Enhancement of branching efficiency by the actin filament-binding activity of N-WASP/WAVE2

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SUMMARY

The actin-related protein (Arp) 2/3 complex is an essential regulator of de novo actin filament formation. Arp2/3 nucleates the polymerization of actin and creates branched actin filaments when activated by Arp2/3-complex activating domain (VCA) of Wiskott-Aldrich syndrome proteins (WASP family proteins). We found that the branching of actin filaments on pre-existing ADP filaments mediated by the Arp2/3 complex is twice as efficient when Arp2/3 was activated by wild-type neural WASP (N-WASP) or WASP-family verprolin-homologous protein (WAVE) 2 than when activated by the VCA domain alone. By contrast, there was no difference between wild-type N-WASP or WAVE2 and VCA in the branching efficiency on de novo filaments, which are thought to consist mainly of ADP-phosphate filaments. This increased branching efficiency on ADP filaments is due to the basic region located in the center of N-WASP and WAVE2, which was found to associate with ADP actin filaments. Actin filaments and phosphatidylinositol bisphosphate (PIP_2) associate with N-WASP at different sites. This association of N-WASP and WAVE2 with actin filaments enhanced recruitment of Arp2/3 to the pre-existing filaments, presumably leading to efficient nucleation and branch formation on pre-existing filaments. These data together suggest that the actin filament binding activity of N-WASP and WAVE2 in the basic region increases the number of barbed ends created on pre-existing filaments. Efficient branching on ADP filaments may be important for initiation of actin-based motility.

Key words: N-WASP, WAVE, Arp2/3 Complex, Actin, Capping Protein

INTRODUCTION

The actin cytoskeleton is one of the primary determinants of cell shape and motility. In the process of actin cytoskeletal reorganization, the Wiskott-Aldrich syndrome protein (WASP) family plays essential roles downstream of many regulators (Takenawa and Miki, 2001). This family consists of two WASP proteins (Derry et al., 1994; Miki et al., 1996) and three verprolin-homologous proteins (WAVEs) (Miki et al., 1998; Suetsugu et al., 1999). The key role of WASP family proteins is to activate the actin-related protein (Arp) 2/3 complex in response to signals (Fukuoka et al., 2001; Miki et al., 2000; Rohatgi et al., 1999).

The architecture of WASP family proteins, WASP, neural WASP (N-WASP) and WAVEs are similar not only in their C-terminal regions but also in several other domains (Fig. 1a). All WASP family proteins have a basic region and a proline-rich region in their center as well as verprolin-homology, cofilin-homology and acidic domains (VCA domain) at the C terminus. The proline-rich region binds many signaling molecules such as Ash/Grb2 and WASP-family-interacting SH3 protein (WISH), as well as actin regulatory molecules such as profilin (Carlier et al., 2000; Fukuoka et al., 2001; Miki et al., 1996; Suetsugu et al., 1998). The VCA domain is the activating domain for Arp2/3. Basic amino acids are clustered throughout the mid-region of WASP family proteins and this basic domain was recently found to bind phosphoinositides (Prehoda et al., 2000; Rohatgi et al., 2000). However, the entire basic domain is not necessary for binding to phosphoinositides (Rohatgi et al., 2000).

Experiments performed in vitro with pure Arp2/3 complex and actin show that the C-terminal VCA domain of WASP family proteins is the activating domain of the Arp2/3 complex, which results in rapid de novo nucleation and subsequent burst of actin polymerization (Blanchoin et al., 2000; Pantaloni et al., 2000; Rohatgi et al., 1999). Therefore, the VCA peptide fragment is thought to be a constitutive activator of actin polymerization in vitro. In this process, the verprolin-homology (V) domain directly associates with actin, and the cofilin-homology acidic (CA) region interacts with the Arp2/3 complex, forming a nucleating machine for actin polymerization (Machesky et al., 1999; Miki et al., 1996; Miki et al., 1998; Rohatgi et al., 1999; Suetsugu et al., 1999).

Activated Arp2/3 not only causes actin polymerization but also induces formation of branched filaments, which are similar in morphology to actin filaments seen in the lamellipodia and actin comet of motile bacteria (Blanchoin et al., 2000; Pantaloni et al., 2000). This activity is due to an actin filament side-binding activity of Arp2/3 itself (Mullins et al., 1998; Mullins et al., 1997), because pre-existening actin filaments increased their actin polymerization even when their barbed ends were protected from elongation (Amann and

Pollard, 2001), and antibody against the actin-filament-binding interface of Arp2/3 inhibited branching (Bailly et al., 2001).

In studies of actin-based motility, the formation of actin comets by intracellular pathogens such as *Shigella* and *Listeria* are well-established models for understanding the event at the leading edges of motile cells. This actin comet formation can also be reconstituted with plastic beads coated with N-WASP and five other proteins: F-actin, Arp2/3, barbed-end capping protein, profilin and actin depolymerizing factor (ADF)/cofilin (Loisel et al., 1999; Pantaloni et al., 2001; Suetsugu et al., 2001b). This observation clearly shows that N-WASP is the minimum essential molecule for actin-based movement. Previously, N-WASP itself was shown to associate with actin filaments (Egile et al., 1999). Therefore, the association of F-actin with N-WASP is likely to be important. However, the significance of this filament-binding activity is still unclear.

We previously investigated which region of N-WASP is essential for actin comet formation (Suetsugu et al., 2001b). Whereas actin comets formed on beads coated with full-length N-WASP, no comets formed on beads coated with only VCA domain, the minimum domain required for Arp2/3 activation (Suetsugu et al., 2001b). VCA-coated beads were able to induce actin clouds around the beads, but few grew to actin comets. Thus, some other region is likely to be important for actin-based movement besides the VCA domain (Suetsugu et al., 2001b). Various mutant studies indicated that the basic region is also required for actin comet formation (Suetsugu et al., 2001b).

In the present study, we investigated how full-length N-WASP/WAVE2 induces filament branching. We found that the basic domain located in the mid-region of N-WASP and WAVE2 binds with actin filaments. Increased association of Arp2/3 with filaments was achieved by the actin-filament-binding activity of N-WASP and WAVE2. The increase in association between Arp2/3 and filaments was independent of the N-WASP activators Cdc42 and PIP_2. In N-WASP, the basic region is reported to be the PIP_2-binding region. However, PIP_2 and actin filaments bind to different sites in N-WASP, and recruitment of Arp2/3 was not prevented by PIP_2. Furthermore, filament branching induced by wild-type N-WASP was more efficient than branching induced by VCA fragments, presumably owing to recruitment of Arp2/3 to filaments.

MATERIALS AND METHODS

Proteins

Mutant or wild-type N-WASP and WAVE2 with an N-terminal six-His tag were expressed in SF9 cells with the Bac-to-Bac baculovirus expression system (Gibco BRL). Recombinant virus-infected Sf9 cells were lysed, clarified and affinity-purified with Ni-NTA beads (Qiagen). VCA was purified as described previously (Miki et al., 1996). Recombinant Cdc42 was purified and loaded with GTPγS as described previously (Suetsugu et al., 1998). Arp2/3 was affinity purified as described previously with the CA fragment of N-WASP (Egile et al., 1999). Actin was purified from rabbit skeletal muscle and monomeric actin (G-actin) was isolated by gel filtration on Superdex 200 (Amersham Pharmacia) in G buffer (2 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$, 0.5 mM DTT, 0.2 mM ATP). Capping protein β1 (CapZ) was purified as described previously (Soeno et al., 1998). Barbed-end capping activity was determined by assaying for lowering of the end point of polymerization and pointed-end nucleating activities not inhibited by cytochalasin b.

Pyrene-actin assay

The pyrene-actin assay was performed in XB as described previously (Rohatgi et al., 1999). Final concentrations of Arp2/3, G-actin and pyrenyl-actin were 60 nM, 0.46 μM and 0.04 μM, respectively. The wild-type or mutant N-WASP concentration was 100 nM unless otherwise indicated. PIP_2-containing vesicles were prepared as previously described (Rohatgi et al., 1999). Components without G-actin were mixed and allowed to form complexes for 5 minutes at room temperature and then G-actin was added. The generated barbed-end concentration was calculated at the point where polymerization is 80% complete, assuming that pointed elongation is minimum (Higgs et al., 1999) with the equation,

$$\text{elongation rate} = k_+ \times [\text{actin monomers}] \times [\text{barbed ends}]$$

where k_+ is the rate constant for the association of G-actin with filament ends.

Actin filament preparation

The G-actin solution was first clarified by ultracentrifugation at 100,000 ***g*** for 20 minutes. To make ordinary filaments, G-actin (40 μM) was polymerized for 1 hour at room temperature by addition of 5× G-to-F conversion buffer (80 mM Tris-HCl, 250 mM KCl, 1 mM ATP, 2 mM $MgCl_2$). For generation of ADP-F-actin, the F-actin obtained was treated with 20 units ml^{-1} hexokinase and 0.2 mM glucose overnight, as described previously (Pollard, 1984). The amount of polymerized actin was confirmed by ultracentrifugation. ADP-phosphate (ADP-P_i) filaments were made by polymerizing G-actin in the presence of phalloidin (4 μM). ADP filament was mixed with phalloidin and also used in the assays. Gelsolin-capped filaments were prepared as described (Mullins et al., 1998). The molar ratio of gelsolin and actin monomer was 1:200.

F-actin binding assay

The binding of F-actin to N-WASP was analyzed by co-sedimentation assay. Wild-type or mutant N-WASP and WAVE2 solutions were clarified by ultracentrifugation to remove any aggregates. These F-actin and N-WASP/WAVE2 solutions were then mixed and incubated at room temperature for 1 hour. The mixture was then centrifuged at 70,000 ***g*** for 20 minutes. The supernatants and pellets were then analyzed by SDS-PAGE.

Visualization of F-actin

After 15 minutes of actin polymerization, 1 μl of the reaction mixture was diluted in 100 μl of staining solution composed of 0.002% BSA, 0.5% methylcellulose, 3 mg ml^{-1} glucose, and 4 units ml^{-1} Alexa 488 phalloidin (Molecular Probes) in XB. Then, 2 μl of solution containing stained actin filaments was placed between a polylysine-coated glass slide and an 11×22 mm cover glass. Samples were observed on a Zeiss Axiovert S100 with an intensified charge-coupled device camera (Photometrics). F-actin for pre-incubation was generated by co-polymerization of G-actin and rhodamine-labeled G-actin (Theriot and Fung, 1998) at a ratio of 7:3. This labeled F-actin was added to the reaction mixture to a final concentration of 300 nM. Capping protein was added at a final concentration of 100 nM during pre-incubation.

RESULTS

Identification of the basic region as an actin filament-binding region

The basic clusters are often the sites for actin filament binding. Therefore, to determine the role of the basic region in detail, we tested the binding of wild-type or Δbasic N-WASP, in which the basic region is deleted, and WAVE2 to actin filament (F-actin). We performed co-sedimentation assays of wild-type or

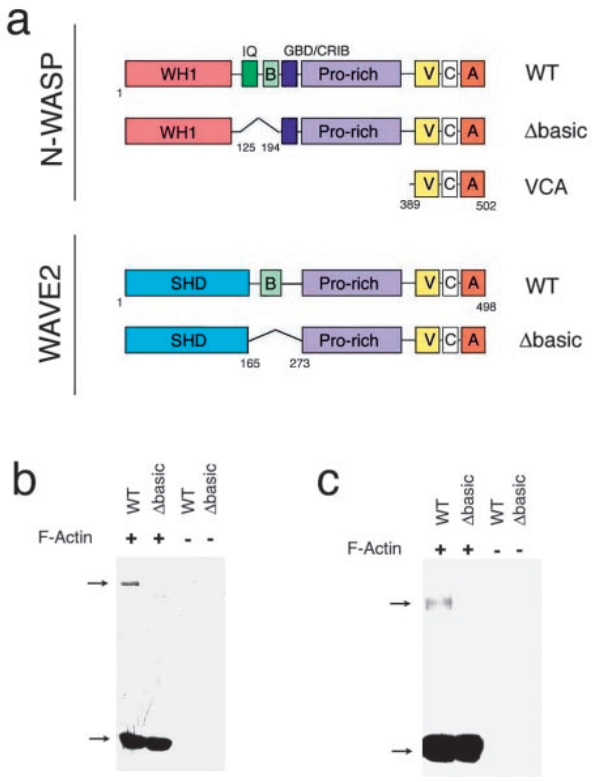

Fig. 1. F-actin binding ability of N-WASP/WAVE2. (a) Schematic structure of N-WASP and WAVE2. The number of amino acids from rat N-WASP or human WAVE2 is indicated. Abbreviations: A, acidic region; B, basic region; C, cofilin homology domain; CRIB, Cdc42/Rac interactive binding region; IQ, IQ motif; Pro, proline-rich region; SHD, SCAR/WAVE homology region; V, verprolin homology domain; WH1, WASP homology 1 domain. (b,c) Association of F-actin with the basic region of N-WASP (b) and WAVE2 (c). Wild-type protein and Δbasic N-WASP (1 μM) or WAVE2 (0.3 μM) were mixed with F-actin (2 μM). The association with F-actin was assayed by co-sedimentation with F-actin after ultracentrifugation. The precipitates were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining. The upper arrows indicate N-WASP or WAVE2, and the lower arrows indicate actin.

Δbasic N-WASP and F-actin. Whereas wild-type N-WASP co-sedimented with F-actin, Δbasic mutant N-WASP did not. These results indicates that the basic region of N-WASP binds with F-actin (Fig. 1b). The Δbasic WAVE2 also did not associate with F-actin through the basic region (Fig. 1c).

To determine how N-WASP or WAVE2 associates with actin filament through the basic region, we assayed for association of actin filaments with isolated basic region by co-sedimentation (Fig. 2). We first tested actin filament binding of N-WASP fragment spanning amino acids 125-273, which contains the entire basic region. As shown in Fig. 2a, this fragment associated with actin filaments. Basic clusters at amino acids 186-195 were previously shown to be the PIP_2 binding region (Rohatgi et al., 2000). Therefore, we next searched for a region in the basic clusters that contributes to actin filament association. N-WASP amino acids 125-165 (first half of the basic region) strongly associated with filaments. By contrast, N-WASP amino acids 166-275 (latter half of the basic region) had a low affinity for filaments (Fig. 2a).

We next examined whether these basic fragments can compete out with full-length N-WASP for binding with actin filaments. In good agreement with the above experiment, both fragments from the basic region of N-WASP, the two halves of the basic region, inhibited the association of N-WASP with filaments. Also, inhibiting activity was stronger with the first half of the basic region than the second half (Fig. 3). Therefore, association of N-WASP with actin filaments involves the entire basic region. Because the second half of the basic region is involved in PIP_2 binding, the modes of association of N-WASP to filaments and to PIP_2 are different. Indeed, neither PIP_2 nor Cdc42 prevented the association of N-WASP to filaments (not shown).

The isolated basic region of WAVE2 also associated with actin filaments, demonstrating that the basic region is the actin-filament-binding region (Fig. 2a). For both N-WASP and WAVE2, association of the isolated basic region with filaments was saturated as filament concentration increased. From a plot of the association of the basic regions and filaments, the K_d for actin filament association was estimated to be ~1 μM, which agrees with that of a previous study (Fig. 2b) (Egile et al., 1999).

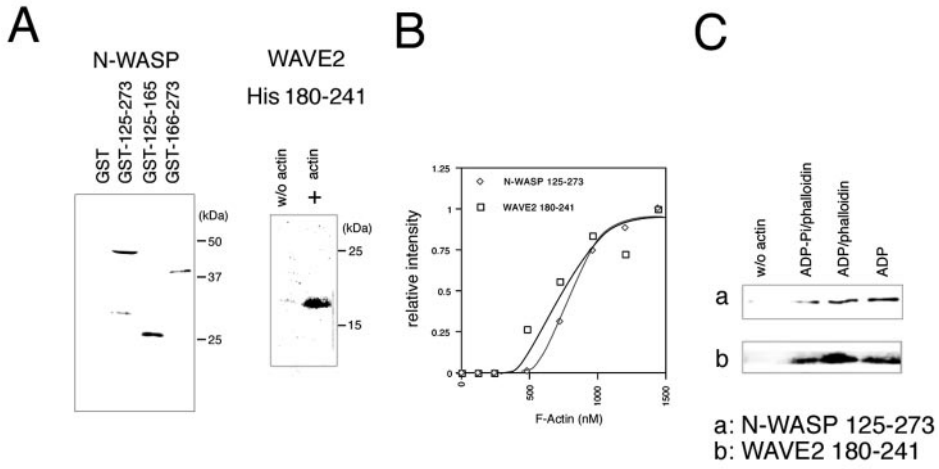

Fig. 2. F-actin binding region of N-WASP/WAVE2. (a) The association of isolated basic region with filaments was analyzed by co-sedimentation assay. The amino acid numbers of these fragments from N-WASP or WAVE2 are shown. Association was analyzed by western blotting to either glutathione-*S*-transferase tag or His tag. (b) Dose dependence of actin filaments (which are composed mostly of ADP filaments and small amounts of filaments with ATP and ADP-P_i caps) against association with the isolated basic region by co-sedimentation assay. The association was monitored by western blotting followed by densitometry. (c) Comparison of the affinity of isolated basic region against ADP-P_i or ADP filaments by co-sedimentation assay. Association was analyzed by western blotting. ADP-P_i filaments were made by polymerizing actin in the presence of phalloidin. ADP filaments were made by hexokinase treatment. To estimate the effect of phalloidin staining, some of the ADP filaments were mixed with phalloidin and also used in the assays.

The actin filaments used in the above experiments were made by polymerization of G-actin (ordinary filaments). When actin polymerizes, the state of the actin filaments changes with time. First, ATP-G-actin polymerizes to form ATP-F-actin. However, the ATP on the filaments is soon hydrolyzed to ADP, but the phosphate generated by ATP hydrolysis is not released from the filaments immediately, resulting in transient ADP-P_i filaments. ADP-P_i filaments, which have a half-life of ~2 minutes, release P_i, becoming the more-unstable ADP filaments. Therefore, most of the filaments used in the above study (ordinary filaments) were ADP filaments with very short ATP and ADP-P_i actin caps at the ends (Carlier et al., 1984). In the place of active actin polymerization, where N-WASP or WAVEs might function, it is thought that significant amounts of ADP-P_i filaments exist, whereas in the resting state, most filaments are thought to be ADP filaments.

Therefore, we examined association of these isolated basic regions with ADP-P_i filaments and ADP filaments (Fig. 2c). Because phalloidin inhibits phosphate release from filaments, actin filaments made by polymerization reaction including phalloidin were used as ADP-P_i filaments. ADP filaments were made by treating ordinary filaments with hexokinase (Pollard, 1984). These ADP filaments were used with and without phalloidin for comparison of ADP-P_i filaments. The affinity against both N-WASP and WAVE2 seems to be stronger for ADP filaments than for ADP-P_i filaments (Fig. 2c). Association of phalloidin with filaments did not significantly affect association with basic regions (Fig. 2c).

Recruitment of Arp2/3 to filaments by N-WASP independent of PIP_2

To examine the meaning of the filament-binding activity of N-WASP/WAVE2, we assessed the influence of N-WASP/WAVE2 on the association of Arp2/3 with filaments. Arp2/3 alone was reported to have actin filament side-binding activity (Mullins et al., 1998; Mullins et al., 1997). Thus, we examined the effect of N-WASP/WAVE2 on the association of actin filaments with Arp2/3 by the co-sedimentation assay. Addition of VCA or Δbasic N-WASP increased the association of Arp2/3 with filaments, as reported previously (Amann and Pollard, 2001), suggesting that activation of Arp2/3 by VCA increases the affinity of Arp2/3 to filaments. In parallel with stronger association with ADP filaments, the amount of Arp2/3 in the actin filament pellet was increased about threefold by addition of wild-type N-WASP/WAVE2 (Fig. 4). This increase was not observed upon addition of the Δbasic mutant or VCA fragment, suggesting that actin filament binding of N-WASP/WAVE2 is essential for recruitment of Arp2/3 to filaments. Association of N-WASP-Arp2/3 with ADP-P_i filaments stabilized with phalloidin is less than that with ordinary filaments stabilized with phalloidin, and ordinary filaments without phalloidin (Fig. 4). Therefore, the ability of N-WASP to recruit Arp2/3 to the F-actin pellet was greater with ADP-F-actin than with ADP-P_i-F-actin. In agreement with a different mode of association for PIP_2 and filaments, addition of PIP_2 at a concentration sufficient to activate N-WASP did not prevent recruitment of Arp2/3 to actin filaments. Therefore, PIP_2 is not likely to compete with actin filaments for the same binding sites on N-WASP. Rather, this result indicates that PIP_2 and actin filaments bind to different sites within the basic region of N-WASP (Fig. 4).

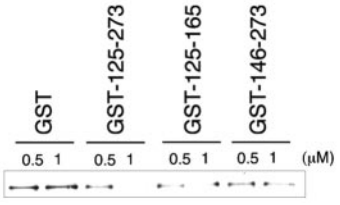

Fig. 3. Basic region of N-WASP competed for actin filament binding. The association of full-length N-WASP with actin filaments in the presence or absence of protein fragments of the N-WASP binding region was analyzed by co-sedimentation assay. Association of full-length N-WASP with actin filaments was monitored with anti-N-WASP antibody. The concentrations of isolated N-WASP fragment are indicated.

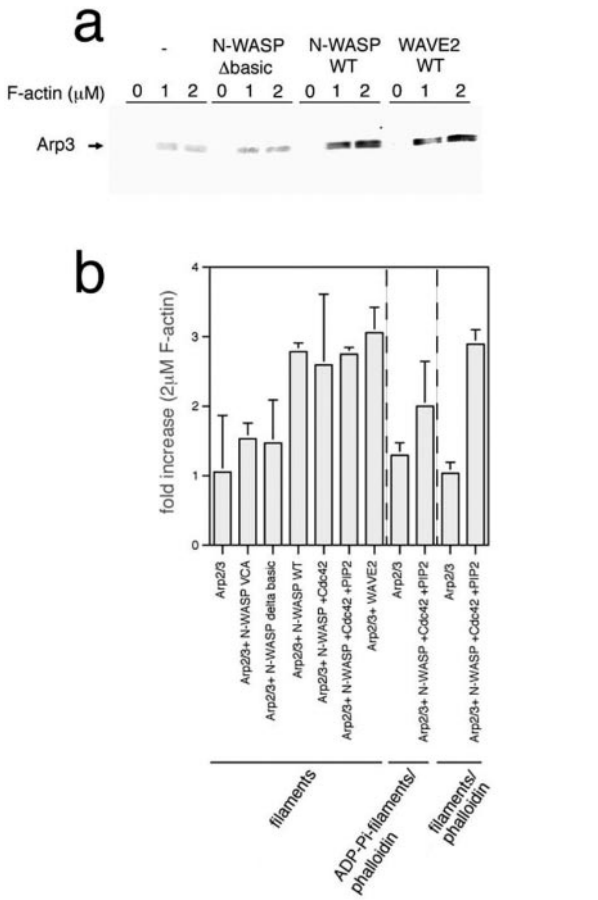

Fig. 4. Increase in association of Arp2/3 with actin filaments by wild-type WASP family proteins. Association of Arp2/3 (0.25 μM) with actin filaments (2 μM) in the presence or absence of N-WASP, its mutants or WAVE2 (1 μM) was analyzed by co-sedimentation. Filaments were made by polymerizing G-actin. Phalloidin filaments were made by adding phalloidin after 1 hour of polymerization. Filaments and phalloidin filaments are composed of ATP actin caps of <100 actin molecules (<0.6 μm per 5 μm (average) filament) and ADP filaments. ADP-P_i/phalloidin filaments were made by polymerizing G-actin with phalloidin. Co-sedimented Arp2/3 complex was monitored by western blotting with anti-Arp3 antibody (a). The extent of association at 2 μM of F-actin was quantified by densitometry and expressed as the fold increase in comparison with Arp2/3 only (b). Bars show the standard deviations.

Essential role of actin filament binding activity in side-branched filament formation

Recent studies showed that VCA in the presence of Arp2/3 simultaneously stimulates nucleation and Y-branching of actin filaments in vitro (Blanchoin et al., 2000; Pantaloni et al., 2000). To assess the effect of binding of actin filaments with N-WASP or WAVE2, we visualized actin filaments formed in the presence of preformed actin filaments. All actin filaments were visualized by staining with green phalloidin and actin filaments used for pre-incubation (preformed filaments), which were made with rhodamine-labeled G-actin, were detected by rhodamine (red) fluorescence (Fig. 5a).

As reported previously (Blanchoin et al., 2000), activation of Arp2/3 by either VCA, Δbasic N-WASP and WAVE2, or wild-type N-WASP and WAVE2 induced branches much more efficiently than did Arp2/3 alone (Fig. 5). However, the branch rate was affected by actin filament binding activity (Fig. 5). We first examined branch formation by wild-type N-WASP/WAVE2 on pre-existing filaments formed from rhodamine-labeled, red G-actin. On these pre-existing filaments, induction of branched filament formation was two times greater with wild-type N-WASP or WAVE2 (0.09-0.10 branches per μm filament) than with Δbasic mutant N-WASP/WAVE2 or VCA fragment (0.03-0.04 branches per μm filament) (Fig. 5d). Decrease in activation of Arp2/3 in the Δbasic mutant is unlikely because Δbasic mutant N-WASP/WAVE2 is a potent Arp2/3 activator (Fig. 6c). Wild-type N-WASP or WAVE2 induced much more branching than did the VCA fragment alone. The similar branch-inducing activity of VCA and the Δbasic mutant N-WASP/WAVE2, which is about half as active as that of wild-type proteins, indicates that the association of N-WASP or WAVE2 with filaments is important for efficient branch formation.

We then measured the lengths of the original filaments (mother filaments) from branch points, which grew in the original direction, and the length of the branched filaments (daughter filaments), which grew at about 70° angles to the direction of mother filament growth (Fig. 5c). Branching on pre-existing filaments occurred randomly with no preference of branch formation near the end of pre-formed filaments (Fig. 6c). This result suggests that actin filament binding activity of N-WASP/WAVE2, which presumably links Arp2/3 and the actin filament, did not create the preference of branch position on pre-existing filaments near either the barbed end or the pointed end.

Filaments polymerized only by G-actin have short ATP- and ADP-P_i-actin caps, which usually consist of several actin monomers (<0.6 μm, or <100 actin monomers) (Carlier et al., 1984). In our preparation, the average length of pre-existing filaments was ~5 μm, so most filaments were thought to be in the ADP form. We confirmed that branching occurs on ADP filaments, which are produced by treatment of ordinary filaments with hexokinase (Pollard, 1984). The extent of branching on ADP filaments made by hexokinase treatment was similar to that on ordinary filaments, confirming the effectiveness of branching on ADP filaments by wild-type N-WASP/WAVE2 (Fig. 5a,d).

We next examined branching on de novo filaments. Actin polymerization ceases within 2 minutes of the burst of polymerization under this condition (2 μM G-actin) (data not shown). Therefore, branches formed on de novo, green filaments are thought to represent branches on ADP-P_i or ATP filaments, because the half-life of phosphate release is ~2 minutes, which is sufficient to cease actin polymerization. The branches on de novo green filaments were observed in all cases at a rate of 0.06 branches per μm filament (Fig. 5a,d). On de novo filaments, which are presumably ADP-P_i filaments, branching also occurred randomly (Fig. 5b). Because wild-type N-WASP and mutant N-WASP lacking the basic domain induced branching equally on ADP-P_i filaments, branching on de novo ADP-P_i filaments does not appear to be influenced by the actin filament binding activity of WASP family proteins, in agreement with the binding assay (Fig. 2).

Therefore, when Arp2/3 is activated by wild-type N-WASP/WAVE2, branching is generated more efficiently on pre-existing filaments than on de novo filaments. This phenomenon is not observed without filament binding activity or with VCA only, suggesting that the filament binding activity of N-WASP/WAVE2 is essential for this preferential branching on ADP filaments.

In all experiments described above, preformed red filaments were pre-incubated with Arp2/3 and N-WASP before addition of G-actin to allow complex formation. After the pre-incubation process, G-actin was added and then polymerization proceeded. Pre-incubation of N-WASP/WAVE2 and filaments might stabilize the filaments, which would increase the rate of branching, because many actin-filament-binding proteins stabilize filaments. However, full-length N-WASP did not stabilize the filaments at all as measured by depolymerization of gelsolin-capped filaments (Fig. 6i). Neither activation of N-WASP with Cdc42 and PIP_2 nor incorporation of Arp2/3 stabilized actin filaments. Therefore, N-WASP and Arp2/3 did not seem to stabilize the pre-existing filaments (Fig. 6i).

Increase of the barbed ends generated in the presence of actin filaments

To elucidate whether the increase of branching by intact N-WASP and WAVE2 accompany the increase of the number of barbed ends, we analyzed the kinetics of actin polymerization in the presence of pre-existing filaments. To minimize the background polymerization at pointed ends, the G-actin concentration was set at 0.5 μM, which is below the pointed end critical concentration. To eliminate polymerization from the free barbed ends of pre-existing filaments, gelsolin-capped filaments were used. Gelsolin binds barbed ends with high affinity against barbed ends (K_d < 1 nM) and blocks barbed end elongation of pre-existing filaments (see control curves in Fig. 6a-c). We measured actin polymerization induced by intact N-WASP (with actin filament binding activity), VCA domain alone or Δbasic mutant (without actin filament binding activity) in the presence and absence of pre-existing filaments. The polymerization curves obtained were differentiated with time (dF/dt) and the kinetics of the increase of actin polymerization were compared (Fig. 6a-g). The differentiated polymerization curve (dF/dt) indicates the rate of increase of actin filaments.

Actin polymerization initiated by Arp2/3 and mutant N-WASP or wild-type N-WASP proceeds in an autocatalytic manner as polymerization proceeds (Pantaloni et al., 2000). By contrast, the polymerization nucleated by actin filament seeds without gelsolin did not display any auto-catalytic polymerization (not shown). In polymerization mediated by VCA, Δbasic N-WASP or wild-type N-WASP, addition of

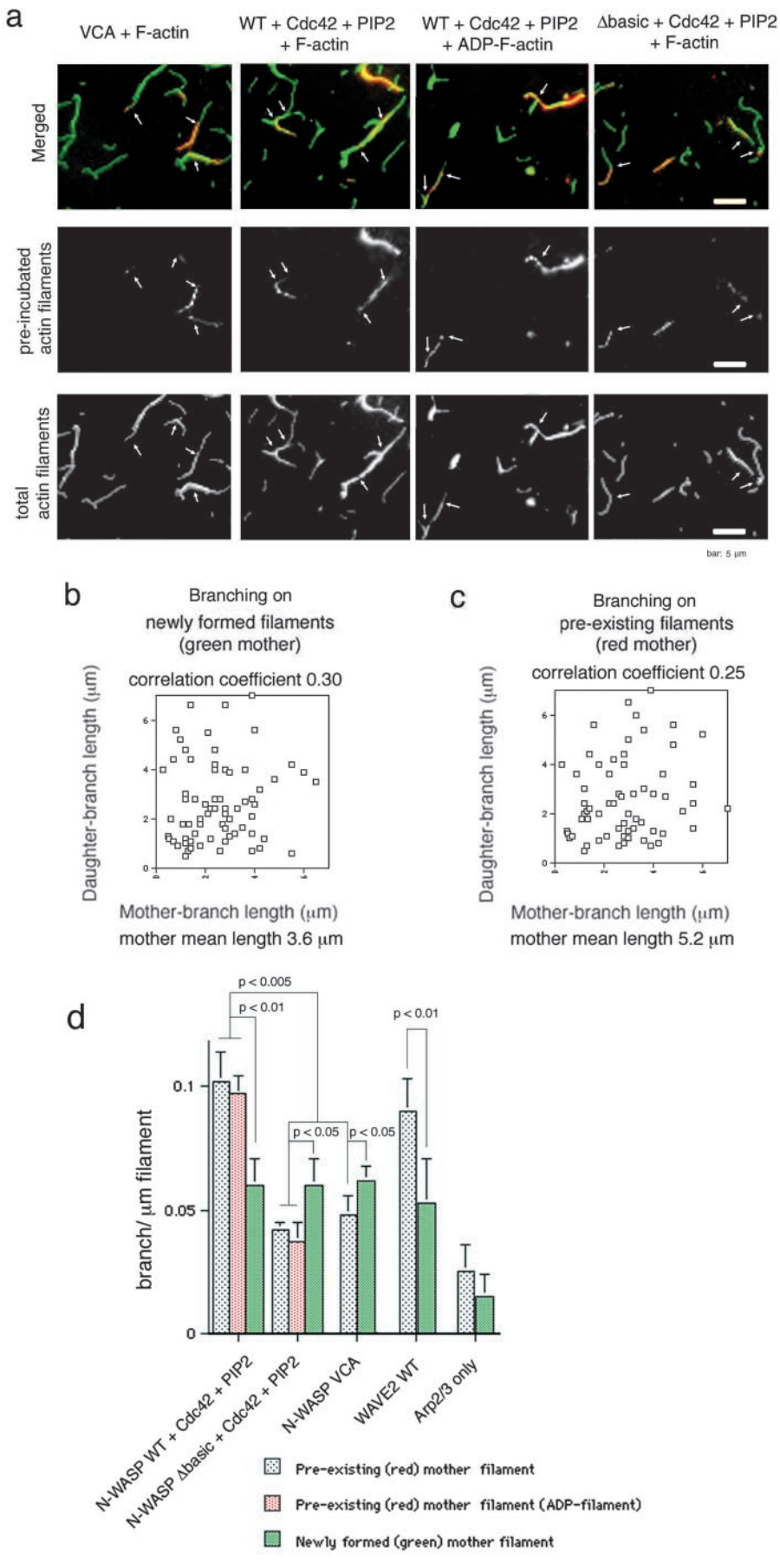

Fig. 5. Essential role of the basic region in branch formation on pre-existing actin filaments. (a) Visualization of actin filaments after actin polymerization for 15 minutes. Total actin filaments were visualized by green phalloidin staining. Preformed pre-incubated actin filaments are shown in red. Filaments were made by polymerizing rhodamine-labeled G-actin, which were composed of ATP actin caps of ~100 actin molecules (<0.6 μm per 5 μm (average) filament) and ADP filaments. ADP filaments were made by treating ordinary filaments with hexokinase overnight to hydrolyze ATP completely. Arrows indicate the points of elongation from pre-existing filaments. Concentration were as follows: VCA, WT and Δbasic N-WASP or WAVE2, 100 nM; GTPγS-loaded Cdc42, 500 nM; PIP_2-containing vesicles, 1 μM; Arp2/3, 60 nM; F-actin, 300 nM; ADP-F-actin, 300 nM; G-actin, 2 μM. Scale bar, 5 μm. (b,c) Plots of length of newly-formed (green) (b) or pre-existing (red) (c) 'mother' filaments between branch points and presumed barbed ends against the length of 'daughter' filaments induced by wild-type N-WASP, Cdc42 and PIP_2. The lengths of red mother filaments do not include end-elongating green filaments. (d) Values for branching per μm filaments are the mean of at least three independent experiments. Values that are significantly different, which are indicated on the graph, were determined by Student's *t* test. Error bars indicate standard deviation.

filaments shifted the peak of polymerization (d*F*/d*t*) earlier, indicating that the lag time of polymerization decreased. Therefore, addition of actin filaments clearly activated Arp2/3-mediated actin polymerization (Fig. 6). Because the barbed ends of these actin filaments are protected by gelsolin, the sides of filaments are important for enhancing actin polymerization, as previously reported (Amann and Pollard, 2001). Addition of pre-existing actin filaments makes the maximum rate of increase of filaments (the height of *dF/dt)* greater for wild-type N-WASP. By contrast, the maximum rate of increase of filaments with VCA or Δbasic was not increased (Fig. 6e-g). Thus, pre-existing filaments confer more co-operativity on polymerization induced by wild-type N-WASP, presumably through actin filament binding activity and recruitment of Arp2/3 to filaments. These results indicate that pre-existing filaments upregulate actin polymerization mediated by wild-type N-WASP by (1) decreasing the lag time (depends on Arp2/3 because the filament-side binding activity of N-WASP is not involved) and (2) increasing the rate of

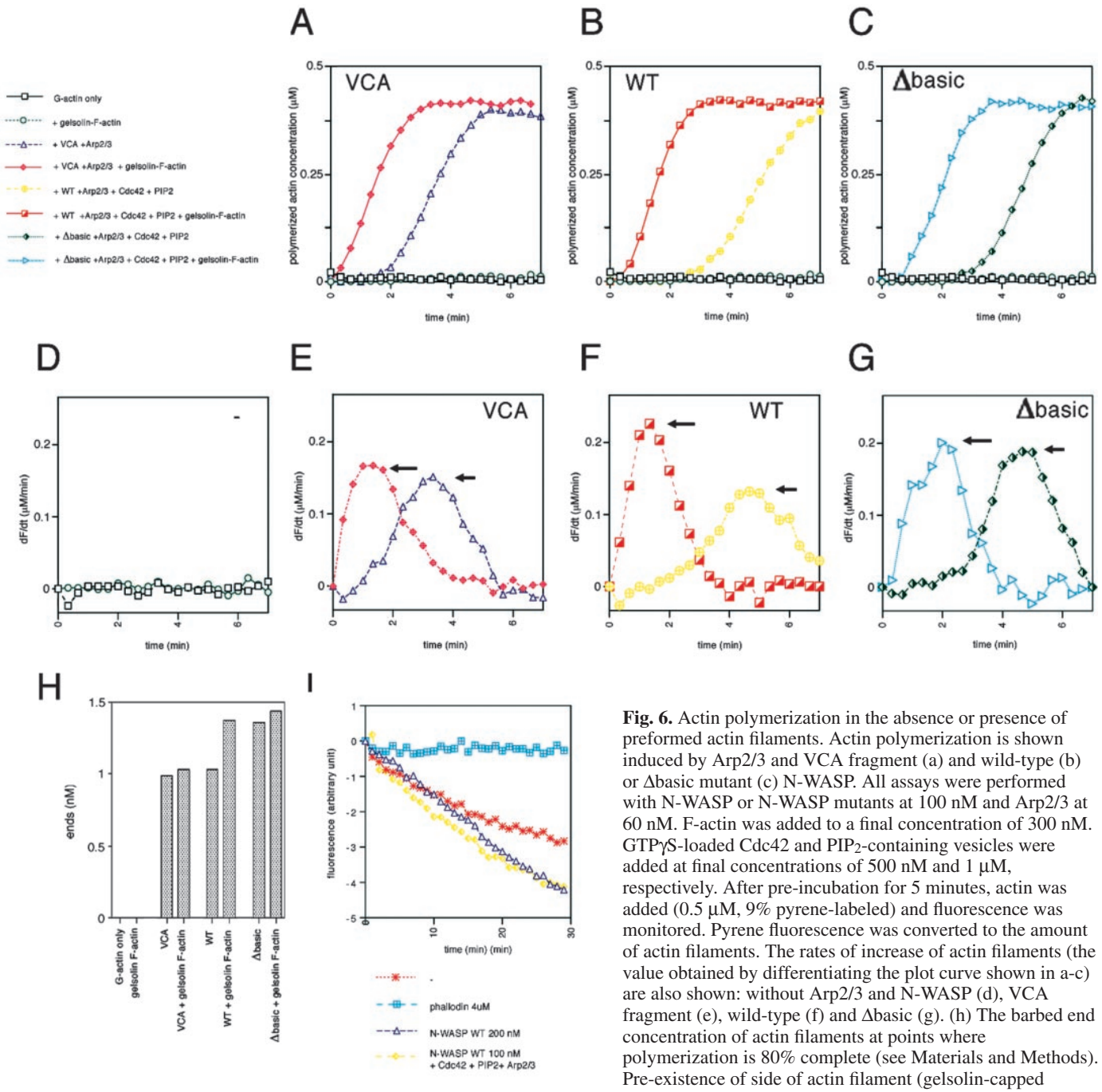

Fig. 6. Actin polymerization in the absence or presence of preformed actin filaments. Actin polymerization is shown induced by Arp2/3 and VCA fragment (a) and wild-type (b) or Δbasic mutant (c) N-WASP. All assays were performed with N-WASP or N-WASP mutants at 100 nM and Arp2/3 at 60 nM. F-actin was added to a final concentration of 300 nM. GTPγS-loaded Cdc42 and PIP_2-containing vesicles were added at final concentrations of 500 nM and 1 μM, respectively. After pre-incubation for 5 minutes, actin was added (0.5 μM, 9% pyrene-labeled) and fluorescence was monitored. Pyrene fluorescence was converted to the amount of actin filaments. The rates of increase of actin filaments (the value obtained by differentiating the plot curve shown in a-c) are also shown: without Arp2/3 and N-WASP (d), VCA fragment (e), wild-type (f) and Δbasic (g). (h) The barbed end concentration of actin filaments at points where polymerization is 80% complete (see Materials and Methods). Pre-existence of side of actin filament (gelsolin-capped filaments) increased the number of the barbed ends only in the case of wild-type N-WASP. (i) Depolymerization of gelsolin-capped actin filaments. Gelsolin-capped filaments were diluted to 0.1 μM in the presence of N-WASP (200 nM) or N-WASP (100 nM) with Cdc42 (500 nM), PIP_2 (1 μM vesicles) and Arp2/3 (60 nM) and the fluorescence was monitored.

polymerization (depends on actin-filament-side binding activity of N-WASP).

We next examined the d*F*/d*t* when 80% of polymerization was completed. At this point, the number of barbed ends is proportional to the rate of increase of actin filaments because pointed end elongation of filaments is assumed to be minimal (Higgs et al., 1999). Pre-existence of actin filaments increased the number of barbed ends by ~30% only with wild type N-WASP, suggesting that actin filament binding activity contributes to the increase in the number of barbed ends (Fig. 6h). The rate increase might be due to a decrease in the rate of depolymerization because many actin-filament-binding proteins stabilize actin filaments. However, N-WASP did not inhibit pointed-end depolymerization (Fig. 6i).

In agreement with preferential binding to ADP filaments, the increase in the number of barbed ends by wild-type N-WASP was specific for ADP-F-actin and was not seen with ADP-P_i-F-actin (actin polymerized in the presence of phalloidin). Association of phalloidin with ADP-F-actin did not affect the rate of polymerization (data not shown).

However it is possible that the rapid formation of ADP-P_i filaments by N-WASP and Arp2/3 might mask the effect of pre-existing ADP-P_i filaments. Although most of the actin filaments used in the above experiment made by polymerization of G-actin are thought to be in the ADP form, enhancement of polymerization rates by ADP-F-actin was confirmed with ADP-F-actin generated by hexokinase treatment (data not shown).

Actin polymerization after the polymerization lag was faster with the Δbasic mutant of N-WASP than with wild-type N-WASP (Fig. 5c,g). This phenomenon is explained in two ways. First, the release of auto-inhibition of N-WASP by the deletion of the basic region contributed to the unmasking of the catalytic VCA domain of N-WASP, because basic-acidic interaction is important for autoinhibition of N-WASP (Suetsugu et al., 2001b). Second, the presence of the WH1 domain further activates Arp2/3 (Suetsugu et al., 2001a). Although the Δbasic mutant of N-WASP is able to activate Arp2/3 more efficiently, neither the efficiency of branching nor the number of barbed ends was increased by the pre-existence of actin filaments, presumably owing to the deficiency in actin filament binding activity (Fig. 5; Fig. 6).

Wild-type WAVE2 but not the Δbasic mutant WAVE2 also increased the number of the barbed ends ~30% in the presence of pre-existing actin filaments, indicating that association of actin filaments plays a similar role among WASP family proteins (data not shown). Therefore, addition of actin filaments in the ADP form enhanced actin nucleation and pre-existing filaments provide the scaffold for induction of rapid actin polymerization.

DISCUSSION

We first examined the role of actin filament binding activity of N-WASP/WAVE2 in formation of actin branching and polymerization. The actin filament binding activity of N-WASP/WAVE2 recruits Arp2/3 more effectively to ADP filaments than to ADP-P_i filaments. Therefore, the pre-existence of ADP filaments increased both the rate of branch formation and the rate of actin polymerization when activated by wild-type N-WASP/WAVE2 but not by VCA or Δbasic. Therefore, the VCA domain, which is the smallest peptide fragment capable of activating Arp2/3-mediated actin polymerization in vitro, does not represent all of the activities of N-WASP/WAVE2 for regulating actin filaments through Arp2/3. ADP filaments are required for optimum activation of Arp2/3 by N-WASP/WAVE2.

Branching on pre-existing filaments and actin cytoskeletal formation

When actin polymerizes, ATP-G-actin first forms ATP filaments. ATP on actin filaments is rapidly hydrolyzed to ADP (half-life is ~10 seconds). The phosphate from ATP is not released from actin filaments immediately after hydrolysis of ATP (dissociation half-life is about 2 minutes). In cells and in extracts, ADF/cofilin enhances the release of phosphate more than 10 times (Blanchoin and Pollard, 1999). Indeed, the most dynamic actin filaments turn over with a half-life of 23 seconds (Theriot and Mitchison, 1991). Therefore, in cells, the filaments formed de novo are likely rapidly to become ADP filaments. Thus, the pre-existing filaments in cells are likely to be in the ADP form. Therefore, branched filament formation at the side of pre-existing ADP filaments is probably important for actin cytoskeletal re-organizations.

There is evidence that supports the importance of efficient side-branching from pre-existing filaments. Actin comet formation in vitro using plastic microspheres coated with N-WASP or its mutant is a good system for studying the mechanism of actin cytoskeletal formation. We previously showed that VCA alone is not sufficient for actin comet formation but VCA-coated beads were able to induce actin filament formation around the beads. The basic region, which we found to be the actin filament binding region, was required to form actin comets (Suetsugu et al., 2001b).

Because N-WASP-coated bead movement was reconstituted by only five proteins [profilin, ADP/cofilin, capping protein, Arp2/3 and actin (Pantaloni et al., 2001)], the lower branching efficiency of VCA or Δbasic mutant of N-WASP is the likely cause for the inability of VCA- or Δbasic-mutant-coated beads in actin comet formation. Before initiation of actin comet formation and directional movement, actin polymerizes randomly around the beads to form a dense actin cloud on which a moving force for beads should be generated (Cameron et al., 1999). After forming an actin cloud, the beads suddenly form actin comets and begin to move. In this period of actin cloud formation, there should be large amounts of ADP-actin filaments around beads. Therefore, higher branching rate on pre-existing ADP filament is thought to be important for formation of dense actin cloud. Because generation of force for movement requires a scaffold, the formation of dense actin clouds by efficient branching, on which many actin filaments can elongate, is likely to be important for initiation of directional movement of actin comets.

Enhanced rate of branching: co-operation by VCA and actin filament binding region

Here, we show that WASP family proteins have two mechanisms to activate Arp2/3-mediated side branching. Activation of Arp2/3 by VCA is the first identified mechanism for actin filament branching (Blanchoin et al., 2000; Pantaloni et al., 2000). Both rate of branching and association of Arp2/3 to filaments were increased by the association of Arp2/3 with VCA (Fig. 3) (Marchand et al., 2001). The enhancement of filament association is presumably due to VCA-induced changes inside Arp2/3, because VCA itself does not associate with actin filaments (Machesky and Insall, 1998; Miki and Takenawa, 1998). Branching induced by VCA-activated Arp2/3 occurs preferentially on newly formed filaments, which are composed mainly of ADP-P_i filaments, because polymerization ceases within the half-life of P_i release (Fig. 6) (Blanchoin et al., 2000). This observation is consistent with the hypothesis that autocatalytic Arp2/3 activation is mediated by ADP-P_i filaments, in which filaments formed de novo by one Arp2/3 complex in turn activate other Arp2/3 complexes (Amann and Pollard, 2001; Pantaloni et al., 2000).

The other mechanism found here is that branching occurs preferentially on ADP filaments when Arp2/3 is activated with wild-type N-WASP or WAVE2. This preference of branching on ADP filaments is due to the recruitment of activated Arp2/3 to filaments or activation of Arp2/3 on filaments, which then increases the rate of branching up to twofold (Fig. 4; Fig. 5).

Thus, recruitment of Arp2/3 to filaments results in greater branching on ADP filaments than on new filaments. The increase in branching by N-WASP/WAVE2 presumably increases the number of elongating filaments near the region of N-WASP/WAVE2 activation and helps to generate the force for movement.

Recently, cortactin has been shown to tighten branches formed by Arp2/3, presumably through filament binding as well as Arp2/3-associating activity of cortactin (Uruno et al., 2001; Weaver et al., 2001; Weed et al., 2000). However, the distributions in actin comets of N-WASP and cortactin are very different. N-WASP is located exclusively at the surface of bacteria, where actin polymerization occurs. In contrast to N-WASP, cortactin is localized throughout the actin comet (Zettl and Way, 2001). So, what is the importance of higher branching efficiency on pre-existing filaments? One possibility is that intact N-WASP or WAVE2 makes more branches on pre-existing filaments, which are presumably tightened by cortactin to make the actin cloud tighter before the formation of actin comets, and thereby promotes the initiation of actin-comet-based movements.

Multiple roles of the basic region of N-WASP

In this study, we identified multiple roles for the basic region of N-WASP. This region was shown to bind PIP_2 and actin filaments (Rohatgi et al., 2000). These two activities are separate in both binding site and activity. The presence of a sufficient amount of PIP_2 for N-WASP activation did not decrease enhancement of Arp2/3 association with filaments (Fig. 4). The PIP_2-binding region is restricted to only 20 amino acids of the latter half of the basic region. However, most of the basic region contributes to association with actin filaments (Fig. 2) (Rohatgi et al., 2000). This region is also proposed to be involved in association of N-WASP with Arp2/3 (Prehoda et al., 2000). However, the association of VCA with Arp2/3 is far greater than that of the basic region (Suetsugu et al., 2001a). Thus, the basic region itself is not likely to be involved in the recruitment of Arp2/3 to filaments. Although the basic region-Arp2/3 complex association will be important for inhibition of Arp2/3 (Prehoda et al., 2000).

Deletion of the basic region of N-WASP leads to constitutively active N-WASP in Arp2/3-mediated actin polymerization (Fig. 4c) (Suetsugu et al., 2001b). This result suggests that the basic region is also involved in the formation of an autoinhibited structure in addition to the region around the CRIB motif (Kim et al., 2000). Positive charges in the basic region and negative charges in the C-terminal acidic region are thought to interact by electrostatic force. In support of this hypothesis, partial deletion of acidic residues led to the activation of N-WASP, indicating that the basic-acidic interaction plays an indispensable role in the formation of the autoinhibited structure (Suetsugu et al., 2001a).

Therefore, the basic region of N-WASP appears to have multiple roles including PIP_2 binding, actin filament binding, Arp2/3 association and autoinhibition. The three-dimensional structure of N-WASP will be required to verify these functions.

Prior to this study, the role of the basic region in WAVE was not known. Like the basic region of N-WASP, the basic region of WAVE binds actin filaments (Fig. 1). Therefore, the binding with actin filaments at the basic region is common to WASP family proteins including N-WASP and WAVE2. The basic region of WAVE, like that of N-WASP, may also be involved in phosphoinositide binding. Further study, however, is needed to determine the detailed role of the basic region of WASP family proteins in regulation of actin filament formation.

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REFERENCES

Amann, K. J. and Pollard, T. D. (2001). The Arp2/3 complex nucleates actin filament branches from the sides of pre-existing filaments. *Nat. Cell Biol.* **3**, 306-310.

Bailly, M., Ichetovkin, I., Grant, W., Zebda, N., Machesky, L. M., Segall, J. E. and Condeelis, J. (2001). The F-actin side binding activity of the Arp2/3 complex is essential for actin nucleation and lamellipod extension. *Curr. Biol.* **11**, 620-625.

Blanchoin, L. and Pollard, T. D. (1999). Mechanism of interaction of *Acanthamoeba* actophorin (ADF/Cofilin) with actin filaments. *J. Biol. Chem.* **274**, 15538-15546.

Blanchoin, L., Amann, K. J., Higgs, H. N., Marchand, J.-B., Kaiser, D. A. and Pollard, T. D. (2000). Direct observation of dendritic actin filament networks nucleated by Arp2/3 complex and WASP/Scar proteins. *Nature* **404**, 1007-1011.

Cameron, L. A., Footer, M. J., van Oudenaarden, A. and Theriot, J. A. (1999). Motility of ActA protein-coated microspheres driven by actin polymerization. *Proc. Natl. Acad. Sci. USA* **96**, 4908-4913.

Carlier, M. F., Pantaloni, D. and Korn, E. D. (1984). Evidence for an ATP cap at the ends of actin filaments and its regulation of the F-actin steady state. *J. Biol. Chem.* **259**, 9983-9986.

Carlier, M.-F., Nioche, P., Broutin-L'Hermite, I., Boujemaa, R., Le Clainche, C., Egile, C., Garbay, C., Ducruix, A., Sansonetti, P. and Pantaloni, D. (2000). Grb2 links signaling to actin assembly by enhancing interaction of neural Wiskott-Aldrich syndrome protein (N-WASP) with actin-related protein (Arp2/3) complex. *J. Biol. Chem.* **275**, 21946-21952.

Derry, J. M., Ochs, H. D. and Francke, U. (1994). Isolation of a novel gene mutated in Wiskott-Aldrich syndrome. *Cell* **78**, 635-644.

Egile, C., Loisel, T. P., Laurent, V., Li, R., Pantaloni, D., Sansonetti, P. J. and Carlier, M. F. (1999). Activation of the CDC42 effector N-WASP by the *Shigella flexneri* IcsA protein promotes actin nucleation by Arp2/3 complex and bacterial actin-based motility. *J. Cell Biol.* **146**, 1319-1332.

Fukuoka, M., Suetsugu, S., Miki, H., Fukami, K., Endo, T. and Takenawa, T. (2001). A novel neural Wiskott-Aldrich syndrome protein (N-WASP) binding protein, WISH, induces Arp2/3 complex activation independent of Cdc42. *J. Cell Biol.* **152**, 471-482.

Higgs, H. N., Blanchoin, L. and Pollard, T. D. (1999). Influence of the C terminus of Wiskott-Aldrich syndrome protein (WASp) and the Arp2/3 complex on actin polymerization. *Biochemistry* **38**, 15212-15222.

Kim, A. S., Kakalis, L. T., Abdul-Manan, N., Liu, G. A. and Rosen, M. K. (2000). Autoinhibition and activation mechanisms of the Wiskott-Aldrich syndrome protein. *Nature* **404**, 151-158.

Loisel, T. P., Boujemaa, R., Pantaloni, D. and Carlier, M. F. (1999). Reconstitution of actin-based motility of *Listeria* and *Shigella* using pure proteins. *Nature* **401**, 613-616.

Machesky, L. M. and Insall, R. H. (1998). Scar1 and the related Wiskott-Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex. *Curr. Biol.* **8**, 1347-1356.

Machesky, L. M., Mullins, R. D., Higgs, H. N., Kaiser, D. A., Blanchoin, L., May, R. C., Hall, M. E. and Pollard, T. D. (1999). Scar, a WASp-related protein, activates nucleation of actin filaments by the Arp2/3 complex. *Proc. Natl. Acad. Sci. USA* **96**, 3739-3744.

Marchand, J.-B., Kaiser, D. A., Pollard, T. D. and Higgs, H. N. (2001). Interaction of WASP/Scar proteins with actin and vertebrate Arp2/3 complex. *Nat. Cell Biol.* **3**, 76-82.

Miki, H. and Takenawa, T. (1998). Direct binding of the verprolin-homology domain in N-WASP to actin is essential for cytoskeletal reorganization. *Biochem. Biophys. Res. Commun.* **243**, 73-78.

Miki, H., Miura, K. and Takenawa, T. (1996). N-WASP, a novel actin-depolymerizing protein, regulates the cortical cytoskeletal rearrangement in a PIP_2-dependent manner downstream of tyrosine kinases. *EMBO J.* **15**, 5326-5335.

Miki, H., Suetsugu, S. and Takenawa, T. (1998). WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac. *EMBO J.* **17**, 6932-6941.

Miki, H., Yamaguchi, H., Suetsugu, S. and Takenawa, T. (2000). IRSp53 is an essential intermediate between Rac and WAVE in the regulation of membrane ruffling. *Nature* **408**, 732-735.

Mullins, R. D., Stafford, W. F. and Pollard, T. D. (1997). Structure, subunit topology, and actin-binding activity of the Arp2/3 complex from *Acanthamoeba*. *J. Cell Biol.* **136**, 331-343.

Mullins, R. D., Heuser, J. A. and Pollard, T. D. (1998). The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments. *Proc. Natl. Acad. Sci. USA* **95**, 6181-6186.

Pantaloni, D., Boujemaa, R., Didry, D., Gounon, P. and Carlier, M.-F. (2000). The Arp2/3 complex branches filament barbed ends: functional antagonism with capping proteins. *Nat. Cell Biol.* **2**, 385-391.

Pantaloni, D., Le Clainche, C. and Carlier, M. F. (2001). Mechanism of actin-based motility. *Science* **292**, 1502-1506.

Pollard, T. D. (1984). Polymerization of ADP-actin. *J. Cell Biol.* **99**, 769-777.

Prehoda, K. E., Scott, J. A., D. R., M. and Lim, W. A. (2000). Integration of multiple signals through cooperative regulation of the N-WASP-Arp2/3 complex. *Science* **290**, 801-806.

Rohatgi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T. and Kirschner, M. W. (1999). The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. *Cell* **97**, 221-231.

Rohatgi, R., Ho, H. Y. and Kirschner, M. W. (2000). Mechanism of N-WASP activation by Cdc42 and phosphatidylinositol 4, 5-bisphosphate. *J. Cell Biol.* **150**, 1299-1310.

Soeno, Y., Abe, H., Kimura, S., Maruyama, K. and Obinata, T. (1998). Generation of functional beta-actinin (CapZ) in an *E. coli* expression system. *J. Muscle Res. Cell Motil.* **19**, 639-646.

Suetsugu, S., Miki, H. and Takenawa, T. (1998). The essential role of profilin in the assembly of actin for microspike formation. *EMBO J.* **17**, 6516-6526.

Suetsugu, S., Miki, H. and Takenawa, T. (1999). Identification of two human WAVE/SCAR homologues as general actin regulatory molecules which associate with Arp2/3 complex. *Biochem. Biophys. Res. Commun.* **260**, 296-302.

Suetsugu, S., Miki, H. and Takenawa, T. (2001a). Identification of another Actin-related protein (Arp) 2/3 complex binding site in Neural Wiskott-Aldrich syndrome protein (N-WASP), that complements actin polymerization induced by the Arp2/3 complex activating (VCA) domain of N-WASP. *J. Biol. Chem.* **276**, 33175-33180.

Suetsugu, S., Miki, H., Yamaguchi, H. and Takenawa, T. (2001b). Requirement of the basic region of N-WASP/WAVE2 for actin-based motility. *Biochem. Biophys. Res. Commun.* **282**, 739-744.

Takenawa, T. and Miki, H. (2001). WASP and WAVE family proteins: key molecules for rapid rearrangement of cortical actin filaments and cell movement. *J. Cell Sci.* **114**, 1801-1809.

Theriot, J. A. and Mitchison, T. J. (1991). Actin microfilament dynamics in locomoting cells. *Nature* **352**, 126-131.

Theriot, J. A. and Fung, D. C. (1998). *Listeria monocytogenes*-based assays for actin assembly factors. *Methods Enzymol* **298**, 114-122.

Uruno, T., Liu, J., Zhang, P., Fan, Y., Egile, C., Li, R., Mueller, S. C. and Zhan, X. (2001). Activation of Arp2/3 complex-mediated actin polymerization by cortactin. *Nat. Cell Biol.* **3**, 259-266.

Weaver, A. M., Karginov, A. V., Kinley, A. W., Weed, S. A., Li, Y., Parsons, J. T. and Cooper, J. A. (2001). Cortactin promotes and stabilizes Arp2/3-induced actin filament network formation. *Curr. Biol.* **11**, 370-374.

Weed, S. A., Karginov, A. V., Schafer, D. A., Weaver, A. M., Kinley, A. W., Cooper, J. A. and Parsons, J. T. (2000). Cortactin localization to sites of actin assembly in lamellipodia requires interactions with F-actin and the Arp2/3 complex. *J. Cell Biol.* **151**, 29-40.

Zettl, M. and Way, M. (2001). New tricks for an old dog? *Nat. Cell Biol.* **3**, E74-E75.