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WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac

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Rac is a Rho-family small GTPase that induces the formation of membrane ruffles. However, it is poorly understood how Rac-induced reorganization of the actin cytoskeleton, which is essential for ruffle formation, is regulated. Here we identify a novel Wiskott–Aldrich syndrome protein (WASP)-family protein, WASP family Verprolin-homologous protein (WAVE), as a regulator of actin reorganization downstream of Rac. Ectopically expressed WAVE induces the formation of actin filament clusters that overlap with the expressed WAVE itself. In this actin clustering, profilin, a monomeric actin-binding protein that has been suggested to be involved in actin polymerization, was shown to be essential. The expression of a dominant-active Rac mutant induces the translocation of endogenous WAVE from the cytosol to membrane ruffling areas. Furthermore, the co-expression of a ΔVPH WAVE mutant that cannot induce actin reorganization specifically suppresses the ruffle formation induced by Rac, but has no effect on Cdc42-induced actin-microspike formation, a phenomenon that is also known to be dependent on rapid actin reorganization. The ΔVPH WAVE also suppresses membrane-ruffling formation induced by platelet-derived growth factor in Swiss 3T3 cells. Taken together, we conclude that WAVE plays a critical role downstream of Rac in regulating the actin cytoskeleton required for membrane ruffling.

Keywords: actin/profilin/Rac/WASP/WAVE

Introduction

The actin cytoskeleton plays critical roles in cell morphological changes and motility (reviewed by Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). Many actin-binding regulatory proteins have been identified and classified according to their effects on actin filaments, including globular actin (G-actin)-sequestering proteins, severing (depolymerizing) proteins, capping proteins and bundling (gelating) proteins. For their special roles in cells, however, these proteins do not function independently, but rather do so in harmony.

Rho-family small GTPases such as Rho, Rac and Cdc42 are thought to be organizers of the actin cytoskeleton. Experiments using dominant-negative and active mutants have shown that Rho, Rac and Cdc42 regulate the formation of stress fibers, lamellipodia (membrane rufflings) and filopodia (microspikes) in mammalian cells, respectively (Ridley and Hall, 1992; Ridley *et al*., 1992; Kozma *et al*., 1995; Nobes and Hall, 1995). In all of these events, actin polymerization has been shown to be an essential step. Furthermore, a recent report clearly demonstrated that in the case of Rac (but not in the case of Rho), newly polymerized actin filaments, visualized by injecting fluorescence-labeled monomeric actin into the cells, accumulated at membrane ruffles (Machesky and Hall, 1997), strongly suggesting that the accumulation of actin filaments itself is the driving force for the formation of the special structures induced by these GTPases.

In this respect, downstream effector molecules that induce actin reorganization are thought to be crucial for the induction of morphological change. In the case of Cdc42-induced filopodium formation, N-WASP appears to play such roles. N-WASP was originally discovered as a 65-kDa protein that binds to the SH3 domains of the Grb2 adaptor protein (Miki *et al*., 1996). However, N-WASP has also been found to associate directly with activated Cdc42 (Miki *et al*., 1998) and regulate the actin cytoskeleton through profilin (Suetsugu *et al*., 1998). In addition, we have shown that N-WASP is recruited to one pole of the surface of the infectious bacteria, *Shigella flexneri*, through direct association with the *Shigella* VirG protein and plays critical roles in the formation of the actin comet tail (Suzuki *et al*., 1998), by which *Shigella* swims in the cytoplasm of infected cells in a rocket-like manner. These results strongly suggest that N-WASP is an inducer of actin reorganization downstream of various stimuli. p140mDia, a mammalian homolog for *Drosophila* diaphanous protein, may also play similar roles downstream of Rho (Watanabe *et al*., 1997). p140mDia also binds directly to both activated Rho and profilin. Indeed, the ectopic expression of p140mDia has been shown to reorganize the actin cytoskeleton. Although the *in vivo* role of p140mDia downstream of Rho has not yet been clearly assigned, it is quite possible that p140mDia may supply the source of actin filaments required for actin cytoskeletal reorganization that proceeds in parallel with stress-fiber formation.

The N-WASP-related molecule WASP was originally identified as the gene product whose mutation is responsible for the human hereditary disease Wiskott–Aldrich syndrome, which is characterized by thrombocytopenia, eczema and immunodeficiency (Derry *et al*., 1994). The ectopic expression of WASP has been shown to induce the formation of actin clusters around the nucleus in a Cdc42-dependent manner (Symons *et al*., 1996). At present, there remain many questions concerning the relationship between actin-cluster formation and the physiological function of WASP. However, it is accepted that WASP

A

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WASP (human)                    RGALLDQIRQGIQLNK
N-WASP (human)                  KAALLDQIREGAQLKK
N-WASP (human)                  RDALLDQIRQGIQLKS
verprolin (S.cerevisiae)        RDALLGDIRKGMKLKK
WAVE (human)                    RSVLLEAIRKGIQLRK

consensus                       R ALLD IR G QLKK
                                K    E         R
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B

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MPLVKRNIDPRHLCHTALPRGIKNELECVTNISLANIIRQLSSLSKYAED

IFGELFNEAHSFSFRVNSLQERVDRLSVSVTQLDPKEEELSLQDITMRKA
            putative leucine zipper motif
FRSSTIQDQQLFDRKTLPIPLQETYDVCEQPPPLNILTPYRDDGKEGLKF

YTNPSYFFDLWKEKMLQDTEDKRKEKRKQKQKNLDRPHEPEKVPRAPHDR
                                    basic region
RREWQKLAQGPELAEDDANLLHKHIEVANGPASHFETRPQTYVDHMDGSY

SLSALPFSQMSELLTRAEERVLVRPHEPPPPPPMHGAGDAKPIPTCISSA

TGLIENRPQSPATGRTPVFVSPTPPPPPPPLPSALSTSSLRASMTSTPPP
                          Pro-rich region
PVPPPPPPPPATALQAPAVPPPPAPLQIAPGVLHPAPPPIAPPLVQPSPPV

ARAAPVCETVPVHPLPQGEVQGLPPPPPPPPLPPPGIRPSSPVTVTALAH

PPSGLHPTPSTAPGPHVPLMPPSPPSQVIPASEPKRHPSTLPVISDARSV

LLEAIRKGIQLRKVEEQREQEAKHERIENDVATILSRRIAVEYSDSEDDS
  VPH domain                                      acidic
EFDEVDWLE
 region
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Fig. 1. Identification of WAVE. (**A**) Alignment of VPH domain sequences. Highly conserved residues are shown in italics under the sequences. Database accession numbers (NCBI) are 695151 (human WASP), 2116984 (human N-WASP), 439289 (*Saccharomyces cerevisiae* verprolin) and 1665805 (human WAVE). (**B**) Sequence of WAVE. A putative leucine-zipper motif, a highly basic region, a proline-rich region, a VPH domain and a highly acidic region are shown as underlined bold letters.

also plays an essential role in actin reorganization downstream of specific signals, a function required for the normal development and/or function of hematopoietic cells.

In order to explore the mechanism of signal-induced actin regulation, we performed a database search for novel WASP-family proteins. As a result, we found a cDNA with an unknown function containing a putative reading frame showing structural similarity to WASP and N-WASP. Here, we report the functional analysis of this product protein, WAVE, and present evidence that WAVE is involved in the actin reorganization required for the formation of membrane ruffles induced by Rac.

Results

Identification of WAVE

We performed a database search for novel WASP-like molecules. For this purpose, we selected the sequence of the Verprolin-homology (VPH) domain (shown in Figure 1A), a sequence that is highly conserved between WASP and N-WASP, and is essential for both direct *in vitro* binding to actin and *in vivo* actin reorganization (Miki and Takenawa, 1998). As a result, one cDNA clone, KIAA0269 (DDBJ/EMBL/GenBank accession number D87459; Nagase *et al*., 1996), was found to possess a similar VPH-domain-coding sequence. The predicted amino acid sequence of the product protein is shown in Figure 1B. The overall structure is similar to WASP-family proteins in that it possesses a long proline-rich region, a VPH domain, a highly acidic region at the C-terminus and a highly basic region (Derry *et al*., 1994; Miki *et al*., 1996). Thus, we named this putative protein WASP-family Verprolin-homologous protein, WAVE. However, the sequence of the N-terminal half shows no similarity to either WASP or N-WASP. In the case of WASP and N-WASP, the N-terminal regions are known to be important for their regulation. For example, GBD/CRIB motifs that exist in the N-terminal half of both WASP and N-WASP have been shown to bind to activated Cdc42, a critical regulator of the actin cytoskeleton (Aspenstrom *et al*., 1996; Symons *et al*., 1996; Miki *et al*., 1998). Thus, WAVE is thought to be involved in actin reorganization, but to be regulated in a manner quite different from WASP and N-WASP.

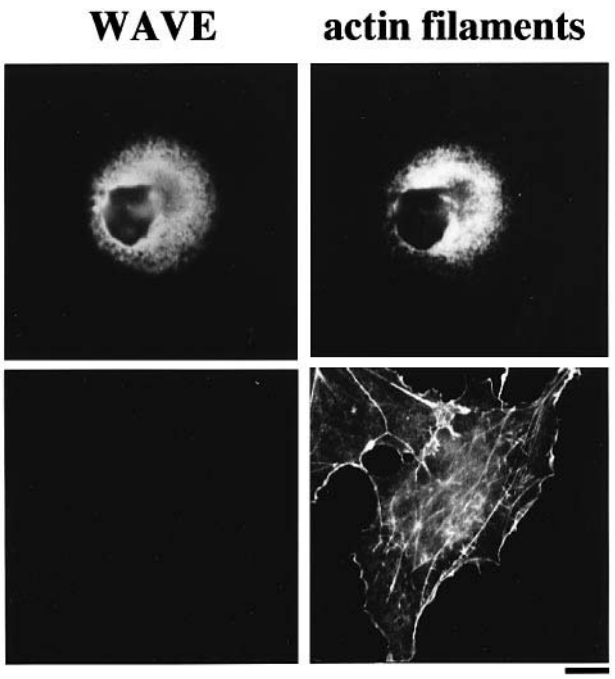

Fig. 2. Ectopic expression of WAVE. Myc-tagged WAVE was expressed transiently in COS 7 cells, and the cells were stained with anti-myc antibody (polyclonal) and phalloidin. Bar, 10 μm.

Ectopic expression of WAVE

In order to examine the physiological function of WAVE, we added a myc-tag to the molecule and then expressed the protein ectopically in COS 7 cells. The transfected cells were doubly stained with anti-myc antibody and phalloidin. As shown in Figure 2, WAVE expression induced the formation of actin filament clusters that co-localized with the expressed WAVE itself. Such actin cytoskeletal reorganization was observed in more than 90% of WAVE-expressing cells. The number of actin filaments that normally exist just beneath the plasma membrane tended to decrease, which may explain why some of the WAVE-expressing cells become rounded as shown in Figure 2.

WAVE induces actin clustering through profilin

As described above, WAVE has a proline-rich region containing several long poly-proline stretches. Long poly-proline stretches are known to be binding sites for

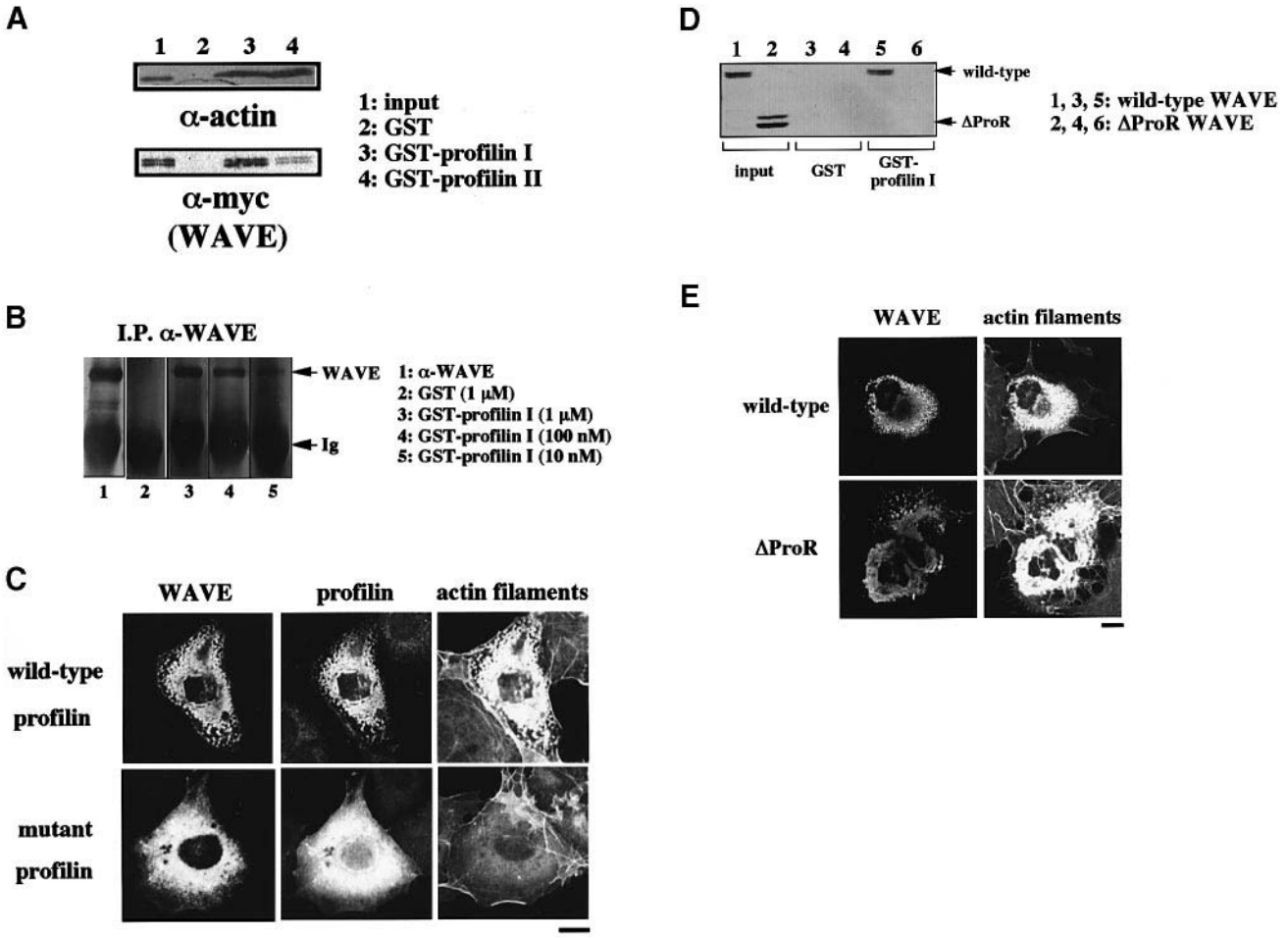

Fig. 3. Induction of actin clustering through profilin. (**A**) Binding to profilin. GST–fusion proteins of profilin I and II were immobilized on beads and mixed with lysates of COS 7 cells expressing myc-tagged WAVE. The bound proteins were analyzed by Western blot with anti-myc antibody and anti-actin antibody. (**B**) Direct binding between WAVE and profilin. WAVE protein was immunoprecipitated with anti-WAVE antibody (characterized in the experiment shown in Figure 4) from WAVE-expressing COS 7 cell lysates and then subjected to Far Western blot assay using GST–profilin I as the probe. The results using GST probe are also shown as negative control. (**C**) Inhibition of WAVE-induced actin clustering by the co-expression of mutant profilin I. FLAG-tagged WAVE and myc-tagged profilin I (wild-type or mutant) were transiently co-expressed in COS 7 cells. The cells were stained with anti-myc antibody (polyclonal), anti-FLAG antibody (monoclonal) and phalloidin. Bar, 10 μm. (**D**) Abolishon of the profilin-binding ability by the deletion of the proline-rich region. ΔProR WAVE protein was checked for profilin-I-binding ability by the same method as in (A). (**E**) Actin clustering by ΔProR WAVE. Myc-tagged ΔProR WAVE protein was expressed in COS 7 cells, and the cells were stained with anti-myc antibody (polyclonal) and phalloidin. Bar, 10 μm.

profilin, a protein that has been strongly suggested to be involved in actin polymerization (Pantaloni and Carlier, 1993; Theriot *et al*., 1994). In the case of N-WASP, we recently showed that profilin associates directly with N-WASP and is essential for the actin-microspike formation induced by Cdc42 and N-WASP (Suetsugu *et al*., 1998). Indeed, glutathione *S*-transferase (GST)-fusion proteins of profilin I and II were shown to bind to WAVE (Figure 3A). In this case, profilin I seemed to bind WAVE more strongly than profilin II. By Far Western blot assay using GST–profilin I probe, direct binding between WAVE and profilin was also confirmed (Figure 3B).

Next, we examined whether profilin participates in the clustering of actin filaments induced by WAVE. We recently succeeded in constructing mutant profilin I (H115E), which lacks completely the ability to bind actin, preserving its ability to bind to other poly-proline-containing proteins, and inhibit N-WASP-induced actin-microspike formation (Suetsugu *et al*., 1998). As shown in Figure 3C, the co-expression of mutant profilin completely blocks the clustering of actin filaments induced by WAVE. In contrast, wild-type profilin has no significant effect. Overall, 80–90% of cells expressing both WAVE and wild-type profilin show actin-filament clustering, but we could not find any single cell bearing actin clusters among cells expressing both WAVE and dominant-negative profilin. This result strongly suggests that the actin clustering is caused by the ectopic polymerization of actin filaments induced by WAVE through profilin.

To determine whether direct binding between WAVE and profilin is required for the actin clustering, we prepared a mutant WAVE that lacks the proline-rich region (ΔProR) where the profilin binding should occur. This mutant WAVE was found to lose the ability to bind profilin (Figure 3D). We then expressed ΔProR WAVE in COS 7 cells and stained the cells with phalloidin. Unexpectedly, ΔPro WAVE could also induce the formation of actin clusters where the expressed ΔProR WAVE localized (Figure 3E). Although the clustering pattern of ΔProR WAVE seems to be a little bit different to that of wild-

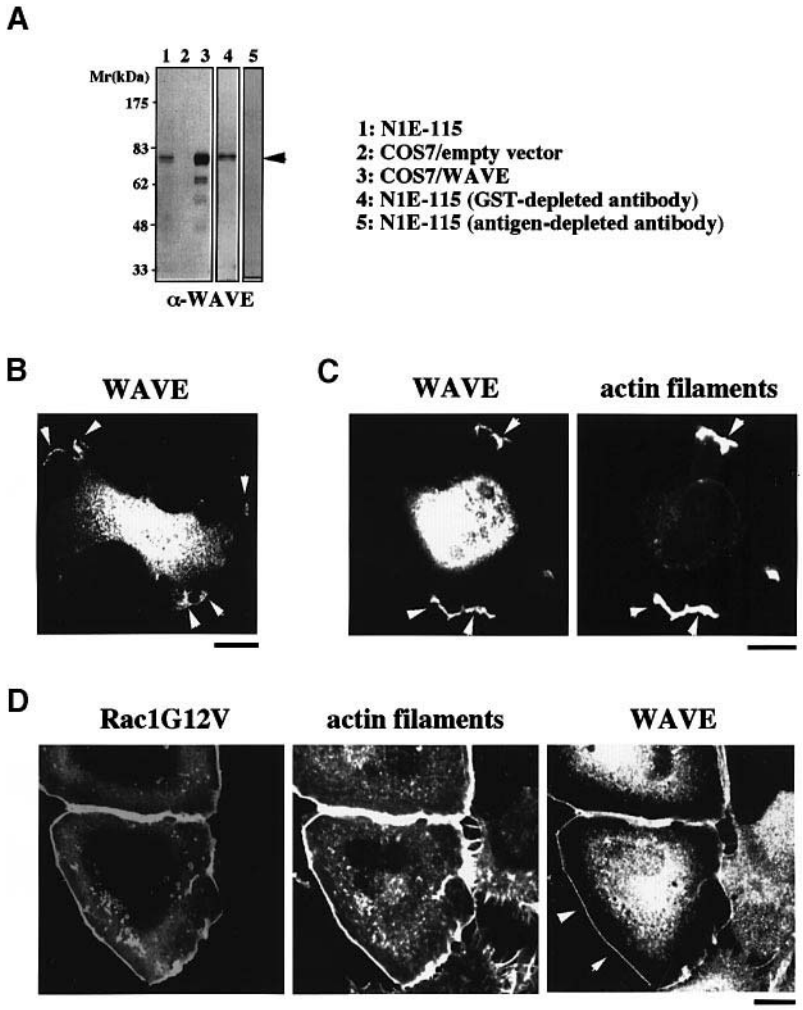

Fig. 4. Intracellular localization of endogenous WAVE. (**A**) Characterization of anti-WAVE antibody. Lysates of N1E-115 neuroblastoma cells and COS 7 cells transfected with control vector alone or myc-tagged WAVE were subjected to SDS–PAGE and Western blot analysis using the purified anti-WAVE antibody described in this study. In order to further confirm the specificity, the antibody was pre-incubated with GST or GST–C (antigen) proteins and then used for Western blotting against N1E-115 cell lysate. (**B**) Localization of endogenous WAVE in N1E-115 cells. N1E-115 cells cultured on coverslips were serum starved for 12 h and then fixed. The cells were stained with anti-WAVE antibody and then observed by confocal microscopy. The accumulation of WAVE at membrane ruffles is indicated by arrowheads. Bar, 10 μm. (**C**) Colocalization of WAVE and actin filaments in areas of ruffling. N1E-115 cells were stained with anti-WAVE antibody and phalloidin. The accumulation of WAVE and actin filaments at membrane ruffles is indicated by arrowheads. Bar, 10 μm. (**D**) Localization of WAVE at membrane ruffles induced by active Rac. Myc-tagged Rac1G12V was expressed transiently in N1E-115 cells which were then stained with anti-myc antibody (monoclonal 9E10), anti-WAVE antibody and phalloidin. The accumulation of WAVE at membrane ruffles is indicated by arrowheads. Bar, 10 μm.

type WAVE (Figure 3E), clustering of actin filaments is clearly observed. Thus, in conclusion, profilin is required for WAVE-induced actin clustering, but the direct binding between WAVE and profilin is not required for the actin clustering induced by WAVE overexpression in COS 7 cells.

Accumulation of endogenous WAVE in membrane-ruffling area

We prepared anti-WAVE antibody using a recombinant protein comprising the C-terminal region of WAVE. The results of the characterization of this antibody are shown in Figure 4A. The antibody recognized an 80-kDa protein in N1E-115 cell lysates and ectopically expressed WAVE in COS 7 cells. We checked for the existence of WAVE in various culture cell lines by Western blotting, and found that neuronal cell lines such as N1E-115 cells and PC12 cells contain larger amounts of WAVE than NIH 3T3 cells, Swiss 3T3 cells, COS 7 cells or MDCK cells (data not shown). This result is consistent with previously reported Northern hybridization analyses that showed the KIAA0269 mRNA level to be highest in brain (Nagase *et al*., 1996).

We then examined the intracellular localization of endogenous WAVE in N1E-115 cells by confocal microscopy. In single-staining experiments, WAVE seems to exist in a dot-like pattern throughout the cytoplasm and also to be concentrated at membrane-ruffling areas (indicated by arrowheads in Figure 4B). Preincubation of the antibody with antigen abolished the positive signal under the same experimental conditions (data not shown), indicating that the staining pattern properly reflects the localization of endogenous WAVE. In order to confirm the localization of WAVE at ruffling areas, we double-stained the cells with anti-WAVE antibody and phalloidin to visualize actin filaments. As a result, a clear colocalization

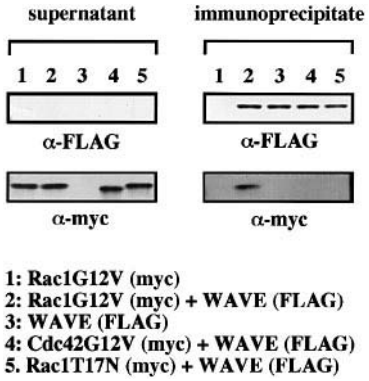

Fig. 5. *In vivo* complex formation between WAVE and active Rac. COS 7 cells were transfected with myc-tagged active Rac alone (lane 1), both myc-tagged active Rac and FLAG-tagged WAVE (lane 2), FLAG-tagged WAVE alone (lane 3), both myc-tagged active Cdc42 and FLAG-tagged WAVE (lane 4), or both myc-tagged negative Rac and FLAG-tagged WAVE (lane 5). The cells were harvested and the cleared lysates were immunoprecipitated with anti-WAVE antibody, and subjected to Western blot with anti-myc antibody and anti-FLAG antibody.

of WAVE with actin filaments in ruffling areas was observed (indicated by arrowheads in Figure 4C).

It is well known that membrane ruffling is regulated by Rac (Ridley *et al*., 1992), a small GTPase belonging to the Rho family. Thus, we transiently transfected N1E-115 cells with a Rac1G12V dominant-active mutant. As reported previously, the expression of active Rac in N1E-115 cells induced ruffling at the margin of the plasma membrane (Leeuwen *et al*., 1997). In this case, WAVE clearly localized in the ruffling areas and the dot-like staining in the cytoplasm became weak (Figure 4D), suggesting that Rac induced the translocation of significant amounts of WAVE from the cytosol to the membrane ruffles.

In vivo association between WAVE and activated Rac

In order to examine the possible interactions between WAVE and Rac, we carried out co-immunoprecipitation analyses. When cell lysates expressing both WAVE and active Rac were immunoprecipitated with anti-WAVE antibody, the immunoprecipitates contained Rac (Figure 5). This positive signal is specific, since anti-WAVE immunoprecipitates from cells expressing only Rac or WAVE showed no anti-myc antibody-positive signal (Figure 5). Furthermore, neither Cdc42G12V dominant-active mutants nor Rac1T17N dominant-negative mutants co-precipitated with WAVE at all (Figure 5).

As described above, WAVE does not possess GBD/CRIB motifs. Indeed, we could not detect any direct interaction between WAVE and Rac by conventional Far Western blot assay (data not shown), strongly suggesting that the interaction between WAVE and activated Rac is indirect.

Construction of ΔVPH WAVE that does not induce actin clustering

The C-terminal region of WAVE is very similar structurally to WASP and N-WASP. In the case of N-WASP, this region binds directly to actin (Miki *et al*., 1996; Miki and Takenawa, 1998). We also examined the C-terminal region

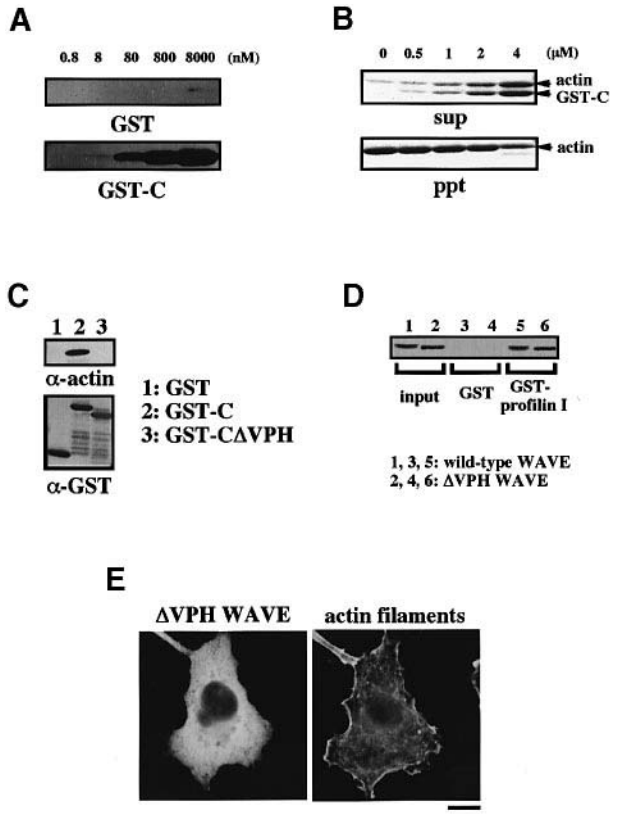

Fig. 6. Construction and characterizarization of ΔVPH WAVE. (**A**) Monomeric actin-binding ability of the C-terminal region of WAVE. The GST–C protein immobilized on beads was mixed with various concentrations of monomeric actin and the amount of bound actin was checked by Western blot with anti-actin antibody. The result using GST is also shown as negative control. (**B**) Actin depolymerization by GST–C protein. Actin was polymerized at 4 μM and then mixed with various concentrations of GST–C protein for 30 min. The monomeric (sup) and filamentous (ppt) actin were separated by ultracentrifugation and subjected to SDS–PAGE. (**C**) Loss of the actin-binding ability by the deletion of the VPH domain. The VPH domain was deleted from the GST–C protein and then checked for its actin-binding ability by the same method as in (A). The results using the original GST–C protein and GST itself are also shown as positive and negative controls, respectively. (**D**) Profilin-binding ability of ΔVPH WAVE. GST–profilin I protein immobilized on beads was mixed with myc-tagged ΔVPH-WAVE-expressing COS 7 cell lysates. The bound proteins were subjected to Western blot with anti-myc antibody. The result using wild-type WAVE is also shown as a positive control. (**E**) Inability of ΔVPH WAVE to induce actin clustering. Myc-tagged ΔVPH WAVE was transiently expressed in COS 7 cells and stained with anti-myc antibody (polyclonal) and phalloidin. Bar, 10 μm.

of WAVE using GST–fusion proteins (GST–C protein). As shown in Figure 6A, GST–C protein binds directly to monomeric actin. A rough estimation of the binding constant from this result is in the range 8–80 nM. Under the same experimental conditions, no significant binding occurred to control GST. Thus, this binding is thought to be a specific interaction. Next, we examined the effects of GST–C protein on actin polymerization. Actin was first polymerized at a concentration of 4 μM, and then various concentrations of GST–C proteins were added and incubated. The mixture was then ultracentrifuged to divide monomeric (supernatant, sup) and filamentous (precipitate, ppt) actin. As shown in Figure 6B, GST–C protein depolymerized actin filaments in a dose-dependent manner. Furthermore, GST–C protein did not precipitate with actin

filaments, indicating that this protein does not remain associated with actin filaments. These results are quite similar to those obtained using the corresponding region of N-WASP (Miki *et al*., 1996), strongly suggesting that WAVE regulates the actin cytoskeketon in the same way as N-WASP.

In order to examine the functional importance of WAVE downstream of Rac, we tried to make dominant-negative mutant WAVE. In the C-terminal actin-binding region, we focused on the VPH domain, which in N-WASP is indispensable for both the direct *in vitro* binding to actin and *in vivo* actin reorganization (Miki and Takenawa, 1998), and produced ΔVPH mutant WAVE lacking the 16 amino acids covering the VPH domain. Indeed, GST–CΔVPH protein, which was made by deleting the VPH domain from GST–C protein, was found to lack actin-binding activity completely (Figure 6C). In contrast, profilin-binding activity, which was examined by incubating ΔVPH WAVE (a mutant WAVE lacking the VPH domain)-expressing COS 7 cell lysates with immobilized GST–profilin I, was not changed compared with wild-type WAVE (Figure 6D). By immunofluorescence microscopy, we confirmed that this mutant lacks the actin-clustering activity *in vivo* (Figure 6E).

WAVE functions downstream of Rac

Next we examined the effect of ΔVPH WAVE expression on Rac-induced membrane ruffling. As shown in Figure 7A, the expression of active Rac in COS 7 cells induced strong membrane ruffling that can be seen in the phalloidin-stained image (actin filaments; upper photos). The expressed active Rac itself also accumulated in the ruffling areas. When ΔVPH WAVE was co-expressed with active Rac, membrane-ruffling formation was inhibited and the localization of active Rac was more diffuse relative to Rac-expressing cells (lower photos). In the co-expressing cells, the phalloidin-stained image is clearly changed compared with the original COS 7 cells, in that some circular-shaped structures that seem to represent macropinocytic vesicles (Ridley *et al*., 1992) are formed not only in Rac-expressing cells but also in Rac- and ΔVPH-expressing cells. This suggests that ΔVPH WAVE specifically inhibits the membrane ruffling among various Rac-induced biological effects. The results of quantitative analysis are shown in Figure 8. To confirm that this dominant-negative effect is specific for Rac, we also performed co-expression analyses using the active Cdc42 mutant Cdc42G12V that induces the formation of actin microspikes through rapid reorganization of the actin cytoskeleton (Kozma *et al*., 1995; Nobes and Hall, 1995). In this case, ΔVPH expression had no marked effect on the microspike formation activity of Cdc42 (Figures 7B and 8), clearly indicating that WAVE functions specifically downstream of Rac, but not Cdc42. Under these experimental conditions, the expression of active Cdc42 seems not to induce significant membrane-ruffling formation in COS 7 cells (data not shown), as is normally seen in protein- or plasmid injection into Swiss 3T3 cells (Kozma *et al*., 1995; Nobes and Hall, 1995).

In order to examine the generality of this effect, we next performed the same co-expression analysis using N1E-115 cells and found that, again, ΔVPH WAVE also inhibits membrane ruffling induced by Rac (Figure 8).

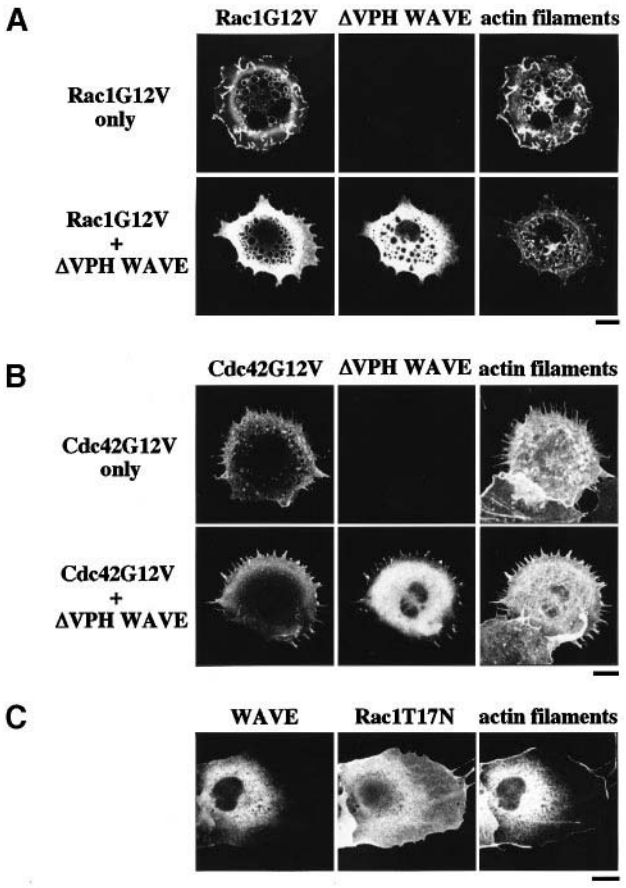

Fig. 7. Specific role of WAVE in actin reorganization downstream of Rac. (**A**) Effect of the co-expression of the ΔVPH WAVE on Rac-induced actin reorganization. FLAG-tagged ΔVPH WAVE was co-expressed transiently with myc-tagged active Rac in COS 7 cells. The cells were stained with anti-FLAG antibody (monoclonal), anti-myc antibody (polyclonal) and phalloidin. The results using active Rac alone are also shown as control (upper photos). Bar, 10 μm. (**B**) Effect of the co-expression of the ΔVPH WAVE on Cdc42-induced actin reorganization. FLAG-tagged ΔVPH WAVE was co-expressed transiently with myc-tagged active Cdc42 in COS 7 cells. The cells were stained with anti-FLAG antibody (monoclonal), anti-myc antibody (polyclonal) and phalloidin. Bar, 10 μm. (**C**) The induction of actin clustering by WAVE in a Rac-independent manner. FLAG-tagged wild-type WAVE was co-expressed transiently with myc-tagged dominant-negative Rac in COS 7 cells. The cells were stained with anti-FLAG antibody (monoclonal), anti-myc antibody (polyclonal) and phalloidin. Bar, 10 μm.

The weakness of the inhibitory activity compared with COS 7 cells may reflect the amount of endogenous WAVE or may indicate the presence of another actin-reorganization mechanism downstream of Rac in N1E-115 cells.

Finally, we co-expressed both wild-type WAVE and dominant-negative Rac (Rac1T17N). Even in this case, WAVE expression induced the formation of actin clusters where the expressed WAVE exists (Figure 7C), also eliminating the possibility that WAVE functions upstream of Rac or in parallel with Rac.

WAVE also functions in growth-factor-induced membrane ruffling in Swiss 3T3 fibroblasts

Rac-induced membrane ruffling has been studied in detail in Swiss 3T3 fibroblasts. In Swiss 3T3 cells, growth factor,

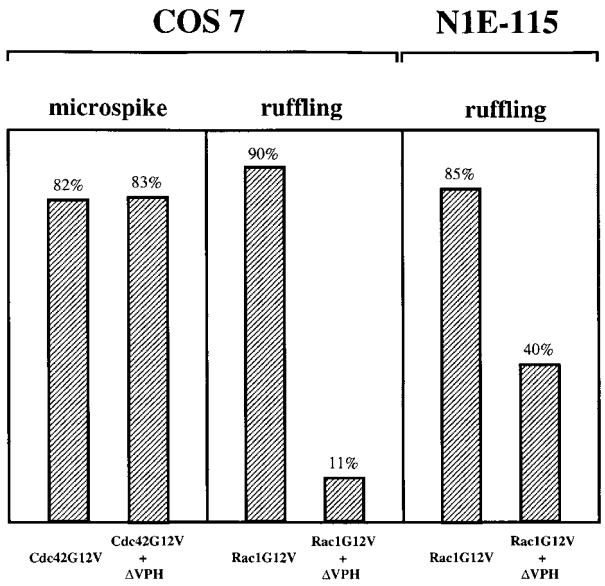

Fig. 8. Quantitative analysis of the effect of ΔVPH WAVE expression. The percentage of cells forming microspikes among active Cdc42-expressing cells, and cells forming ruffles among active Rac-expressing cells were quantitated in COS 7 cell cultures. Results using N1E-115 cells are also shown. More than 100 cells were examined in each experiment.

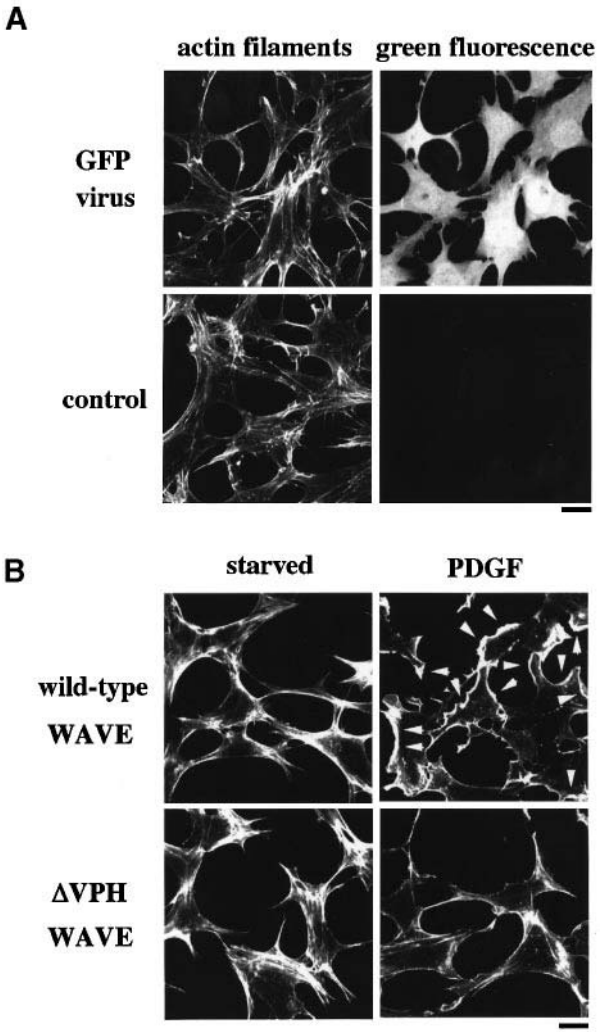

Fig. 9. Inhibitory effect of ΔVPH WAVE on PDGF-induced membrane rufflings in Swiss 3T3 cells. (**A**) Efficiency of ectopic expression using retrovirus vector. Swiss 3T3 cells cultured on coverslips were infected with GFP-expressing recombinant retroviruses. After 48 h, the cells were fixed and stained with phalloidin. Cells not infected with GFP viruses are also shown as negative control. Bar, 10 μm. (**B**) Inhibition of PDGF-induced membrane ruffling by ΔVPH WAVE. Swiss 3T3 cells were infected with recombinant retroviruses expressing wild-type or ΔVPH WAVE. After serum starvation for 24 h, the cells were treated with or without 5 ng/ml PDGF for 10 min and then fixed. Phalloidin-stained images are shown. The expression of each protein was confirmed using anti-myc and anti-WAVE antibody (data not shown). Strong membrane ruffles are indicated by arrowheads. Bar, 10 μm.

such as platelet-derived growth factor (PDGF), stimulation induces membrane ruffling in an endogenous Rac-dependent manner (Ridley *et al*., 1992). In order to examine the possible functional involvement of WAVE in this process, we expressed WAVE (wild-type or ΔVPH mutant) in Swiss 3T3 cells using recombinant retroviruses. As shown in Figure 9A, control green fluorescent proteins (GFPs) are expressed in ~100% of the virus-treated cells. We then expressed wild-type or ΔVPH mutant WAVE in the cells using the same method, and then stimulated the cells with PDGF for 10 min. The wild-type WAVE-expressing cells showed membrane ruffles induced by PDGF treatment (Figure 9B), as in the original Swiss 3T3 cells. In Swiss 3T3 cells, the ectopic expression of wild-type WAVE did not induce the actin clustering that was seen in the case of COS 7 cells. We did not ascertain whether this discrepancy is due to the difference of the cell types used or to the difference in the amount of the expressed proteins. In contrast, cells expressing ΔVPH WAVE showed no membrane ruffling (Figure 9B), clearly indicating that WAVE (or some other closely related molecule) in Swiss 3T3 cells does indeed function in membrane ruffling downstream of PDGF receptors.

WAVE is essential for neurite extension in N1E-115 cells

As described above, the amount of WAVE is richest in N1E-115 cells and PC12 cells among various culture cell lines (data not shown), both of which are of neuronal origin. This fact strongly suggests that WAVE has some important physiological role in neuronal cells. In N1E-115 cells, Rac has been shown to be essential for neurite extension induced by serum starvation using dominant-negative mutant Rac (Kozma *et al*., 1997). Thus, we also examined whether WAVE is required for the neurite extension by expressing ΔVPH WAVE. In serum-containing culture conditions, 80–90% of cells showed a rounded morphology. The ΔVPH WAVE-expressing cells also remained round (Figure 10A). When the culture medium was replaced with a serum-free one, most cells became flattened and about half extended neurites (Figure 10A and B). Under this condition, the ΔVPH-expressing cells also became flattened; however, neurite extension was severely impaired. Only 6% of cells were determined to possess neurites, suggesting that WAVE is specifically required for neurite extension downstream of Rac.

Discussion

Here we identify WAVE as a third member of the WASP family of proteins. Based on the results presented above,

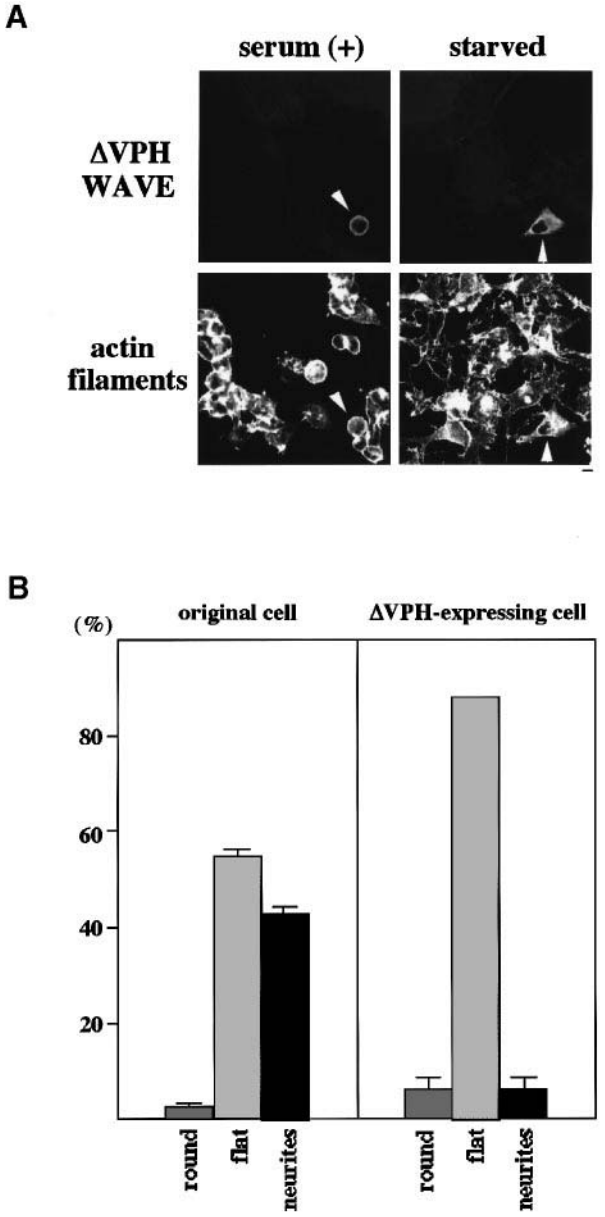

Fig. 10. Inhibitory effect of ΔVPH WAVE on neurite extension in N1E-115 cells. N1E-115 cells cultured on coverslips were transfected with myc-tagged ΔVPH WAVE. The cells were serum starved for 48 h and then fixed. Anti-myc (monoclonal) and phalloidin-stained images are shown in (**A**). ΔVPH-WAVE-expressing cells are shown by arrowheads. Bar, 10 μm. In (**B**), cells were categorized into one of three types according to their morphology. Cells with one or more neurites longer than two times the cell diameter with rounded morphology are considered to be 'neurite-bearing cells'. Error bars represent differences from two independent experiments. Fifty cells were counted in each experiment.

we conclude that WAVE induces actin reorganization downstream of Rac, a reaction required for the formation of membrane ruffles. We find this quite interesting, since the two other WASP family proteins, WASP and N-WASP, have also been shown to induce actin reorganization, but they are regulated by Cdc42, another Rho family small GTPase (Symons *et al*., 1996; Miki *et al*., 1998).

Downstream signaling of Rac

There have been several reports concerning the possible mechanism of the actin reorganization induced by Rac. Very recently, Arber *et al*. (1998) and Yang *et al*. (1998) reported that LIM-kinase, a serine–threonine kinase that has two LIM motifs, plays a critical role in the actin reorganization downstream of Rac. In these reports, it is shown that LIM-kinase is activated by Rac and phosphorylates cofilin, an actin-depolymerizing protein (Nishida *et al*., 1984). This phosphorylation of cofilin shuts off the actin-depolymerizing activity and thus leads to the stabilization of the actin cytoskeleton. However, since actin polymerization is required for ruffling formation, this LIM-kinase–cofilin pathway alone does not explain the mechanism of the induction of membrane ruffling (Rosenblatt and Mitchison, 1998).

Rac has been shown to associate constitutively with type I phosphatidylinositol 4-phosphate (PIP) 5-kinase, which phosphorylates PIP to produce phosphatidylinositol 4,5-bisphosphate (PIP2) (Tolias *et al*., 1995; Rameh *et al*., 1997). PIP2 not only functions as a source of the important second messengers inositol 1,4,5-trisphosphate and diacylglycerol, but also binds directly to several actin-regulating proteins including profilin, cofilin, gelsolin and α-actinin, and modulates their activities (reviewed by Stossel, 1993). Hartwig *et al*. (1995) showed that activated Rac induces the formation of PIP2 in platelets and that the addition of the PIP2-binding peptide derived from gelsolin inhibits the actin polymerization induced by Rac, suggesting that Rac reorganizes the actin cytoskeleton through filament uncapping by replacing capping proteins such as gelsolin. Indeed, gelsolin was recently shown to be essential for Rac-induced membrane ruffling using dermal fibroblasts without gelsolin obtained from gene-targeted mouse (Azuma *et al*., 1998). However, there is another report demonstrating that the Rac-induced replacement of gelsolin from actin filaments occurs in a manner independent of the elevation of PIP2 (or related phospholipids, phosphatidylinositol 3,4,5-trisphosphate) (Arcaro, 1998). In the same report, it was also shown that the uncapping of actin filaments is not sufficient for the induction of actin polymerization. Thus, some other mechanism must regulate actin reorganization after filament uncapping. In this event, WAVE may regulate profilin that has been strongly suggested to be involved in actin polymerization (Pantaloni and Carlier, 1993; Theriot *et al*., 1994). PIP2 might totally regulate Rac-induced actin reorganization by controlling the activity of many actin-regulating proteins, including gelsolin. In a sense, WAVE is the first protein molecule whose positive role in actin reorganization downstream of Rac has been clearly demonstrated using dominant-negative mutant.

Comparison of the Rac–WAVE system with the Cdc42–N-WASP system

We reported previously that N-WASP is an essential factor in Cdc42-induced filopodium formation (Miki *et al*., 1998). In addition, we recently found that N-WASP induces the formation of actin microspikes through profilin (Suetsugu *et al*., 1998). In this case, the expression of dominant-negative profilin clearly suppressed the actin-microspike formation induced by N-WASP. This is quite similar to the case of the WAVE-induced actin reorganization described above. Thus, we suppose that N-WASP and WAVE share a common function as an inducer of actin reorganization through profilin. In experiments using a

COS 7-cell-overexpression system, the direct association between WAVE and profilin was shown not to be required for WAVE-induced actin clustering. However, we can not rule out the importance of the direct binding based only on this result, since it is possible that the direct association may enhance the efficiency of actin reorganization under physiological non-overexpressing conditions. Indeed, in the case of N-WASP, an N-WASP mutant that has no profilin-binding activity can still induce actin-microspike formation at the same rate as wild-type N-WASP, but the length of the microspikes produced by the mutant become shorter (Suetsugu *et al*., 1998).

N-WASP has been shown to bind directly to activated Cdc42 through its GBD/CRIB motif (Miki *et al*., 1998). In contrast, WAVE does not possess a GBD/CRIB motif, and indeed has no direct binding ability to activated Rac in conventional Far Western blot assay (data not shown). However, a recent report on PAK demonstrated that the intracellular localization of PAK is determined not by the direct binding with Cdc42, that is, PAK mutants that can not bind to activated Cdc42 are recruited to focal complexes in a Cdc42-dependent manner, as is wild-type PAK (Zhao *et al*., 1998). Thus, it is not unusual that WAVE also translocates to the plasma membrane in a manner that does not require direct association with activated Rac.

Evolutional conservation of WASP-family proteins

WASP-family proteins have been strongly conserved throughout evolution. WASP-related proteins can be found even in yeast. The putative yeast WASP-homolog protein, Bee1, has also been shown to participate in actin polymerization in experiments using permeabilized yeast cells (Lechler and Li, 1997; Li, 1997). Although the mechanisms for the regulation of WASP-family proteins differ significantly from yeast to mammals, they may share a common function as actin polymerization inducers. We believe that many more WASP-family proteins exist and hold critical clues as to actin cytoskeletal reorganization in response to various external stimuli.

Materials and methods

Ectopic expression in COS 7 and N1E-115 cells

Wild-type, ΔVPH and ΔProR mutant WAVE-expression plasmids were constructed in pEF-BOS (myc-tagged and FLAG-tagged) and pME (FLAG-tagged) plasmid vectors. Profilin (wild-type and H115E mutant) expression plasmids were constructed in pEF-BOS (myc-tagged). Cdc42G12V, Rac1G12V and Rac1T17N were also constructed in pEF-BOS (myc-tagged and FLAG-tagged). Ten micrograms of recombinant plasmid was transfected into COS 7 cells (800 μl of 5×10^6 cells/ml) by electroporation as described previously (Miki *et al*., 1996). After 30 h, the cells were fixed or harvested. For the transfection into N1E-115 cells, 5×10^4 cells were plated on 3.5 cm dishes with coverslips. After 12 h, 4 μg plasmid DNA was introduced by Ca^{2+}-phosphate precipitation as described (Shibasaki and McKeon, 1995). Twenty hours after transfection, the cells were fixed. For the examination of neurite formation, the transfected cells (plated at 2×10^4 in 3.5 cm dishes) were serum starved for 48 h and then fixed.

Retrovirus-mediated transfection of Swiss 3T3 cells

Recombinant retroviruses were produced as follows. The WAVE cDNAs (wild-type and ΔVPH mutant) were ligated in pMX plasmid vector. The recombinant plasmids were transfected into BOSC23 packaging cells with lipofectoamine reagent (Gibco). The resulting supernatants containing retroviruses were collected and used to infect Swiss 3T3 cells. The infected Swiss 3T3 cells were kept for 48 h under serum-containing growth conditions and then starved for 24 h.

Antibodies

Polyclonal anti-WAVE antibody was prepared in rabbits immunized with the C-terminal region of human WAVE (region C: amino acids 436–560) expressed in *Escherichia coli*. The serum was applied to an antigen-immobilized column and the bound antibody was eluted with glycine buffer (pH 2.5). Polyclonal anti-GST antibody was also prepared in rabbits immunized with the GST protein that is expressed in *E.coli*. Polyclonal anti-myc antibody was purchased from MBL. Monoclonal anti-myc antibody (9E10) was from Santa Cruz. Monoclonal anti-FLAG antibody (M2) was from Kodak. Monoclonal anti-actin antibody (MAB1501) was from CHEMICON. Secondary antibodies linked to alkaline phosphatase (used for Western blotting) were from Promega. Secondary antibodies linked to fluorescein and Cy5 (used for immunofluorescence microscopy) were from Capel and Amersham, respectively.

Construction of ΔVPH and ΔProR mutant WAVE

The ΔVPH mutant (lacking amino acids 498–513) and the ΔProR mutant (lacking amino acids 275–435) were constructed by polymerase chain reaction (PCR). The nucleotide sequence of the PCR products was confirmed by DNA sequencing of both strands.

GST–fusion proteins

GST–profilin I and II (human) were prepared as described elsewhere (Suetsugu *et al*., 1998). GST–C (GST–CΔVPH)-expressing plasmids were constructed in pGEX plasmids (Pharmacia) by inserting PCR-amplified DNA fragments encoding each region. Expression and purification of the GST–fusion proteins were carried out as described (Miki *et al*., 1994).

Binding assay

GST–fusion proteins were immobilized on 20 μl glutathione beads and then mixed with various protein samples such as cell lysates. After incubation for 2 h with rotation, the beads were washed with phosphate-buffered saline (PBS) plus 0.1% Triton X-100 and suspended in 20 μl SDS sample buffer. In Far Western blot assay, the anti-WAVE immunoprecipitates were separated by SDS–PAGE and transferred to membranes. After blocking, the membranes were first incubated with GST–profilin I protein (or control GST) in PBS, and then with anti-GST antibody to detect the membrane-bound GST–profilin I.

Actin assay

Actin was prepared from chick muscle according to a method described previously (Miki *et al*., 1996). In the monomeric actin-binding assay, various concentrations of monomeric actin were mixed with immobilized GST–C protein in G-buffer (5 mM Tris pH 8.0, 0.1 mM $CaCl_2$, 0.1 mM ATP and 10 mM 2-mercaptoethanol) plus 0.1% Triton X-100. Washing was also carried out in the same buffer. In the filamentous actin-binding assay, actin was first polymerized at 4 μM by adding 50 mM KCl, 1 mM ATP and 1 mM $MgCl_2$, and incubating for 60 min at room temperature. Various concentrations of GST–C protein were then added. After incubation for 30 min at room temperature, the mixture was ultracentrifuged at 100 000 *g* for 60 min. The resultant supernatant and precipitated fractions were separated and subjected to SDS–PAGE.

Immunofluorescence microscopic analysis

Cells cultured on coverslips were fixed and stored in 3.7% formaldehyde in PBS. For immunofluorescence staining, cells were first permeabilized with 0.2% Triton X-100 for 5 min and then incubated with primary antibodies such as anti-myc, anti-FLAG and anti-WAVE antibodies for 60 min. After washing, they were incubated with secondary antibodies linked to fluorescein or Cy5 (Cy5-conjugated antibody was used only in triple-staining experiments). In order to visualize actin filaments, rhodamine-conjugated phalloidin (Molecular Probe) was also added during the incubation with secondary antibodies. After 30 min incubation, coverslips were washed and mounted on slide glasses. They were observed with a confocal laser scanning microscope (Bio-Rad, model MRC 1024). Throughout this study, we confirmed that no bleed-through occurred between different channels by comparing the results obtained by depleting one primary antibody.

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Recently, Bear *et al*. identified a novel protein named SCAR (suppressor of cAR) in *Dictyostelium* [*J. Cell Biol*., **142**, 1325–1335 (1998)]. The SCAR protein is probably the *Dictyostelium* homolog of WAVE. Indeed, they showed in the paper that the disruption of the SCAR gene resulted in defects in the accumulation of actin filaments at the leading edge in chemotactic movements.