The EMBO Journal Vol.17 No.22 pp.6516–6526, 1998

The essential role of profilin in the assembly of actin for microspike formation

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Profilin was first identified as an actin monomer binding protein; however, recent reports indicate its involvement in actin polymerization. To date, there is no direct evidence of a functional role *in vivo* for profilin in actin cytoskeletal reorganization. Here, we prepared a profilin mutant (H119E) defective in actin binding, but retaining the ability to bind to other proteins. This mutant profilin I suppresses actin polymerization in microspike formation induced by N-WASP, the essential factor in microspike formation. Profilin associates both *in vivo* and *in vitro* with N-WASP at proline-rich sites different from those to which Ash/Grb2 binds. This association between profilin and N-WASP is required for N-WASP-induced efficient microspike elongation. Moreover, we succeeded in reconstituting microspike formation in permeabilized cells using profilin I combined with N-WASP and its regulator, Cdc42. These findings provide the first evidence that profilin is a key molecule linking a signaling network to rapid actin polymerization in microspike formation.
Keywords: actin/Cdc42/microspike/N-WASP/profilin

Introduction

Cells respond to many extracellular stimuli by activating signaling cascades that lead to gene expression and cytoskeletal organization affecting cell shape and motility. Actin is the major cytoskeletal protein in most eukaryotic cells, and filamentous actin structures are often the primary determinants of cell shape and movement (Lauffenburger and Hortwitz, 1996; Mitchson and Cramer, 1996).

The actin-binding protein profilin is thought to be a key regulator of actin polymerization in cells (Theriot and Mitchison, 1993). Profilin was first identified as a G-actin sequestering protein (Carlsson *et al.*, 1977). Now, however, profilin is thought to act as a nucleotide exchange factor, charging ATP to actin after binding to actin monomers (Goldschmidt-Clermont *et al.*, 1991). *In vitro* experiments show that profilin promotes actin assembly from the G-actin–thymosin-β4 pool when barbed filament ends are free (Pantoloni and Carlier, 1993). Furthermore, profilin is involved in the actin-based motility of *Listeria monocytogenes* (Theriot *et al.*, 1994). It has also been demonstrated that profilin binds not only to actin, but also to phosphatidylinositol 4,5-bisphosphate (PIP2) (Lassing and Lindberg, 1985) and poly-L-proline (Tanaka and Shibata, 1985), and to proteins with proline rich-sequences, which are thought to be important in regulating the actin cytoskeleton. Proteins such as vasodilator-stimulated phosphoprotein (VASP) (Reinhard *et al.*, 1995), MENA (Gertler *et al.*, 1996), p140mDia (Watanabe *et al.*, 1997) and Arp2/3 complex (Machesky *et al.*, 1994; Welch *et al.*, 1997) have been shown to bind to profilin. These proteins appear to recruit the profilin–actin complex to sites where actin polymerization is induced. However, there is no direct evidence that profilin is functionally important to these proteins. Profilin exists in two isoforms in mammals, profilins I and II. Profilin I is more ubiquitous and abundant than profilin II, but the difference in their function is not clear (Honore *et al.*, 1993; Lambrechts *et al.*, 1997; Witke *et al.*, 1998).

Rho-family small G-proteins, such as Cdc42, Rac and Rho, are essential regulators in the rapid actin reorganization leading to the formation of filopodia, lamellipodia and stress fibers (Kozma *et al.*, 1995; Nobes and Hall, 1995; Zigmond 1996; Hall, 1998). Rho-family small G-proteins appear to activate many kinases including p160ROCK/Rho-kinase/ROK (Ishizaki *et al.*, 1996; Matsui *et al.*, 1996), myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) (Leung *et al.*, 1998), Cdc42/Rac-binding p21-activated kinase (PAK) (Manser *et al.*, 1994), PKN (Amano *et al.*, 1996a; Watanabe *et al.*, 1996), Ack (Manser *et al.*, 1993) and mixed lineage kinase (MLK) (Burbelo *et al.*, 1995; Nagata *et al.*, 1998). The first two have been shown to activate myosin by regulating its phosphorylation status (Amano *et al.*, 1996b; Kimura *et al.*, 1996; Leung *et al.*, 1998), and are strongly suggested to be involved in the actin cytoskeletal reorganization induced by Rho and Cdc42, respectively (Leung *et al.*, 1996, 1998; Amano *et al.*, 1997). However, the direct mechanism of actin polymerization downstream of these kinases is unclear.

Some proteins that associate directly with profilin also interact with small G-proteins. p140mDia binds to activated Rho (Watanabe *et al.*, 1997). Indeed, the overexpression of p140mDia in COS-7 cells induces homogeneous fine actin filaments and p140mDia is thought to function through profilin.

N-WASP, which was first characterized as a protein that binds an adaptor protein Ash/Grb2 through its SH3 domain (Miki *et al.*, 1996; Miura *et al.*, 1996), has many proline-rich sequences, suggesting some interaction with profilin other than through Ash/Grb2. N-WASP functions as an effector of activated-Cdc42 in filopodium formation. Furthermore, N-WASP has Cdc42-dependent actin-depolymerizing activity in its cofilin homology domain (Miki and Takenawa, 1998; Miki *et al.*, 1998) which seems to be necessary for the creation of barbed ends. However, the role of N-WASP in microspike extension including actin polymerization remains unclear.

Here we report that mutant profilin I deficient in actin-binding suppresses N-WASP-induced microspike formation in COS-7 cells, clearly showing the role of profilin in N-WASP-induced microspike formation. Profilin and N-WASP associate both *in vitro* and *in vivo*, and this binding is necessary for efficient microspike formation. Furthermore, we show that three proteins, profilin I, N-WASP and activated Cdc42, are essential for microspike formation through reconstitution experiments using permeabilized Swiss 3T3 cells.

Results

Profilin mutants deficient in actin binding

To clarify the role of profilin in the rapid reorganization of actin filaments, we attempted to make profilin mutants that lacked actin-binding ability while retaining the ability to associate with other profilin-binding proteins. We constructed several mutants based on the three-dimensional structure of the actin–profilin I co-crystal (Schutt *et al.*, 1993), and investigated their ability to associate with actin and other binding proteins by constructing glutathione *S*-transferase (GST)-fusion proteins, immobilizing them on glutathione–Sepharose beads, and then mixing them with various bovine tissue homogenates. One mutant (H119E), in which His119 is replaced with glutamate, lacked the ability to bind actin but not other profilin binding proteins (Figure 1A). Peptide sequencing and Western blotting of the bound proteins showed them to include known profilin binding proteins such as p140mDia (Watanabe *et al.*, 1997), gephyrin (Mammoto *et al.*, 1998), MENA (Gertler *et al.*, 1996), N-WASP (this paper) and VASP (Reinhard *et al.*, 1995), suggesting that this system functions well. Other proteins at 170, 120 and 70 kDa had unknown peptide sequences. Western blotting showed that the only band not present in the H119E mutant profilin-binding proteins compared with the binding proteins of wild-type profilin is actin. This observation suggests that this mutant acts as a dominant-negative regulator of profilin-binding proteins. The H119E mutant profilin II exhibited slight changes in the profilin II binding protein pattern compared with wild-type profilin II (not shown).

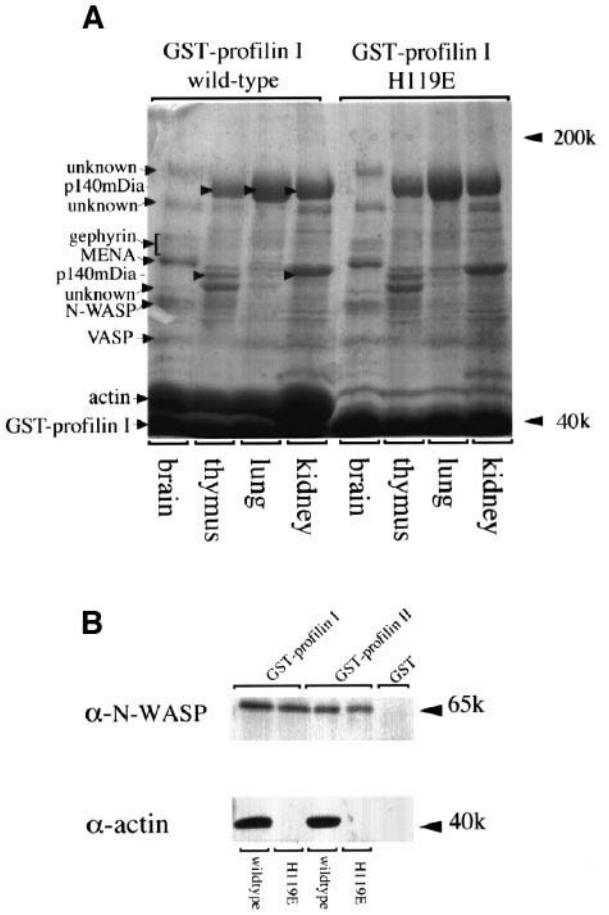

Fig. 1. The complete loss of actin binding ability by the H119E mutant profilin. (**A**) Equal amounts of wild-type or mutant GST–profilin I were immobilized on glutathione–Sepharose 4B beads and mixed with the cytosol fractions of various bovine tissues. After washing, the bound proteins were visualized by Coomassie Brilliant Blue staining. (**B**) Equal amounts of wild-type or H119E mutant GST–profilin I or II were mixed with 3Y1 cell lysates as in (A). The bound proteins were analyzed by Western blot with anti-N-WASP and anti-actin antibodies. GST protein was subjected to the same treatment as a negative control.

Profilin I mutant suppresses the microspike formation caused by N-WASP

We previously reported that N-WASP regulates the formation of filopodia downstream of Cdc42, probably by severing pre-existing actin filaments, leading to actin uncapping. However, it is not known how the severing of actin filaments leads to filopodium formation.

N-WASP has a proline-rich sequence to which the Ash/Grb2 SH3 domain binds. Since profilin also binds to a proline-rich sequence, we examined the physical association between profilin and N-WASP in binding assays using 3Y1 cell lysates against immobilized GST–profilins I and II. Western blot analysis of co-precipitants showed that N-WASP associates with both profilin I and II (Figure 1B).

Next, we investigated whether H119E profilins also bind to N-WASP. H119E profilins were found to bind N-WASP as well as the wild-type profilins, but their actin binding was not as expected (Figure 1B). In order to examine the functional commitment of profilin in N-WASP-induced actin-cytoskeletal reorganization, we expressed N-WASP mixed with mutant or wild-type profilins I or II (myc-tagged) ectopically in COS-7 cells. COS-7 cells are shown to produce microspikes under EGF stimulation when N-WASP is overexpressed (Miki *et al.*, 1996). EGF stimulation of cells transfected with N-WASP was found to induce microspike formation in 52% of transfected cells (Figure 2A). Of cells co-transfected with wild-type profilin I and N-WASP, 55% showed microspike formation, suggesting that wild-type profilin I does not affect the microspike formation by N-WASP. In contrast, only 6% of cells co-transfected with mutant profilin I and N-WASP showed microspike formation. This marked decrease in microspike formation by mutant profilin I strongly suggests that profilin I is an essential factor in actin cytoskeletal rearrangement downstream of N-WASP. Western blot analysis using an anti-profilin I antibody and a transfection efficiency of ~15–20% showed the level of ectopically expressed profilin I in transfected cells to be 5- to 10-fold higher than the level of endogenous profilin I. Endogenous profilin II levels are quite low as estimated

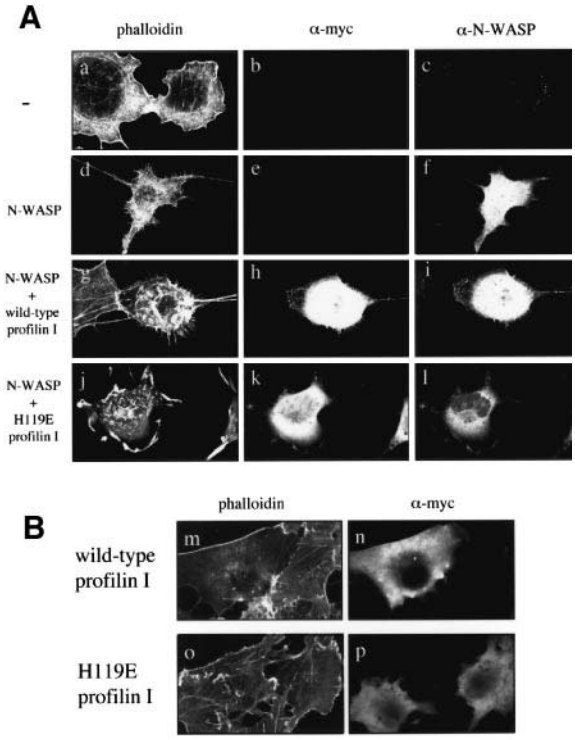

Fig. 2. Inhibition of N-WASP-induced microspike formation by H119E mutant profilin I. (**A**) Inhibition of N-WASP-induced microspike formation by the H119E mutant profilin I. COS-7 cells were transfected with plasmids expressing wild-type profilin I, the H119E mutant (myc-tagged) or N-WASP. Transfected cells were stimulated with EGF for 10 min. After fixation and permeabilization, the cells were stained with phalloidin (a, d, g and j), anti-myc antibody (b, e, h and k) and anti-N-WASP antibody (c, f, i and l). (**B**) Effect of profilin expression on actin structure in COS-7 cells cultured with serum. COS-7 cells transfected with plasmid expressing wild-type profilin I or H119E profilin I were fixed and stained with phalloidin (m and o) and anti-myc antibody (n and p). Myc staining represents ectopically profilin-expressing cells. The ectopically expressed profilin I level was 5- to 10-fold higher than the endogenous profilin I level from Western blotting (not shown).

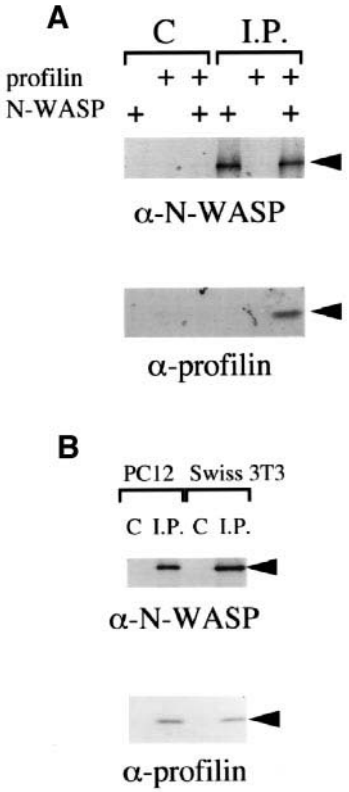

Fig. 3. Profilin associates with N-WASP both *in vitro* and *in vivo*. (**A**) Direct *in vitro* association of N-WASP with profilin. N-WASP (recombinantly expressed using baculovirus) and profilin (purified from bovine spleen) were mixed at final concentrations of 10 μM for each protein and anti-N-WASP antibody was added (I.P.). The immune complexes were precipitated with protein A–agarose beads and subjected to Western blot analysis with anti-N-WASP and anti-profilin antibodies. N-WASP and profilin are indicated by arrowheads. Control experiments (C) without anti-N-WASP antibody were also carried out. (**B**) *In vivo* association of N-WASP with profilin. PC12 or Swiss 3T3 cell lysates were mixed with (I.P.) or without (C) anti-N-WASP antibody immobilized on protein A–agarose beads. The precipitates were analyzed by Western blot with anti-N-WASP and anti-profilin antibodies.

by Western blot analysis using anti-profilin II antibody. Therefore, we did not examine the effects of profilin II and its mutant.

In cells overexpressing profilin I (Figure 2B), thick stress fibers disappeared in wild-type profilin I-expressing cells, especially in the areas of high profilin I expression as reported previously (Finkel *et al.*, 1994). The mutant profilin I overexpression had no effect on stress fibers.

N-WASP and profilin associate both in vitro and in vivo

To confirm and investigate further the association between profilin and N-WASP, we performed a series of experiments. The mixing of native profilin purified from bovine spleen and recombinantly expressed N-WASP caused them both to form complexes at concentrations of 10 μM, indicating direct interaction between N-WASP and native profilin (Figure 3A). Lysates of Swiss 3T3 and PC12 cells were subjected to immunoprecipitation using anti-N-WASP antibody. As shown in Figure 3B, the co-precipitation of profilin was observed, indicating an *in vivo* interaction. These cells were shown to possess only profilin I by Western blotting analysis (not shown).

N-WASP has a higher affinity for profilin I than profilin II

To clarify the difference between profilins I and II, we determined the affinity of both profilin isoforms for N-WASP. We mixed various amounts of N-WASP with equal amounts of GST–profilins I and II immobilized on agarose beads, and examined the association. GST–profilin I formed complexes with N-WASP at lower concentrations than GST–profilin II (Figure 4A). Next, we prepared profilins without GST and labeled the N-terminal α-amino group with succinimide–fluorescein. These labeled profilins were incubated with various amounts of N-WASP to measure fluorescent polarization. The association between the proteins can be observed by the rise in the polarization values of the labeled proteins. By measuring polarization at various protein concentrations, we estimated profilin–N-WASP binding constants.

The polarization of profilin I rose at a lower N-WASP concentration than that of profilin II, indicating that profilin I has a higher affinity for N-WASP. The polarization values were saturated at high N-WASP concentrations, indicating a specific association. The calculated K_d values

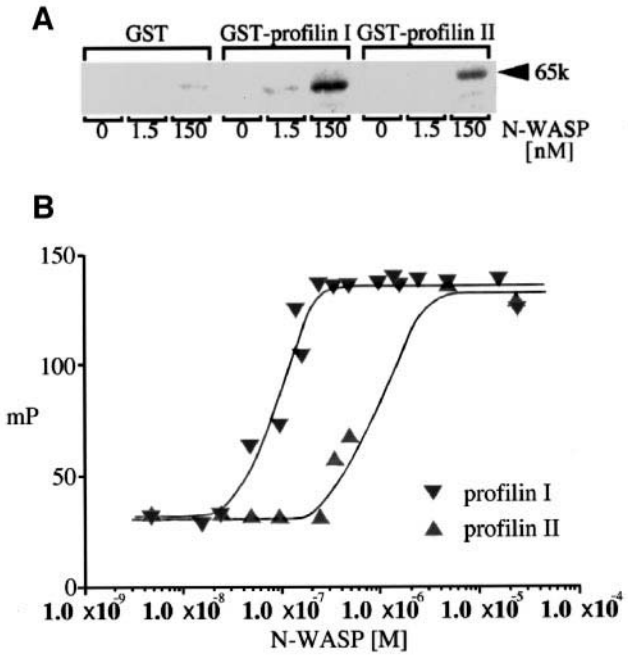

Fig. 4. Difference in the affinities between profilin I or II and N-WASP. (**A**) Profilin I shows a higher affinity for N-WASP than profilin II. Various concentrations of N-WASP were mixed with the immobilized GST fusion proteins of profilin I and II. After washing, the bound proteins were analyzed by Western blot with anti-N-WASP antibody. GST protein was subjected to the same treatment as a control. (**B**) Determination of the binding constants between profilin and N-WASP. Fluorescein (FS)-labeled profilin I or II (50 nM) was mixed with various concentrations of N-WASP. After 2 h, the polarization values of the FS-labeled proteins were measured. The increase in polarization values reflects the association between proteins. The K_d value for profilin I and N-WASP was determined from curve fitting to be 60 nM while that between profilin II and N-WASP was determined to be 400 nM. mP, milli-polarization.

between profilins I and II and N-WASP were 60 and 400 nM, respectively (Figure 4B). A rough calculation of the K_d from Western blotting and densitometry as shown in Figure 4A correlated with the data obtained from polarization analysis. Considering that it is more ubiquitous and abundant than profilin II (Witke *et al.*, 1998), profilin I seems to be the *in vivo* target of N-WASP.

Profilin I and Ash/Grb2 bind different sites in the proline-rich region of N-WASP

N-WASP was first identified as an Ash/Grb2 SH3 domain binding protein (Miki *et al.*, 1996; Miura *et al.*, 1996) through its proline-rich sequence where profilin-binding should occur. There are many poly-proline stretches in N-WASP. To confirm whether profilin really binds through its proline-rich region and to investigate whether profilin and Ash/Grb2 share binding sites in N-WASP, we constructed mutants of N-WASP completely deleted of the proline-rich region (Δppro1) or partly deleted (Δppro2 and Δppro3), as shown in Figure 5A. Δppro2 lacks all of the putative Ash/Grb2 SH3 domain binding motifs (Yu *et al.*, 1994; Feng *et al.*, 1995; Rickels *et al.*, 1995), whereas putative profilin I binding motifs exist in both Δppro2 and Δppro3 mutants (Purich *et al.*, 1997). N-WASP and its mutants were expressed in COS-7 cells and resulting cell lysates were subjected to binding assay using GST–profilin I and Ash/Grb2. As shown in Figure 5B, profilin I did not associate with the Δppro1 mutant, but weakly associated with the Δppro2 and Δppro3 mutants. However, Ash/Grb2 did not associate with the Δppro1 and Δppro2 mutants, but associated with the Δppro3 mutant to a similar extent as full-length N-WASP. The bands at full-length N-WASP observed in all lanes in Figure 5B were due to the endogenous N-WASP in COS-7 cells. This result suggests that profilin I and Ash/Grb2 bind different poly-proline stretches.

We next examined the association between Ash/Grb2 and N-WASP and its influence on profilin binding. To investigate the affinity of Ash/Grb2 for N-WASP, Ash/Grb2 was labeled with fluorescein. The polarization of Ash/Grb2 rose specifically with the increase in N-WASP concentration, yielding a K_d value of 2 μM (Figure 5C). Next, labeled profilin I or Ash/Grb2 together with unlabeled competitor proteins were mixed with N-WASP until the polarization value rose to the saturated or half-saturated state if competitor was absent. If profilin and Ash/Grb2 compete for the same sites, then the polarization value should decrease because the amount of free, non-complex-forming labeled protein will increase. However, no decrease in polarization was observed, also indicating strongly that Ash/Grb2 and profilin bind through different sites in the proline-rich region (Figure 5D).

The cells overexpressing mutants of N-WASP that lack the proline-rich region can form short microspikes

To investigate whether mutants of N-WASP lacking the proline-rich region retain the ability for microspike formation, we transfected COS-7 cells with these mutants and allowed them to form microspikes with EGF stimulation. Surprisingly, the N-WASP mutants deficient in profilin binding still formed microspikes (Figure 6). The percentages of microspike-forming cells among the cells transfected with N-WASP mutants were similar to those of cells transfected with full-length N-WASP and cells co-transfected with N-WASP and profilin I (Figure 7A). However, the characteristics of the microspikes in cells expressing mutants of N-WASP were different from those in the cells expressing full-length N-WASP. The longest microspike in a spike-forming cell was photographed, and the length was measured and plotted. The length of microspikes was obviously less in cells transfected with N-WASP mutants lacking the proline-rich region (Figure 7B).

On average, the lengths of microspikes in N-WASP transfected cells and in N-WASP and profilin I co-transfected cells were 8.8 and 9.4 μm, respectively. On the other hand, they were 4.4, 4.8 and 6.2 μm in Δppro1, Δppro2, and Δppro3 transfected cells, respectively. Thus, binding between profilin and N-WASP is probably required for rapid and efficient microspike formation.

Reconstitution of microspike formation in permeabilized Swiss 3T3 cells

Our previous study (Miki *et al.*, 1998) showed that Cdc42 is an upstream regulator of N-WASP, that is, that Cdc42 binds directly to N-WASP and activates its actin-depolymerizing activity. Therefore, we examined the functional involvement of three components in microspike formation, profilin I, N-WASP and Cdc42. We permeabilized Swiss

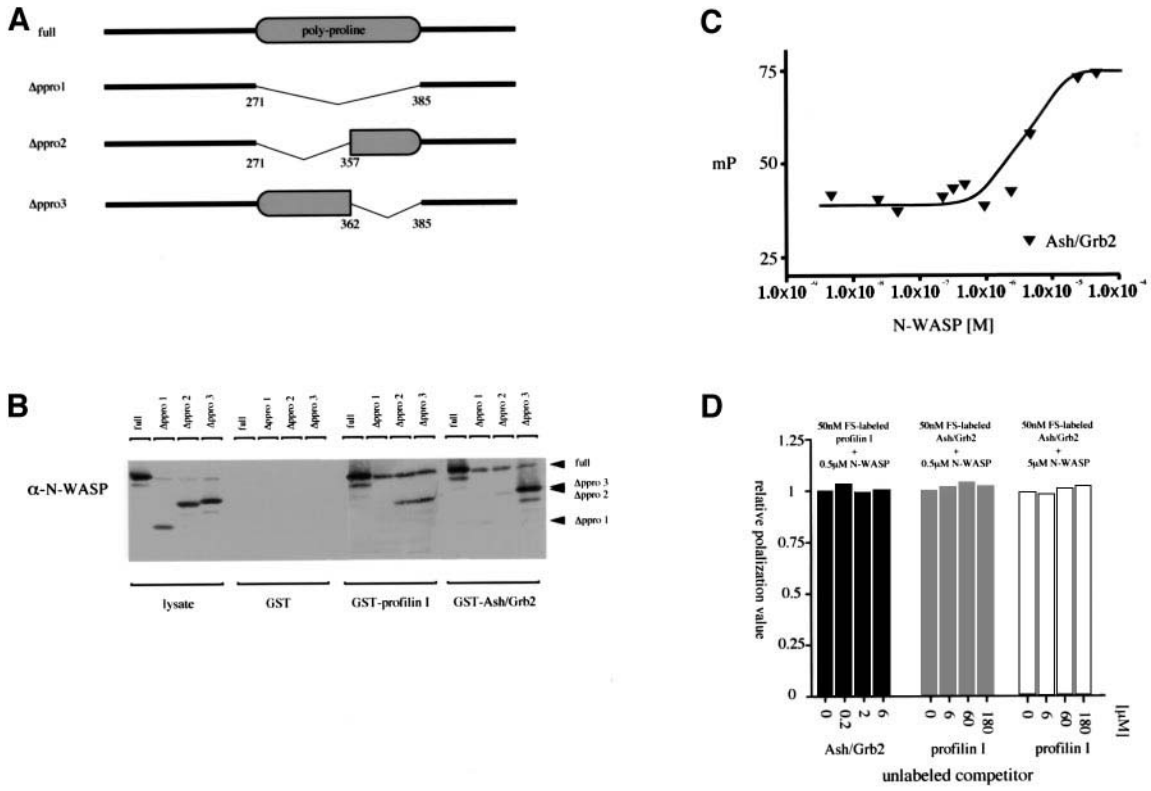

Fig. 5. Determination of profilin I and Ash/Grb2 binding sites in N-WASP. (**A**) The deleted regions in N-WASP mutants used in this study are shown. The N-terminus half of the proline-rich region, which is included in Δppro3, contains all putative Ash/Grb2 binding motifs. Whereas putative profilin binding motifs exist in both the N- and C-terminus half of the proline-rich region. (**B**) Δppro1, Δppro2, Δppro3 mutants and full-length N-WASP were transfected into COS-7 cells and resulting cell lysates were subjected to binding assay using GST–profilin I and GST–Ash/Grb2. The bound proteins were analyzed by Western blot analysis with anti-N-WASP antibody. The results using GST protein are also shown as negative controls. (**C**) Determination of the binding constant between Ash/Grb2 and N-WASP. The binding constant between Ash/Grb2 and N-WASP was determined as in Figure 4B against 50 nM FS-labeled Ash/Grb2. The K_d for Ash/Grb2 and N-WASP was determined to be 2 μM from curve fitting. (**D**) Competition between Ash/Grb2 and profilin I. Constant amounts of labeled profilin I or Ash/Grb2 and N-WASP were mixed with unlabeled Ash/Grb2 or profilin I as a competitor and the polarization values were measured.

3T3 cells and then added these components to examine whether actin-microspike formation would be reconstituted. Swiss 3T3 cells are routinely used in studies of actin-cytoskeletal reorganization events such as the formation of filopodia, lamellipodia and stress fibers, and were previously shown to require N-WASP for filopodia formation through microinjection of anti-N-WASP antibody (Miki *et al.*, 1998). Permeabilized cells were stimulated by adding profilin I, N-WASP, Cdc42 loaded with GTPγS [Cdc42(GTPγS)], and GTPγS in combination or alone. Only all three components in combination, profilin I, N-WASP and Cdc42(GTPγS), induced microspike formation (Figure 8A, n). Active or inactive Cdc42 alone (Figure 8A, g and h), N-WASP alone (Figure 8A, i), GTPγS alone (Figure 8A, b), profilin I wild-type or mutant alone (Figure 8A, c and d), profilin I and N-WASP with or without GTPγS (Figure 8A, l and m), profilin I wild-type or mutant and GTPγS (Figure 8A, e and f), Cdc42 and N-WASP (Figure 8A, j), or Cdc42 and profilin I (Figure 8A, k) were not sufficient to induce microspike formation, strongly suggesting the importance of profilin. Under the same conditions, mutant profilin I used in place of wild-type profilin I also suppressed microspike formation (Figure 8A, o). Furthermore, the addition of cytochalasin D, an inhibitor of actin polymerization, to the mixture of profilin I, Cdc42 and N-WASP abolished actin polymerization, and microspike formation was not observed (Figure 8A, p), suggesting that this microspike formation is due not to retraction, but to actin polymerization.

We incorporated Cy3-labeled actin under stimulation by the three components to allow newly polymerized actin filaments to be visualized. Cy3-actin was incorporated into the microspikes, showing clearly that microspike formation was induced after permeabilization, indicating that these microspikes are quite similar to filopodia (Figure 8B). In the case of mutant profilin I, Cy3-actin was not incorporated into actin filaments, indicating the essential function of profilin I in actin polymerization. As shown in Figure 8C, profilin I, Cdc42 and N-WASP were largely eliminated from the cells by permeabilization, although most of the actin was retained. The amounts of profilin I, Cdc42 and N-WASP following permeabilization were estimated to be one-tenth, one-third and one-quarter the amounts in non-permeabilized cells, respectively. This explains why Cdc42 alone, N-WASP alone, or Cdc42 and N-WASP without profilin I were unable to induce microspike formation. These results indicate that three components, profilin I, N-WASP and Cdc42, are essential factors in microspike formation. However, some other unknown factors in the cell may also be required.

Importance of profilin in neurite extension

All the experiments mentioned above focused on the actin reorganization processes induced by exogenous N-WASP.

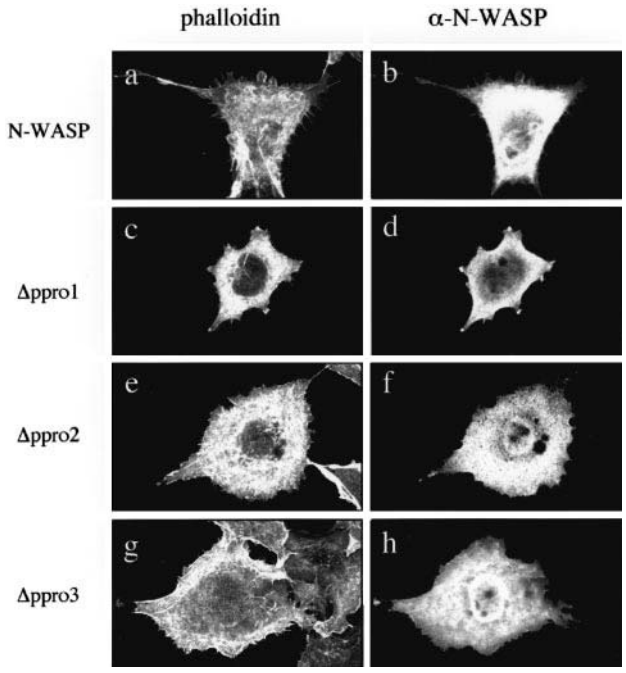

Fig. 6. Expression of the N-WASP mutants in COS-7 cells. COS-7 cells were transfected with plasmids expressing Δppro1, Δppro2 or Δppro3 mutants of N-WASP, or full length N-WASP. Transfected cells were stimulated with EGF for 10 min. After fixation and permeabilization, the cells were stained with phalloidin (**a**, **c**, **e** and **g**) and anti-N-WASP antibody (**b**, **d**, **f** and **h**).

To investigate the role of profilin in the more physiological processes of actin reorganization, we examined the role of profilin in neurite extension using N1E-115 cells which are known to extend neurites under serum starvation in an endogenous Cdc42- and Rac-dependent manner (Kozma *et al.*, 1997). The mutant and the wild-type profilin I were introduced into N1E-115 cells and the effect on neurite extension was observed (Figure 9). In wild-type profilin I-expressing cells, the rate of neurite-bearing cells was not significantly changed compared with original N1E-115 cells, whereas in H119E mutant profilin I-expressing cells, formation of neurites was severely impaired, and the cells remained round. This indicates the importance of profilin for actin polymerization in neurite extension.

Discussion

This is the first conclusive demonstration that profilin plays an important role in the regulation of the actin cytoskeleton downstream of actin regulatory protein.

Profilin-binding proteins in various tissues seem to be mainly actin-regulatory proteins

Major profilin-binding proteins were characterized by peptide sequencing or Western blotting. These proteins include p140mDia (Watanabe *et al.*, 1997), gephyrin (Mammoto *et al.*, 1998), MENA (Gertler *et al.*, 1996), N-WASP and VASP (Reinhard *et al.*, 1995), all of which

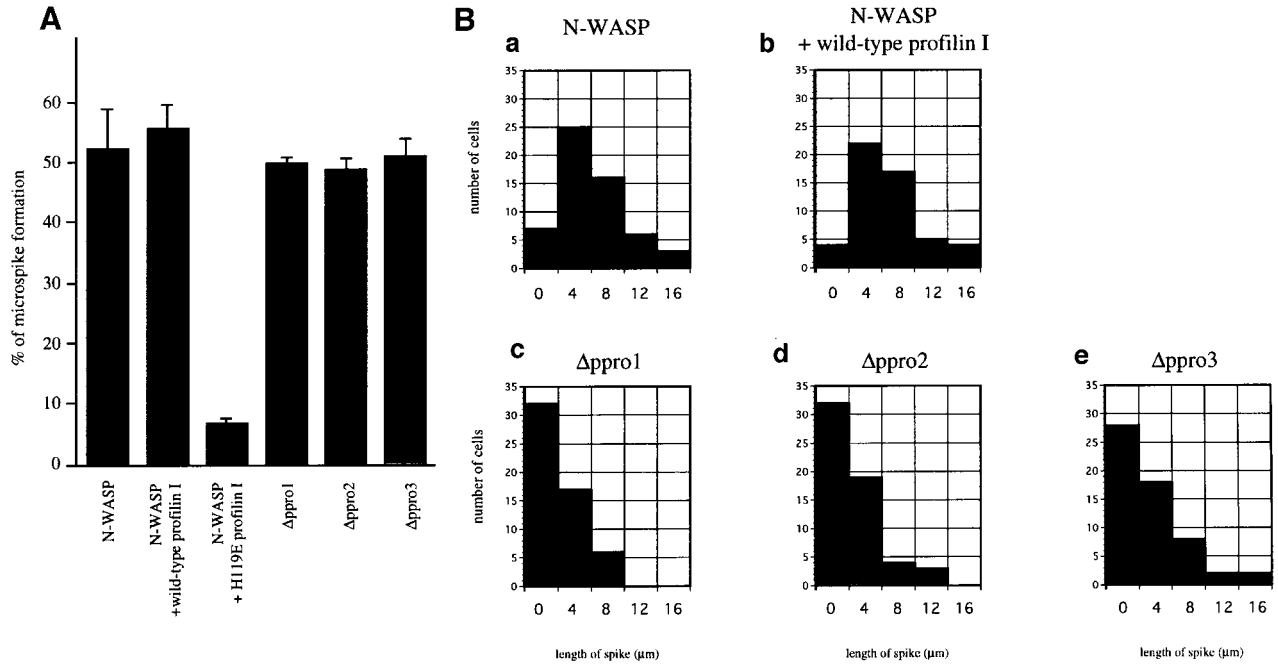

Fig. 7. Quantification of spike-forming rate and length of microspikes among COS-7 cells expressing various types of N-WASP. (**A**) Percentage of spike-forming cells among transfected cells. The cells were stained and the percentage of spike-forming cells was calculated. Cells giving strong FITC (myc-profilin) and Cy5 (N-WASP) signals (in Figure 2A) or strong FITC (N-WASP) signals (in Figure 6) compared with surrounding cells were considered to be expressing the recombinant protein and accounted for ~15–20% of total cells. Cells with spikes lining the edge were considered to be spike-forming cells. At least two transfections were performed in each case and ~100 cells were counted. Error bars represent SEM. (**B**) Length of microspikes in spike-forming cells. The length of the longest microspike in a spike-forming cell was measured by photographing a cell and putting a ruler onto the picture. The distributions of spike length were plotted. In each case, ~50 spike-forming cells were measured. In each transfection, similar distributions were observed. (a) N-WASP alone. (b) Co-expression of N-WASP and wild-type profilin I. (c, d and e) Δppro1, Δppro2 and Δppro3 mutants of N-WASP, respectively.

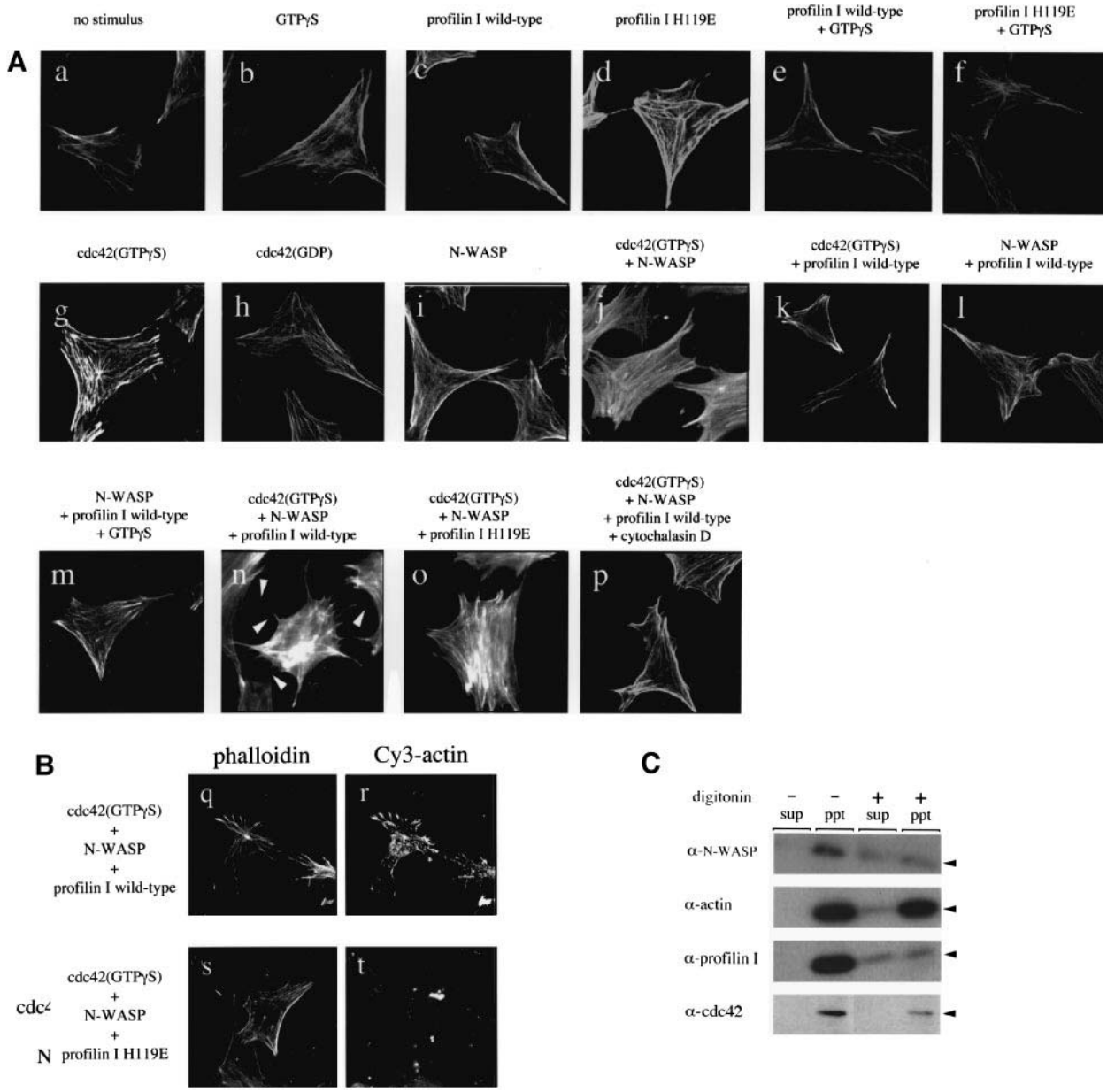

Fig. 8. Reconstitution of microspike-formation by Cdc42, N-WASP and profilin I in permeabilized Swiss 3T3 cells. (**A**) Permeabilization of Swiss 3T3 cells with or without various stimuli. The cells on coverslips were permeabilized with 0.003% digitonin for 6 min at room temperature, and then transferred to buffer solutions containing the stimuli stated below, but without digitonin, and incubated for 20 min at 37°C. Incubation at 37°C was performed (a) in the absence of stimulants, or in the presence of (b) 100 μM GTPγS, (c) 10 μM wild-type profilin I, (d) 10 μM H119E profilin I, (e) 10 μM wild-type profilin I and 100 μM GTPγS, (f) 10 μM H119E profilin I and 100 μM GTPγS, (g) 5 nM Cdc42 loaded with GTPγS [Cdc42(GTPγS)], (h) 5 nM Cdc42 loaded with GDP [Cdc42(GDP)], (i) 50 nM N-WASP, (j) 5 nM Cdc42(GTPγS) with 50 nM N-WASP, (k) 5 nM Cdc42(GTPγS) and 10 μM wild-type profilin I, (l) 50 nM N-WASP and 10 μM wild-type profilin I, (m) 50 nM N-WASP, 10 μM wild-type profilin I and 100 μM GTPγS, (n) 10 μM wild-type profilin I, 5 nM Cdc42(GTPγS), (o) 50 nM N-WASP, 10 μM H119E mutant profilin I, 5 nM Cdc42(GTPγS) and 50 nM N-WASP, or (p) 10 μM wild-type profilin I, 5 nM Cdc42(GTPγS), 50 nM N-WASP and 3 μM cytochalasin D. The cells were fixed and F-actin was visualized with rhodamine-conjugated phalloidin. (**B**) Permeabilization of Swiss 3T3 cells with exogenously added Cy3-labeled actin to visualize newly polymerized actin filaments. Following permeabilization, the cells were incubated at 37°C in the presence of 0.5 μM Cy3-labeled actin and the same stimuli as in n (q and s) and o (s and t). F-actin was visualized using fluorescein-conjugated phalloidin (q and s). Cy3-actin was visualized directly (r and t). (**C**) Retention of components after permeabilization. Cells on coverslips were permeabilized as described above with (+) or without (–) 0.003% digitonin for 6 min, and the supernatant (sup) and precipitate, i.e. cell pellets (ppt) were collected. Precipitates were removed from coverslips with 30 μl of 2× SDS–PAGE sample buffer. Equal volumes of samples were subjected to Western blot analysis using anti-N-WASP, anti-actin, anti-profilin I and anti-Cdc42Hs antibodies. Signals are indicated by arrowheads.

are known to regulate the actin cytoskeleton and to associate with profilin, strongly supporting the utility of this assay. In addition, our results correlate well with the results obtained by Mammoto *et al.* (1998) who used a similar experimental procedure. Witke *et al.* (1998) showed a profilin-binding protein pattern different from the pattern obtained by our GST–profilin-bound glutathione–Sepharose method using profilins cross-linked to agarose beads. We also used covalently-linked GST–profilin beads and obtained results similar to those of Witke *et al.* (1998) (data not shown). With the GST–profilin-bound glutathione–Sepharose methods, profilins themselves are

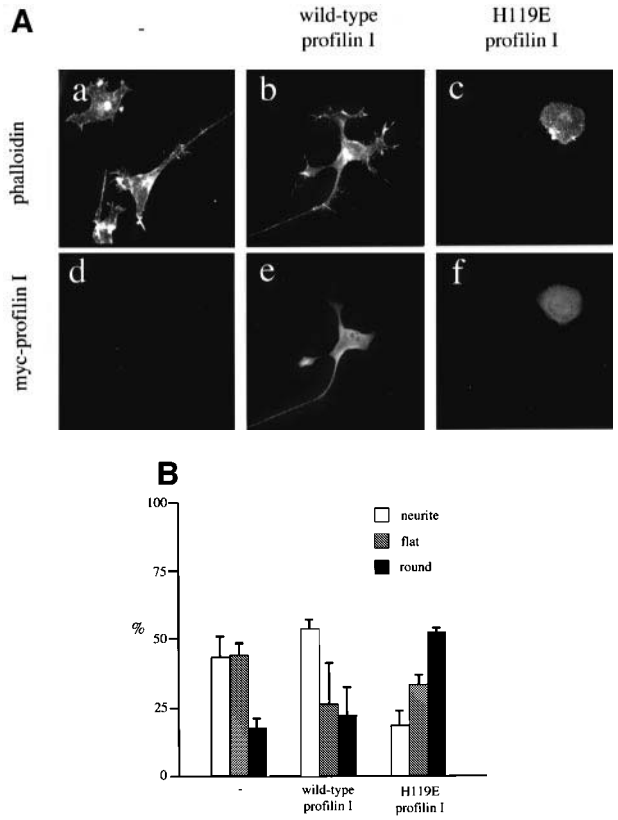

Fig. 9. Inhibition of neurite extension by H119E mutant profilin I in N1E-115 neuroblastoma cells. (**A**) N1E-115 cells were transfected with plasmids expressing wild-type profilin I or the H119E mutant profilin I. Transfected cells were serum starved for 48 h to allow cells to extend neurites. After fixation and permeabilization, the cells were stained with phalloidin (a, b and c) and anti-myc antibody (d, e and f). (**B**) Percentage of neurite-bearing cells among transfected cells. The morphology of cells with strong FITC signals (myc-profilin I) was scored as either round, flat or neurite bearing. Cells with at least one process greater than one cell diameter were considered as neurite bearing. Usually 100 cells were counted and the values presented are the mean percentages of two independent transfections. Error bars represent SEM.

thought to retain their normal conformations, although some interference by the GST part of GST–profilin in the protein–protein interaction may occur. With profilin-crosslinked agarose methods, the possibility of a conformational change in profilin caused by cross-linking can not be excluded.

H119E mutant profilin I, a valuable tool for the study of actin filament regulation

We made a profilin mutant that is defective only in actin binding. This mutant suppresses the microspike formation induced by N-WASP and Cdc42 both in COS-7 cells and permeabilized Swiss 3T3 cells. Thus, this mutant is thought to repel the endogenous profilin required for the G-actin recruitment that leads to actin polymerization. As shown in Figure 1, this mutant profilin I is thought to retain its ability to bind to all other proteins except actin. Thus, this mutant profilin represents a valuable tool for characterizing actin cytoskeletal reorganization events into two categories, processes requiring profilin recruitment probably for the *de novo* actin polymerization, and processes that proceed independently of profilin such as the modification of previously existing actin structures or unknown profilin-independent actin polymerization mechanisms.

Profilin I and Ash/Grb2 bind different sites in the proline-rich region of N-WASP

Both profilin and Ash/Grb2 associate with N-WASP through its proline-rich region. The results shown in Figure 5 demonstrated that profilin and Ash/Grb2 bind different poly-proline stretches and thus do not compete for association with N-WASP. This characteristic of N-WASP seems to be important for it to induce actin filament reorganization rapidly at the sites where signals are received. N-WASP constitutively associates with Ash/Grb2 (Miki *et al.*, 1996). This constitutive association of N-WASP with Ash/Grb2 may be required for the recruitment of N-WASP at the sites where rapid cytoskeletal reorganization should occur.

Reconstitution of microspike formation using permeabilized cells

Microspikes and filopodia, which are thin cylinders that can extend tens of microns from the main cortex of a cell and contain a tight bundle of long actin filament, are the simplest protrusive cellular structures (Mitchson and Cramer, 1996). Thus, the mechanisms of their formation may be the basis for actin-based cell motility. We succeeded in reconstituting microspike formation in permeabilized cells using a combination of activated Cdc42, N-WASP and profilin I.

The initiation of actin polymerization requires seeds from which actin filaments can elongate. *In vitro*, N-WASP depolymerizes actin filaments in a Cdc42-dependent manner (Miki *et al.*, 1998). This may generate free barbed ends for actin polymerization, although much about the actin-filament cutting mechanism of N-WASP remains to be investigated. It is also possible that the seeds result from the generation of free barbed ends due to the removal of barbed-end capping protein. It has been suggested that gelsolin replacement is the trigger for actin polymerization in platelets (Hartwig *et al.*, 1995). However, cells that lack gelsolin appear to form filopodia as effectively as cells containing gelsolin (Azuma *et al.*, 1998). We also have to consider the involvement of *de novo* nucleation in actin polymerization. In this case, the seeds from multiprotein complexes that serve as templates elongate actin filaments. Candidate proteins include the Arp2/3 complex, reported to induce an F-actin cloud around *L.monocytogenes* in platelet extracts and to localize at the leading edge of motile cells (Machesky *et al.*, 1994; Welch *et al.*, 1997). It may also be that N-WASP somehow regulates or cooperates with the Arp2/3 complex in the formation of microspikes.

Elongation of actin filaments from free barbed ends requires the recruitment of G-actin necessary for actin polymerization. N-WASP is possibly involved in this process through binding to profilin, concentrating G-actin in the area of newly formed barbed ends. Since the use of mutant profilin instead of wild-type profilin suppressed the formation of microspikes, profilin itself elongates actin filaments in microspikes. We performed an additional experiment to ascertain whether profilin I is essential for actin incorporation into microspikes. Cy3-labeled actin, which is exogenously added in Figure 8B, was incorporated into microspikes in the case of wild-type profilin I,

but not in that of H119E profilin I. Therefore, it is clear that profilin I mediates the incorporation of actin into microspikes.

The role of profilin in N-WASP-induced microspike formation

Profilin was first identified as an actin filament depolymerizing factor. However, recent reports have indicated functions of profilin in actin polymerization such as the enhancement of ATP–ADP exchange on G-actin and the decrease of critical concentration in the barbed end actin assembly. These contradictory characteristics make it difficult to understand the role of profilin in actin filament dynamics. Our report supports a positive role for profilin in actin polymerization *in vivo*. In this sense, it is a surprise that the cells overexpressing N-WASP deficient in profilin binding can form microspikes. However, this phenomenon does not negate the importance of profilin proved through mutant profilin experiments. Since profilin is abundant and especially enriched at the periphery of cells (Finkel *et al.*, 1994), formation of microspikes may be possible without recruiting profilin to the sites of microspike formation by utilizing profilin which already exists. However, the microspikes induced by N-WASP mutants were not the same as those induced by wild-type N-WASP. The length of microspikes was reduced greatly in N-WASP mutant-expressing cells. This result strongly supports the importance of the association between profilin and N-WASP, which has a very low K_d value, for the rapid and efficient reorganization of actin in microspikes.

Materials and methods

Recombinant protein expression and purification

Profilin I and II cDNAs were amplified by the reverse transcription–polymerase chain reaction (RT–PCR) from human fibroblast total RNA using synthetic oligonucleotide primers and then inserted into the *Bam*HI site of pBluescript (Stratagene) for mutagenesis, pGEX-2T (Pharmacia) for GST-fusion protein expression, or pEF-BOS with myc-tag for transient expression in COS-7 cells. The expression and purification of GST-fusion proteins were carried out as described previously (Miki *et al.*, 1994). Profilins without GST were obtained from GST–profilins by thrombin digestion as described (Self and Hall, 1995). Recombinant N-WASP was expressed using recombinant baculovirus and purified as described previously (Miki *et al.*, 1998). GST–Ash/Grb2 was obtained by the methods of Miki *et al.* (1994).

Ash/Grb2 without GST was produced by digesting GST–Ash/Grb2 with Factor Xa (Danax Biotek) and purified by gel filtration. Cdc42 was expressed and purified from *Escherichia coli* as described (Self and Hall, 1995).

Mutagenesis of profilin

Point mutations (His119 changed to glutamate in both profilins I and II) were introduced by PCR using mutated oligonucleotide primers. The mutated cDNA fragments were amplified and ligated into the same vectors. The effects of mutations on actin binding were assayed using GST-fusion proteins immobilized on glutathione–Sepharose 4B beads. The immobilized beads were mixed with crude cell lysates or bovine tissue homogenates. After washing, the beads were suspended in SDS sample buffer and subjected to Western blot analysis and Coomassie Brilliant Blue staining. The peptide sequences were determined as described (Miki *et al.*, 1996).

Deletion mutants of N-WASP

The N- and C-terminus regions (shown in Figure 5A) of Δppro1, Δppro2 and Δppro3 mutants of N-WASP were amplified by PCR, and these regions were ligated through *Kpn*I sites added in the PCR primers. The resulting mutants were ligated to pcDL-SRα for transient expression in COS-7 cells. These mutants were expressed in COS-7 cells as described below and the cell lysates were collected. Association between these mutants and profilin I or Ash/Grb2 was examined as described above.

Transient expression in COS-7 cells and N1E-115 cells

Recombinant plasmids for transfection such as myc-profilin in pEF-BOS and N-WASP in pcDL-SRα, were prepared from *E.coli* using a Plasmid Midi kit (Qiagen).

For COS-7 cells, 10 μg of each plasmid was mixed with 1×10^7 cells and the mixtures were subjected to electroporation (Bio-Rad). The cells were re-plated and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. After 24 h cultivation, the cells were serum-starved for 15 h before stimulation with 100 ng/ml human EGF (Gibco-BRL) for 10 min. The stimulated cells were analyzed by immunofluorescence microscopy and Western blotting. The length of microspikes was measured by photographing the cell and then putting a ruler onto the picture.

For N1E-115 cells, DNA transfections were performed as described previously (Shibasaki and McKeon, 1995). Then, the cells were serum starved for 48 h before fixation.

Purification of profilin and binding assay with N-WASP

Profilin was partially purified from bovine spleen using a polyproline peptide column as described previously (Janmey, 1991). The purity was checked by SDS–PAGE (~80–90% pure). N-WASP and profilin were mixed to final concentration 10 μM in phosphate-buffered saline (PBS) for 2 h at 4°C and immunoprecipitated with anti-N-WASP antibody. The immunoprecipitates were subjected to Western blot analysis using an anti-profilin antibody.

Measurement of protein–protein associations by fluorescence polarization

Profilin I, profilin II and Ash/Grb2 were labeled with succinimide–fluorescein at pH 7.0 using a kit provided by Pan Vera Corporation. Since succinimide–fluorescein reacts best with unprotonated primary amines, the selectivity for the N-terminal α-amino group ($pK_a = 7$) over side-chain lysine ε-amino groups ($pK_a = 9.5$) is greatly increased at neutral pH. To determine binding constants, constant amounts of labeled proteins and various amounts of N-WASP were mixed in buffer (40 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol) for 2 h at room temperature. The polarization values were measured by BEACON™ (Pan Vera) and plotted against N-WASP concentration. Fluorescence polarization (Dandliker *et al*., 1981) has often been used recently to measure the binding affinities of protein–protein interactions (Schindler *et al.*, 1995; Kwok *et al*., 1996). When excited with plane-polarized light, fluorescein attached to the smaller protein, in this case profilin, initially emits polarized light. Rapid depolarization occurs, however, because of the free rotation of the smaller protein. Upon binding to N-WASP, the fluorescein-labeled profilin becomes attached to a much larger molecule. The rotation of the bound profilin is thus slowed, thereby reducing the rate of depolarization of the emitted light and leading to a high steady-state polarization. Thus, polarization values are proportional to molecular volume. Increased polarization reflects an increase in the association between proteins. K_d values were calculated by a curve-fitting program (GraphPad PRISM™).

In competition experiments, constant amounts of labeled proteins, various amounts of unlabeled proteins (as competitors) and N-WASP were mixed and measured as described above.

Permeabilization

Swiss 3T3 cells were cultured in DMEM supplemented with 10% fetal bovine serum. Cells were seeded on 11×22 mm coverslips and cultured for 2 days. The medium was changed to serum-free type and the cells further cultured for 24 h before use. Permeabilization was performed based as described previously (Mackay *et al.*, 1997) with modification. Permeabilization buffer composed of 150 mM glutamate, 10 mM HEPES, 5 mM glucose, 2 mM $MgCl_2$, 0.4 mM EGTA (pH adjusted to 7.6 with KOH) was stored at –80°C until use. Immediately before the experiment, complete buffer was prepared by adding the ATP regenerating system composed of 1 mM ATP, 1 mM UTP (Pharmacia), 5 mM creatine phosphate, and 10 μg/ml creatine phosphokinase (Sigma), and protease inhibitor cocktail of 10 μg/ml of chymostation, leupeptin, aprotinin, antipain, pepstain (Peptide Institute, Japan) and 1 mM benzamidine hydrochloride (Sigma) to the permeabilization buffer. Cells on coverslips were permeabilized and then incubated with various stimuli which are indicated in the figure legends). Permeabilization was performed for 6 min at room temperature in 100 μl complete buffer containing 0.003% digitonin (Sigma). Only in the case of incubation with profilin, was

permeabilization performed by adding wild-type or mutant profilin I at the same concentration as for incubation. Then, incubation with stimulus was performed for 20 min at 37°C in 100 μl of complete buffer containing one of the stimuli (as indicated in the figures), but without digitonin. The cells were fixed in 3.7% formaldehyde in PBS for 20 min. After washing fixed cells with PBS, the coverslips were incubated with phalloidin for 1 h, and the cells were observed by fluorescence microscopy.

Cdc42 was loaded with GTPγS as follows. The loading mixture was composed of 0.4 μM Cdc42, 50 μM GTPγS, 50 mM Tris–HCl pH 7.5 and 5 mM EDTA. This mixture was incubated for 10 min at 30°C, and the reaction was stopped by adding $MgCl_2$ to a final concentration of 100 mM. The resulting GTPγS-loaded Cdc42 was directly added to complete buffer as stimulus.

Cy3-actin was prepared as described (Machesky and Hall, 1997). After the final step of the labeling procedure, Cy3-labeled G-actin solution was gel-filtrated using Sephadex G-50 (Pharmacia) to completely remove unreacted dye and concentrated by ultrafree C3LGC (Millipore). The polymerizing activity was checked by subjecting Cy3-labeled actin to polymerizing conditions (50 mM KCl, 1 mM $MgCl_2$ and 1 mM ATP) and incubating for 1 h at room temperature. Cy3-actin in polymerizing and unpolymerizing conditions was then ultracentrifuged at 100 000 r.p.m. (Beckman type 45 Ti) for 20 min and the resultant supernatant and pellet were analyzed by SDS–PAGE. The labeling was checked by directly visualizing the gel under UV light.

Antibodies

The anti-myc antibody, anti-Cdc42 antibody, and phalloidin conjugated to rhodamine or fluorescein were from Santa Cruz Biotechnology. The anti-N-WASP antibody was prepared as described (Fukuoka *et al.*, 1997). The anti-profilin antibody, which recognizes both profilins I and II, was a generous gift from Dr S.Obinata (Chiba University School of Science). Anti-profilin I and II antibodies were produced by immunizing rabbits with synthetic oligopeptides as antigens. The peptide sequences were VPGKTFVNITPAEVG for profilin I and TAGGVFQSITPIEID for profilin II. The secondary antibodies linked to alkaline phosphatase were from Promega. The secondary antibodies linked to peroxidase were from Cappel. The secondary antibodies linked to fluorescein were also from Cappel. The secondary antibodies linked to Cy5 were from Amersham.

Western blotting

Samples from immunoprecipitation, binding assay and so on were subjected to SDS–PAGE and electrically transferred to a polyvinylidene (PVDF) membrane. After blocking with PBS with 0.2% Tween 20 and 5% skimmed milk, the membrane was incubated with the primary antibody and then with alkaline phosphate-conjugated or peroxidase-conjugated antibody suitable for the primary antibody. Detection of alkaline phosphatase was with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate. Detection of peroxidase was with ECL system (Amersham).

Immunofluorescence microscopy

Immunofluorescence microscopy except for permeabilized Swiss 3T3 cells was performed as follows. Cells fixed in 3.7% formaldehyde in PBS for 20 min were permeabilized with 0.2% Triton X-100 in PBS for 5 min. After incubation with the anti-N-WASP antibody and/or anti-myc antibody followed by anti-rabbit antibody linked to fluorescein and/or anti-mouse antibody linked to Cy5, the cells were observed by fluorescence microscopy.

Acknowledgements

We thank Dr S.Obinata of the Chiba University School of Science for providing us with the anti-profilin antibody. This study was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan, and by a Grant-in-Aid for Research for the Future Program from the Japan Society for the Promotion of Science.

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Received April 9, 1998; revised August 6, 1998; accepted September 18, 1998