WASP and WAVE family proteins: key molecules for rapid rearrangement of cortical actin filaments and cell movement

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Summary

Reorganization of cortical actin filaments plays critical roles in cell movement and pattern formation. Recently, the WASP and WAVE family proteins WASP and N-WASP, and WAVE1, WAVE2 and WAVE3 have been shown to regulate cortical actin filament reorganization in response to extracellular stimuli. These proteins each have a verprolin-homology (V) domain, cofilin-homology (C) domain and an acidic (A) region at the C-terminus, through which they activate the Arp2/3 complex, leading to rapid actin polymerization. N-WASP is usually present as an inactive form in which the VCA region is masked. Cooperative binding of Cdc42 and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) exposes the VCA region, activating N-WASP. In addition to this activation mechanism, WISH also activates N-WASP independently of Cdc42 and PtdIns(4,5)P_2, by binding to the proline-rich region of N-WASP. N-WASP activation induces formation of filopodia in vivo. In contrast, the ubiquitously expressed form of WAVE2 is activated downstream of Rac, leading to formation of lamellipodia. In this case, IRSp53 transmits a signal from Rac to WAVE2 through formation of a ternary Rac-IRSp53-WAVE2 complex. Thus, N-WASP, which is activated downstream of Cdc42 or independently by WISH, induces formation of filopodia and WAVE2, which is activated via IRSp53 downstream of Rac, induces formation of lamellipodia.

Key words: WASP, WAVE, Arp2/3 complex, Actin polymerization, Signal transduction

Introduction

The mechanisms of cell polarity, patterning and movement remain a compelling mystery, but reorganization of the cortical actin cytoskeleton in response to extracellular stimuli is thought to be a key event in these phenomena. Reorganization of cortical actin filaments is regulated by a highly integrated signaling cascade that transduces extracellular stimuli to the actin filaments. This signaling cascade is governed by small GTPases of the Rho family. Among them, RhoA, Rac and Cdc42 play critical roles in the formation and organization of cortical actin networks (Hall, 1998; Bishop and Hall, 2000). RhoA controls assembly of stress fibers, Rac regulates formation of lammellipodia, and Cdc42 controls extension of filopodia and microspikes. During cell movement, Rac and Cdc42 stimulate formation of protrusions at the leading edges of cells, and RhoA induces retraction at the tail ends of cells. This coordinated reorganization permits cells to move towards a target. Although Rac and Cdc42 are known to be essential for cell movement, the downstream molecules involved directly in actin filament reorganization were unknown until the Wiskott-Aldrich syndrome (WAS) protein (WASP) family of WASP and N-WASP proteins was identified. These proteins have been the subject of a great deal of attention as the link between small GTPases and the actin cytoskeleton. The WASP family proteins are classified into two structural groups: WASPs (Derry et al., 1994; Miki et al., 1996; Fukuoka et al., 1997; Winter et al., 1999) and WAVEs (Miki et al., 1998b; Suetsugu et al., 1999).

The barbed ends of actin filaments are capped by large redundant proteins to prevent spontaneous elongation of the filaments in resting cells. Thus, to trigger rapid actin polymerization for rearrangement of the cortical cytoskeleton and movement in response to extracellular stimuli, cells must expose these barbed ends. Three mechanisms of initiation of actin polymerization have been proposed (Higgs and Pollard, 1999): (1) uncapping of barbed ends of the filaments; (2) severing of existing filaments to create new barbed ends; and (3) de novo nucleation of actin filaments by the Arp (actin-related protein) 2/3 complex. Although these three mechanisms are active in various cellular processes, Arp2/3-complex-dependent de novo actin nucleation appears to be critical for rapid formation of an actin network at the leading edge of the cell (Mullins et al., 1998; Bailly et al., 1999; Higgs and Pollard, 1999; Weiner et al., 1999). The resulting reorganization of the actin skeleton provides the protrusive force required for the extension of filopodia and lamellipodia observed during cell movement.

Structural homology of WASP and WAVE family proteins

In 1994, WASP was identified as the causative gene product of WAS, which is characterized by eczema, bleeding and recurrent infections (Derry et al., 1994). This protein is expressed exclusively in hematopoietic cells. Lymphocytes from WAS patients show cytoskeletal abnormalities and a reduction in the number of cell surface microvilli (Kenney et

al., 1986; Molina et al., 1992). WASP binds to Cdc42 through its GBD/CRIB (GTPase-binding domain/Cdc42 and Rac interactive binding) domain, and overexpression of WASP induces formation of actin clusters, which suggests that it has a role in actin polymerization (Symons et al., 1996). Although these data suggested that WASP is involved in the organization of actin filaments, its physiological function was unclear until the isolation of neural WASP (N-WASP). N-WASP was first purified as a protein that bound to the Ash/Grb2 SH3 domain (Miki et al., 1994; Miura et al., 1996), and the corresponding cDNA was isolated in 1996 (Miki et al., 1996). N-WASP shares ~50% sequence similarity with WASP, and it contains several multifunctional domains including the WH1/EVH1 (WASP homology/Ena VASP homology) domain, IQ motif, a basic region, a GBD/CRIB domain, a proline-rich region, a verprolin-homology (V) region, a cofilin-like (C) region, and an acidic (A) region (Fig. 1).

The WH1 domain binds phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2; Miki et al., 1996; Imai et al., 1999), and N-WASP is thought to be anchored to the membrane through this region. The WH1 domain also binds to WASP-interacting protein (WIP; Ramesh et al., 1997). The IQ motif, which is contained within the WH1 domain, may be a calmodulin-binding site (Miki et al., 1996). The GBD/CRIB domain is a Cdc42-binding site, through which N-WASP is regulated by Cdc42 (Miki et al., 1998a). The proline-rich region binds various SH3 domains, including those present in Ash/Grb2 (Miki et al., 1996; She et al., 1997; Carlier et al., 2000), Nck (Rivero-Lezcano et al., 1995), proline, serine, threonine phophatase interacting protein (PSTPIP; Wu et al., 1998), phospholipase Cγ1 (Finan et al., 1996), the phosphoinositide 3-kinase (PI3K) 85-kDa subunit (Finan et al., 1996), Fyn (Banin et al., 1996), syndapins (Qualmann et al., 1999), Itk/Tsk (Bunnell et al., 1996), Btk (Morrogh et al., 1999) and WISH (Fukuoka et al., 2001), as well as profilin (Suetsugu et al., 1998). The V domain is a G-actin-binding site, and the CA domain binds the Arp2/3 complex (Miki and Takenawa, 1998; Machesky et al., 1999; Rohatgi et al., 1999). Through these domains, N-WASP is thought to be regulated by and to regulate many signaling molecules.

In addition to WASP and N-WASP, another WASP homologue, WAVE, was identified as a novel protein that possesses a V domain (Miki et al., 1998b; Suetsugu et al., 1999). At the same time, a *Dictyostelium* homolog of WAVE was identified and named Scar (Bear et al., 1998). These proteins have a WAVE homology/Scar homology domain (WHD/SHD), a basic region, a proline-rich region and a VCA region (Fig. 1). Therefore, the C-terminal regions of these proteins are highly homologous to that of N-WASP. WAVE, however, does not have the GBD/CRIB domain, and therefore, it is likely that WAVE is regulated in a different way from WASP/N-WASP. Furthermore, two additional homologous but unique proteins have been identified: WAVE2 and WAVE3 (the original WAVE was then termed WAVE1; Suetsugu et al., 1999).

N-WASP induces filopodium formation downstream of Cdc42

In unstimulated cells, ectopically expressed N-WASP is present at the plasma membranes and does not cause significant morphological change in the cell. Since N-WASP contains a GBD/CRIB domain, a putative Cdc42-binding site, it was

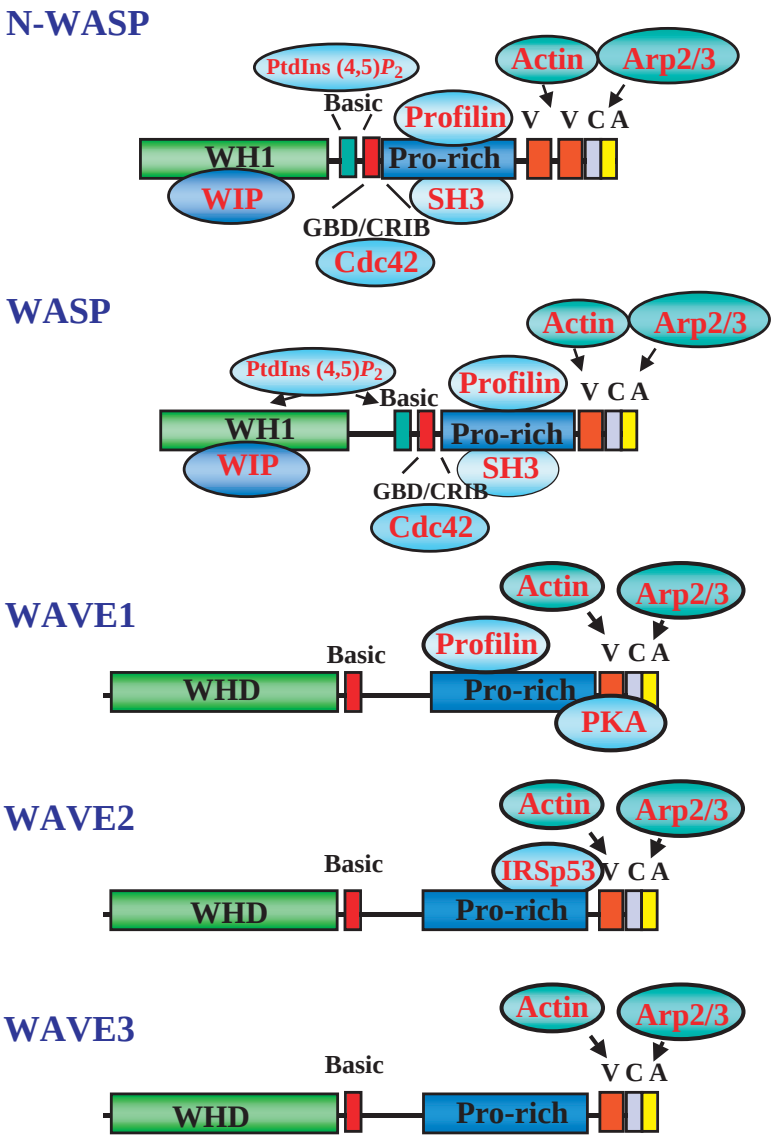

Fig. 1. WASP and WAVE family proteins and molecules to which they bind. The WH1 domain at the N-terminus of N-WASP and WASP binds to WIP (Ramesh et al., 1997) and F-actin (Egile et al., 1999). Calmodulin binds to the IQ domain. The basic region of N-WASP is a PtdIns(4,5)P_2-binding site (Rohatgi et al., 2000). The GBD/CRIB domain is a Cdc42-binding site (Miki et al., 1996). Several SH3-containing proteins, including Ash/Grb2 (Miki et al., 1996; Carlier et al., 2000), Nck (Rivero-Lezcano et al., 1995), PSTPIP (Wu et al., 1998), phospholipase Cγ1 (Finan et al., 1996), the phosphoinositide 3-kinase 85-kDa subunit (Finan et al., 1996), Fyn (Banin et al., 1996), syndapins (Qualmann et al., 1999), Itk/Tsk(Bunnell et al., 1996), Btk (Morrogh et al., 1999), WISH (Fukuoka et al., 2001) and profilin (Suetsugu et al., 1998), bind to the proline-rich region. The V domains of N-WASP, WASP and WAVEs bind to G-actin (Miki et al., 1998), and the CA domains bind to the Arp2/3 complex (Machesky et al., 1999). The V domain of WAVE1 binds to the PKA RII subunit (Westphal et al., 2000). The proline-rich region of WAVE2 binds to IRSp53 (Miki et al., 2000).

supposed that Cdc42 regulates the function of N-WASP. Thus, the cooperative effect of these two proteins was investigated. Expression of Cdc42 in Cos7 cells induced protrusion of the membrane. Interestingly, coexpression of N-WASP and Cdc42 induced formation of long filopodia (Miki et al., 1998a). Real-time observations confirmed that these protrusions are filopodia and not retractive fibers. These data implied that N-WASP acts downstream of Cdc42. In addition, Cdc42 binding was found to be essential for N-WASP-induced formation of filopodia, because a mutant of N-WASP that cannot bind Cdc42 does not induce formation of filopodia in the presence of Cdc42 (Miki et al., 1998a). The involvement of N-WASP in filopodia formation was further supported by observations that filopodia formation in response to epidermal growth factor (EGF) is inhibited in Cos7 cells expressing V-deleted N-WASP (Miki and Takenawa, 1998) and that bradykinin-induced filopodia formation can be inhibited by microinjection of an anti-N-WASP antibody into Swiss 3T3 cells (Miki et al., 1998a).

Several investigators have reported possible Cdc42 effectors that bind directly to activated Cdc42, which include ACK, PAK, IQGAP, S6 kinase and MLK (reviewed by Boettner and Aelst, 1999). However, most of these also associate with activated Rac, and only ACK and WASP/N-WASP are specific binding proteins for Cdc42. In addition, no protein among them can account for the rapid actin polymerization that is induced by Cdc42 and is the most important basis for Cdc42-induced filopodium formation. In this sense, N-WASP is the first Cdc42 effector for which importance in filopodium formation has been clearly established.

The VCA region is critical for ARP2/3 complex-induced actin polymerization

All WASP and WAVE proteins have VCA regions. The V domain is a G-actin-binding site and is essential for N-WASP-induced formation of microspikes (Miki and Takenawa, 1998). N-WASP has two V domains, whereas WASP and WAVE proteins have only one. The CA region contains a sequence homologous to the ActA protein of the pathogenic bacterium *Listeria monocytogenes* (Machesky, 1999). *L. monocytogenes* moves within eukaryotic cells through ActA-mediated generation of actin comets. ActA directly binds to the Arp2/3 complex and stimulates it to nucleate and polymerize actin, and this generates the force to propel the bacterium (Welch et al., 1997; Welch et al., 1998).

The Arp2/3 complex consists of seven protein subunits, which include actin-related protein, Arp2 and Arp3. It was first discovered in *Acanthamoeba* as a component of the actin cortex (Machesky et al., 1994) and is essential for reconstitution of both in vitro actin-based motility and actin polymerization on the surface of *L. monocytogenes* (Welch et al., 1997; May et al., 1999; Machesky, 1999). However, the CA domain (the Arp2/3-binding site) of WASPs or WAVEs alone is not sufficient to enhance Arp2/3-complex-induced nucleation and polymerization of actin in vitro; the V domain (G-actin-binding site) is also required (Banzai et al., 2000; Egile et al., 1999; Machesky et al., 1999; Rohatgi et al., 1999; Yarar et al., 1999, Sasaki et al., 2000; Yamaguchi et al., 2000). Thus, the VCA region, containing both the G-actin-binding site and the Arp2/3-complex-binding site appears to be the minimal essential region for Arp2/3 complex activation by WASP and WAVE proteins.

All WASPs and WAVEs have VCA regions, but is there any difference between the potencies of their VCA regions in the Arp2/3 complex activation? Recent work indicates that this is indeed the case (Yamaguchi et al., 2000). The N-WASP VCA region is the strongest activator of the Arp2/3 complex, whereas that of WAVE1 is the weakest. Experiments involving chimeric proteins containing the N-WASP and WAVE1 VCAs yielded unexpected results. Neither the actin-binding affinity of the V domain nor the Arp2/3-complex-binding affinity of the CA domain is related directly to Arp2/3 complex activation potency. Instead, the number of V domains is important. N-WASP has two V domains, whereas other proteins have only one. This is probably the reason that N-WASP is the strongest activator of actin polymerization. In fact, a chimeric protein that has two WAVE1 V domains has an activity similar to that of the VCA region of N-WASP. The presence of three V domains, however, reduces the Arp2/3 complex activation potency, which suggests that tandem G-actin binding is most appropriate for actin nucleation by the Arp2/3 complex.

Profilin accelerates actin polymerization induced by WASP and WAVE

All WASPs and WAVEs bind directly to profilin through a proline-rich region. Profilin is a G-actin-binding protein that accelerates the exchange of ADP/ATP on actin and promotes actin polymerization. Interestingly, a profilin mutant that cannot bind actin but can bind other profilin-binding proteins suppresses N-WASP- or WAVE-induced actin reorganization in cells. This suggests that profilin plays a critical role in actin reorganization induced by WASPs and WAVEs (Suetsugu et al., 1998; Miki et al., 1998b). In addition, an N-WASP mutant that lacks the proline-rich region can still induce extension of filopodia, but these filopodia are shorter than those induced by wild-type N-WASP. In a recent report, Yang et al. (Yang et al., 2000) also emphasized the importance of profilin in the actin-nucleation process and suggested that WASPs and WAVEs probably stimulate rapid actin polymerization through not only the Arp2/3 complex but also profilin.

A model for activation of N-WASP by Cdc42 and PtdIns(4,5)P_2

How is N-WASP activated downstream of Cdc42? We first hypothesized that, in resting conditions, the N-WASP VCA region is masked by an intramolecular interaction between the C-terminal VCA region and the GBD/CRIB domain (Miki et al., 1998a). When cells are stimulated, activated Cdc42 may bind to the GBD/CRIB motif, thus exposing the VCA region. Indeed, activated Cdc42 inhibits binding of a protein fragment containing the basic region and GBD/CRIB domain to the C-terminal VCA region (Miki et al., 1998a). This hypothesis is supported by data from experiments on full-length N-WASP protein. Full-length N-WASP does not activate the Arp2/3 complex as strongly as does the VCA region, which suggests that the VCA region is masked in inactive N-WASP. In the presence of Cdc42 and PtdIns(4,5)P_2, however, N-WASP activates the Arp2/3 complex to a level comparable to that of a VCA fragment (Rohatgi et al., 1999). These data support the

idea that the folded N-WASP molecule is unfolded by binding of Cdc42 and PtdIns(4,5)P_2 and that the VCA region is then exposed. An N-WASP fragment containing only the basic region and neighboring GBD/CRIB domain of N-WASP appears to be sufficient for PtdIns(4,5)P_2 binding (Rohatgi et al., 2000). In contrast, although direct binding assays were not carried out, fragments containing the basic region but not the WH1 region of WASP do not block activation of WASP by PtdIns(4,5)P_2, which suggests that the basic region of WASP is not sufficient for a response to PtdIns(4,5)P_2 in the case of WASP (Higgs and Pollard, 2000). These results suggest that N-WASP and WASP are regulated by different mechanisms.

A recent NMR study has provided a structural model for the mechanism of autoinhibition and activation of N-WASP (Kim et al., 2000). In the autoinhibited state, the GBD/CRIB domain is in contact with the C-terminal region, and this contact blocks the Arp2/3-complex-binding site. Binding of Cdc42 to the GBD/CRIB domain causes a dramatic conformational change that results in disruption of the hydrophobic core and release of the C-terminus, thereby enabling interaction with the Arp2/3 complex. Rohatgi et al. showed that the interaction between the GBD/CRIB domain and the C-terminal region must be intramolecular because N-WASP is present as a monomer in solution (Rohatgi et al., 2000). They further showed that PtdIns(4,5)P_2 influences the activity of N-WASP through a conserved basic sequence located near the Cdc42-binding site. As in the case of Cdc42, PtdIns(4,5)P_2 reduces the interaction between the N-terminal and C-terminal regions. Finally, Prehoda et al. reported that the Arp2/3 complex binds to N-WASP even in the inactive closed conformation (Prehoda et al., 2000). They used partial constructs comprising the GBD/CRIB domain and the basic region connected to the VCA region through a short linker. The Arp2/3 complex associates with inactive N-WASP through the A region in the C-terminus and the basic region, but in a manner that precludes Arp2/3 complex activation. In this state, both the Cdc42- and PtdIns(4,5)P_2-binding sites are masked. Binding of either molecule destabilizes the closed state and enhances binding of the other molecule, which thus provides a means of integrating the molecular signals for activation of N-WASP. The concentration of each molecule required to activate N-WASP is reduced by 90% if they are added in combination.

Binding of the Arp2/3 complex to N-WASP, however, can barely be detected when full-length N-WASP is used (Rohatgi et al., 1999), which suggests that the Arp2/3-binding site is masked in the inactive state. It is hard to compare the results of experiments on partial fragments to those using native N-WASP, because the three-dimensional structure may be changed when a short linker without the WH1 and proline-rich regions is used. Thus, it remains unclear whether inactive N-WASP is associated constitutively with the Arp2/3 complex.

WISH activates N-WASP independently of Cdc42 and PtdIns(4,5)P_2

Recently a new aspect of the mechanism of N-WASP activation has emerged. WASP-interacting SH3 protein (WISH), which is an SH3-containing protein identified originally as an Ash/Grb2-binding protein, was found to stimulate N-WASP-induced Arp2/3 complex activation strongly by binding to the proline-rich region of N-WASP (Fukuoka et al., 2001). The level of activation was similar to that induced by a VCA fragment. Interestingly, this activation occurred independently of Cdc42 and PtdIns(4,5)P_2 binding. A construct containing the WISH SH3 domain alone was able to activate N-WASP as strongly as does full-length WISH. Another SH3 protein, Ash/Grb2, also activated N-WASP, although its activation was weak and caused only partial exposure of the VCA region (Carlier et al., 2000). Other SH3 proteins, however, such as Nck, the PI3K 85-kDa subunit, phospholipase Cγ1 and Fyn, do not influence N-WASP-induced activation of the Arp2/3 complex (Fukuoka et al., 2001). Therefore, WISH appears to be a unique SH3 protein that can activate N-WASP with unusual potency. Furthermore, expression of WISH in Cos7 cells can induce filopodia formation even in the presence of a Cdc42-binding-defective mutant form of N-WASP (Fukuoka et al., 2001). Thus, it is clear that the proline-rich region receives activation signals as well as localization signals from proteins containing SH3 domains. These data indicate that several mechanisms regulate activation of N-WASP and that Cdc42 and PtdIns(4,5)P_2 might not be essential upstream signals for activation. Therefore, any protein or lipid that can bind to N-WASP may have the potential to expose the VCA region and activate the Arp2/3 complex (Fig. 2).

The role of N-WASP in actin comet formation

The pathogenic bacterium *Shigella flexneri* propels itself in the cytoplasm of mammalian cells by nucleating actin filaments on its surface membrane, and this process is similar to the nucleation observed in *L. monocytogenes*. Newly assembled actin filaments are crosslinked to form a comet tail structure that generates the force necessary for movement. The formation of actin comets induced by *Shigella* proceeds in a fashion similar to that of N-WASP-induced actin polymerization. The N-WASP–Arp2/3 complex is used to trigger actin nucleation and polymerization on the surface of its membrane. The *Shigella* VirG/IcsA protein, which has no sequence homology to the *Listeria* ActA protein, binds to N-WASP but not WASP and activates N-WASP in a cooperative fashion with Cdc42 (Suzuki et al., 1998; Egile et al., 1999; Suzuki et al., 2000). As a result, formation of a VirG–N-WASP–Arp2/3 ternary complex is induced, and explosive actin polymerization occurs. In contrast, *Listeria* does not use this system to generate actin comets. ActA recruits two critical factors in motility (Cameron et al., 2000): (1) the Arp2/3 complex, which interacts with the N-terminal domain of ActA and leads to activation of actin nucleation without N-WASP (Welch et al., 1997; Welch et al., 1998); and (2) VASP, which interacts with the proline-rich domain of ActA (Chakraborty et al., 1995; Smith et al., 1996) and links the host cell actin cytoskeleton with the surface of the bacterial cell (Laurent et al., 1999). Vaccinia virus also stimulates formation of actin comets by N-WASP. In this case, recruitment of N-WASP to vaccinia is mediated by WASP-interacting protein (WIP), which was originally identified as a protein that binds to the WH1 domain of WASP (Ramesh et al., 1997). WIP binding to WASP activates the Arp2/3 complex and stimulates actin-based motility of vaccinia virus in a Cdc42-independent manner (Moreau et al., 2000), and polystyrene beads coated with WASP can generate actin comets and movement in cytoplasmic extracts (Yarar et al., 1999).

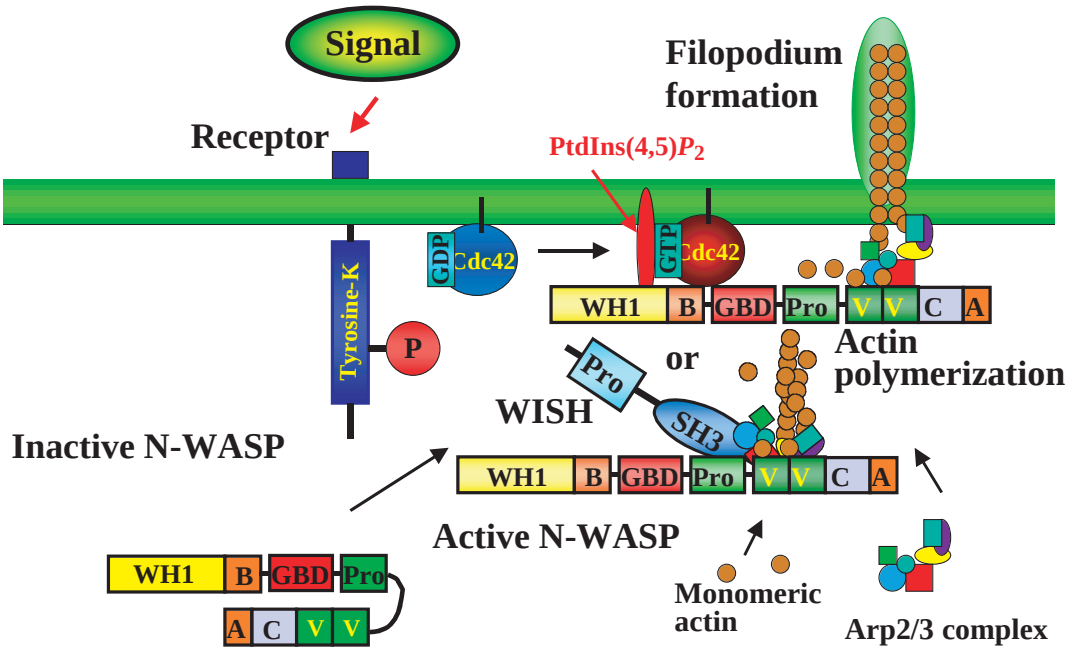

Fig. 2. Mechanism of activation of N-WASP. In the inactive state, N-WASP is folded by an interaction between the GBD/CRIB domain and the cofilin-homology (C) domain that masks the VCA region. Cooperative binding of PtdIns(4,5)P_2 to the basic (B) region and of active Cdc42 to the GBD/CRIB domain exposes the VCA region, resulting in Arp2/3 complex activation. Alternatively, WISH binds to the proline-rich region of N-WASP and exposes the VCA region independently of PtdIns(4,5)P_2 and Cdc42 binding. N-WASP activation stimulates formation of filopodia and appears to require additional factors that bundle and knit the actin filaments into straight filaments.

Formation of dynamic actin comet tails also occurs on a subset of cytoplasmic vesicles in vivo. Actin comets appear within minutes of activation of *Xenopus* eggs through the recruitment of N-WASP to the surface of vesicles (Ma et al., 1998; Taunton et al., 2000). Overexpression of type I phosphatidylinositol phosphate 5-kinase, which synthesizes PtdIns(4,5)P_2, also promotes N-WASP–Arp2/3-dependent actin polymerization from membrane-bound vesicles to form actin comets (Rozelle et al., 2000). Such actin comet formation also occurs in pervanadate-treated cells and PDGF-stimulated cells, which suggests that tyrosine phosphorylation is important for actin comet formation. Comet generation appears to occur at vesicles enriched in lipid rafts; rafts might therefore be important for actin-based movement of vesicles (Qualmann et al., 2000).

WAVE-induced membrane ruffle formation downstream of Rac

To date, three isoforms of the WAVE/Scar proteins have been isolated (Suetsugu et al., 1999). These proteins have a WHD/SHD domain (WAVE homology domain/Scar homology domain), a basic region, a proline-rich region and a VCA region. Half of the C-terminus shows high homology to that of WASP family proteins. The WAVE/Scar proteins, however, do not contain GBD/CRIB domains, which suggests that these proteins probably are not downstream of Cdc42. Endogenous WAVE localizes to membrane ruffles induced by treatment with PDGF or expression of dominant active Rac (Miki et al., 1998b). A recent study using 3Y1 fibroblasts also indicated that endogenous WAVE localizes at the tips of membrane ruffles, whereas endogenous N-WASP accumulates at the bundled actin-microspikes (Fig. 3; Nakagawa et al., 2001). The

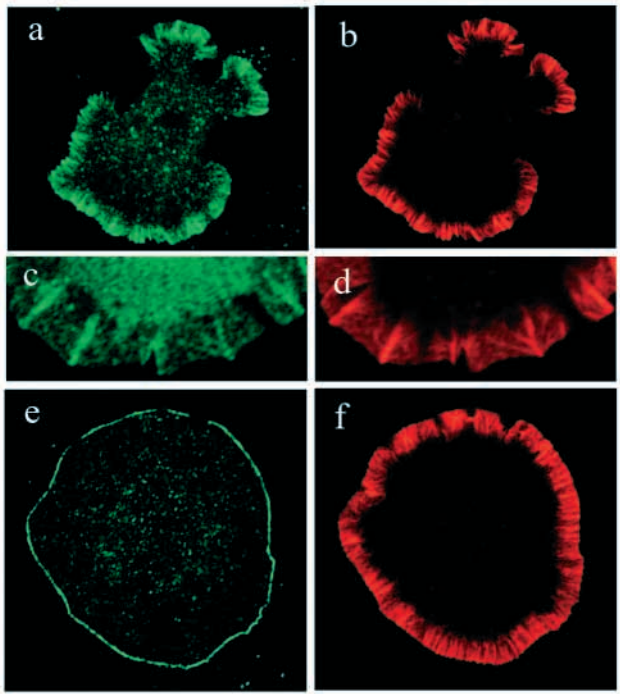

Fig. 3. Distinct localization of endogenous N-WASP and WAVE in 3Y1 cells. Rat 3Y1 fibroblasts were seeded on polylysine-coated glass coverslips. Cells were then fixed and stained with anti-N-WASP antibody (a and c), anti-WAVE antibody (e), and phalloidin (b, d, and f). c and d are magnified images of a and b, respectively.

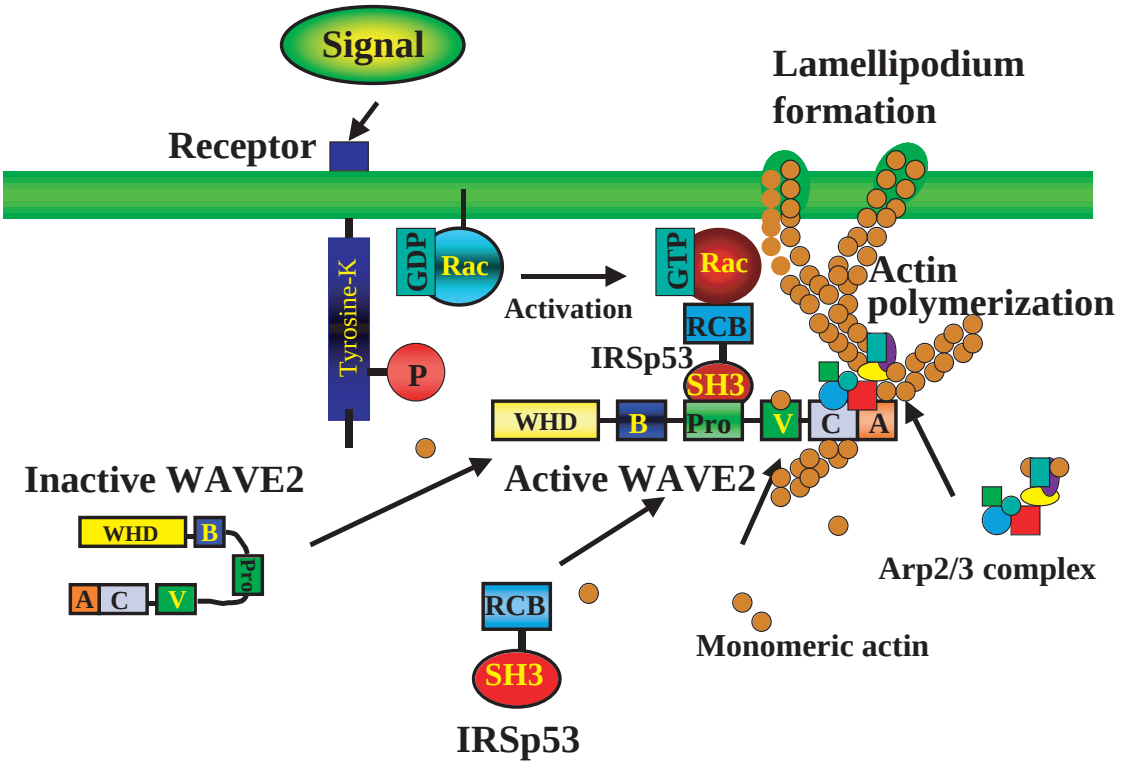

Fig. 4. Mechanism of activation of WAVE2. WAVE2 is probably inactive in the resting state, although WAVEs purified from a baculovirus system are already activated. In response to extracellular stimuli, Rac is activated. IRSp53 is recruited through its Rac-binding domain (RCB) and binds to the proline-rich region of WAVE2. As a result, the VCA region of WAVE2 is exposed and causes Arp2/3 complex activation. This leads to assembly of a meshed network of actin filaments and the formation of lamellipodia.

difference in the localization of WAVE and N-WASP strongly suggests that WAVE is regulated by a different signal from that which regulates N-WASP. Indeed, a dominant negative mutant WAVE protein in which the V domain is deleted blocks the formation of membrane ruffles induced by Rac but does not inhibit Cdc42-induced filopodia formation. These data suggest that WAVE regulates membrane ruffle formation by activating the Arp2/3 complex downstream of Rac. WAVE forms complexes with Rac when both molecules are expressed in Cos7 cells (Miki et al., 1998b). However, this binding does not appear to be direct; at least one additional factor is therefore probably required to link Rac and WAVE. Because all WAVEs have the WHD/SHD in the N-terminal region, this domain was predicted to be the site that receives the signal from Rac.

Yeast two-hybrid experiments using the WHD as bait failed to identify a missing link. Similar experiments, using the proline-rich region as bait, however, identified several WAVE-interacting proteins. Of these, IRSp53, an insulin receptor substrate of unknown function (Yeh et al., 1996), associated with WAVE most strongly. Further investigation revealed that activated Rac binds to the Rac-binding domain (RCB) in the N-terminus of IRSp53 and that the C-terminal SH3 domain of IRSp53 binds to WAVE to form a trimolecular complex (Miki et al., 2000). Surprisingly, IRSp53 binds specifically to WAVE2, a ubiquitously expressed WAVE.

Purified IRSp53 stimulated WAVE2-dependent Arp2/3-complex-induced actin polymerization in vitro, but the effect was partial because the WAVE2, which was purified from a baculovirus system, was already activated. In addition, WASP, purified from a baculovirus system, was activated. In contrast, WASP purified from bovine thymus was shown to be inactive (Higgs and Pollard, 2000), which suggests that WASP and WAVEs become activated during purification and concentration. Even in vivo, overexpressed WASP and WAVEs form clusters with actin filaments (Kato et al., 1999; Suetsugu et al., 1999). If binding of IRSp53 and WAVE2 is critical for Rac-induced membrane ruffling, inhibition of binding should show a dominant negative effect. Indeed, expression of the proline-rich region of WAVE2, which associates with the SH3 domain of IRSp53, suppresses Rac-induced membrane ruffling (Miki et al., 2000). Furthermore, expression of SH3-deleted IRSp53 also inhibits Rac-induced membrane ruffling, indicating that the IRSp53-WAVE2 complex has a crucial role in this process (Fig. 4). However, several other SH3-domain-containing proteins, such as phospholipase Cγ1, the PI3K 85-kDa subunit, Fyn and WISH, do not bind to WAVE2, although Ash/Grb2 does weakly (Miki et al., 1999; Fukuoka et al., 2001). Thus, the IRSp53 SH3 domain recognizes the proline-rich region of WAVE2 specifically. The binding region of WAVE2 is a 17-residue region that contains a WAVE2-specific sequence.

Rac has also been shown to associate with several effector molecules, such as POR1, p67phox, S6 kinase, IQGAP, PAK and MLK (reviewed by Boettner and Aelst, 1999). Several pathways are shown to be important for inducing membrane ruffles. However, as in the case of Cdc42-induced filopodium formation, such molecules cannot explain the rapid actin polymerization induced by activated Rac. Furthermore, as

discussed later, membrane ruffles contain branched networks of actin filaments, which should be formed by the Arp2/3 complex. In this sense, the Rac-IRSp53-WAVE2 pathway seems to be the main route for formation of membrane ruffles, since only this signaling can clearly explain how the Arp2/3 complex is activated downstream of Rac.

WAVE as a cAMP-dependent protein kinase anchoring protein (AKAP)

Scar, a *Dictyostelium* homolog of WAVEs, was originally isolated in a genetic screen for proteins downstream of the chemotaxis receptor for cAMP (Bear et al., 1998). Recently, Westphal et al. (Westphal et al., 2000) reported that WAVE1 associates specifically with the regulatory subunit (RII) of protein kinase A (PKA) through a region overlapping with the V domain (the G-actin-binding site). This association was originally identified during a survey of ABL-SH3-interacting and RII-binding proteins. Thus, WAVE1 was classified as an A-kinase-anchoring protein (AKAP) that tethers PKA to defined sites (Diviani and Scott, 2001). Actin can compete with PKA-WAVE1 at the V domain, which suggests that PKA anchoring is regulated dynamically at sites of actin reorganization. The WAVE-PKA-ABL complex might be tethered to the cytoskeleton through actin-based interactions. Interestingly, such interactions were observed only with WAVE1 but not WAVE2 or WAVE3. These results suggest that each WAVE isoform functions differently. In addition, another protein, AMY-1 (activator of MYC transcription), associates with WAVE1 through the V domain (Taira et al., 1998; M. Furusawa et al., unpublished). This protein binds to AKAP84/140 as well. Therefore, WAVE1 may be present in a huge complex of AKAPs and act as a spatiotemporal PKA regulator that is important in actin reorganization, cell polarity and morphogenesis (Colledge and Scott, 1999; Westphal et al., 2000).

The configuration of actin filaments induced by the ARP2/3 complex

Nucleation of actin filaments induced by VCA proteins and the Arp2/3 complex results in formation of branched actin filaments (Higgs and Pollard, 1999; Pantaloni et al., 2000; Blanchoin et al., 2000), which are the main structures involved in membrane ruffling and lamellipodia (Bailly et al., 1999; Svitkina et al., 1999). The Arp2/3 complex is present at the branch points of branching actin filaments but not in straight-bundled actin filaments. Pantaloni et al. claimed that the Arp2/3 complex interacts with the barbed ends of actin filaments to induce branching (Pantaloni et al., 2000). However, Mullins et al. have proposed a dendritic nucleation model in which the Arp2/3 complex nucleates actin polymerization from the side of older filaments (Mullins et al., 1998). In this model, the Arp2/3 complex is recruited to the leading edge of the cell and activated to form new actin nuclei, which branch from existing filaments.

Arp2/3-complex-asssociated actin polymerization may induce not only membrane ruffling but also filopodia composed of straight-bundled actin filaments. In this case, it is understandable how WAVE induces formation of mesh-like actin networks by activating the Arp2/3 complex. It is unclear, however, why N-WASP induces filopodia formation in vivo, even though it also uses the Arp2/3 complex to stimulate polymerization of actin filaments. How does N-WASP lead to polymerization of straight actin filaments, such as microspikes? One possibility is that N-WASP-induced formation of straight actin structures requires another factor that bundles branching actin filaments, releases the Arp2/3 complex and straightens the filaments.

Filamin may be a good candidate for a molecule that reorganizes actin filaments into straight bundles. Filamin was originally identified as an actin-crosslinking protein, and Ohta et al. (Ohta et al., 1999) showed that filamin plays an essential role in Cdc42-induced filopodia formation and speculated that filamin bundles polymerized actin into straight filaments. WIP also associates directly with WASP and N-WASP through the WH1 domain (Ramesh et al., 1997). Thus, WIP might also participate in formation of straight actin filaments by bundling actin filaments or releasing the Arp2/3 complex from branch sites to knit the branched filaments to form straight filaments.

Conclusion

In rapid reorganization of cortical actin filaments induced in response to extracellular stimuli, WASP and WAVE family proteins play critical roles. These proteins have a VCA region at the C-terminus, through which they bind G-actin and the Arp2/3 complex, causing rapid actin nucleation and polymerization. N-WASP is key to formation of filopodia downstream of Cdc42; WAVE2 is key to formation of lamellipodia downstream of Rac. However, it is still unclear whether WASP also induces formation of filopodia (as N-WASP does) and whether WAVE1 and WAVE3 have functions similar to those of WAVE2. Another interesting question is how N-WASP constructs straight actin filaments, even though it uses the Arp2/3 complex. Since formation of filopodia and lamellipodia is essential for cell movement and morphogenesis, WASP and WAVE family proteins are thought to be crucially involved in these processes. The production of knockout mice that lack these proteins will provide some answers to the intriguing questions about the functions of WASP and WAVE family proteins in cell movement and morphogenesis.

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