ-expressing or wild-type N-WASP-expressing neural cells develop neurites. Thus, also in primary cultured neural cells, the expression of Δcof inhibits neurite outgrowth, whereas the expression of wild-type N-WASP does not. Proportions of neurite-bearing cells were also calculated in control, wild-type, and Δcof N-WASP-expressing cells (Fig. 5C). Among LacZ-expressing control cells, 74% showed neurite extension. Wild-type N-WASP-expressing cells showed a similar percentage, 76%, but in Δcof N-WASP-expressing cells, the percentage was reduced to 29%.

Taken together, our results strongly suggest that N-WASP is a crucial and general component in neurite extension. Furthermore, this phenomenon is probably caused through Cdc42-triggered N-WASP activation and the consequent activation of Arp2/3 complex-dependent actin polymerization.

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