Signalling to actin: the Cdc42-N-WASP-Arp2/3 connection

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The molecular link between the signalling pathway regulating the formation of filopodia and the initiation of local actin polymerization has been elucidated: N-WASP, a close homologue of WASP, which is the product of the gene responsible for the Wiskott-Aldrich syndrome, mediates a direct connection between the small G-protein Cdc42 and the Arp2/3 complex.

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Actin polymerization occurs in eukaryotic cells in response to a variety of extracellular stimuli (chemotactic peptides, growth factors, insulin and so on) and is essential for the diverse motile activities of the cells, including locomotion, cell growth and morphogenesis, cytokinesis and phagocytosis. Actin polymerization is also responsible for the formation of cellular protrusions that adopt distinct morphologies — either flat (lamellipodia) or spiky (filopodia). These extensions allow the cell to engage in more complex processes.

The notion that actin polymerization alone can develop a force sufficient to cause the deformation of the plasma membrane and change the shape of the cell in a stimulus-directed fashion raises a number of challenging mechanistic issues to cell physicists and biologists. How are the external signals transduced from receptors at the surface of the cell to the actin cytoskeleton? How is actin polymerization spatially and temporally controlled? How can the free energy of ATP hydrolysis linked to the steady state cycle of actin assembly be used to produce force and movement in processes generally called 'actin-based motility'?

In the past six months, ten years of research using different approaches to tackle the problem from its two poles — the actin field and the signalling field — have come to fruition, providing functional and structural insights into the molecular link between signalling molecules and actin polymerization, and therefore providing a new perspective on cell mechanics.

In vitro assays for actin-based motility

In a search for systems suitable for investigating the molecular basis of cell motility, cell biologists were attracted by the amazing ability of some pathogens (*Listeria monocytogenes*, *Shigella flexneri*, the vaccinia virus and so on) to induce polarized, constitutive actin assembly at their surface, and use the propulsive force to move in infected cells at rates of 1–50 μm/min, which is comparable to the rate of forward movement of the leading edge of stimulated motile cells (Figure 1). The successful *in vitro* reconstitution of bacterial propulsion in acellular extracts allowed the identification of cellular effectors of actin polymerization downstream of the signalling pathway. The ActA protein, which is expressed at the surface of *Listeria*, was shown to interact with the Arp2/3 complex [1], and activate Arp2/3 for its function in nucleating actin polymerization [2].

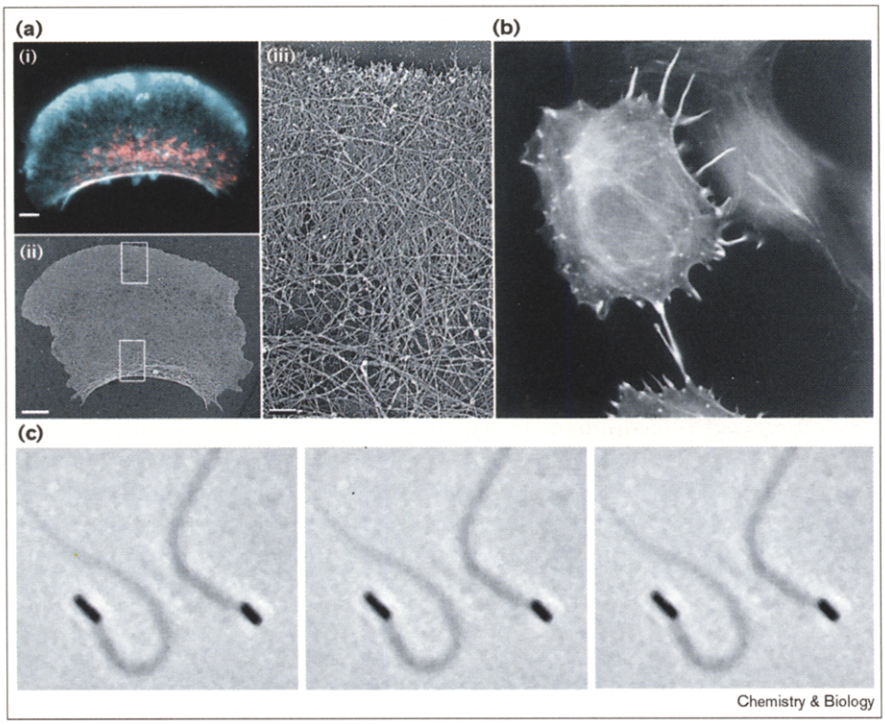
The Arp2/3 complex: the downstream effector of actin polymerization

The Arp2/3 complex (Figure 2a) is formed by the macromolecular stable assembly of seven different proteins comprising the actin-related proteins Arp2 and Arp3, and five other proteins called ARC (p41, p34, p21, p20 and p16). Initially discovered in *Acanthamoeba castellanii*, the Arp2/3 complex appears ubiquitous, conserved from yeast to higher eukaryotes, and generally localized to motile regions of the cell (see [3] for review). *In vitro*, the Arp2/3 complex stimulates spontaneous polymerization of actin and is thought to act by nucleating filaments with free barbed ends (the rapidly growing ends of the actin filaments), by capping the pointed ends and by initiating barbed end growth from the sides of actin filaments [4]. The finding that the intrinsic actin nucleating activity of Arp2/3 is low but can be stimulated by ActA (Figure 2b) raised great interest because it suggested that Arp2/3 could be a stimulus-responsive target of signalling pathways leading to site-directed actin assembly in lamellipodia, a view supported by the subcellular distribution of Arp2/3 [5].

Effectors of Cdc42

Cdc42 is one of the Rho family GTPases that control the assembly and organization of actin filaments in all eukaryotic cells, and is more specifically involved in the formation of filopodia in response to agonists such as bradykinin or epidermal growth factor (EGF) [6]. Like other G proteins, Cdc42 works as a molecular switch, cycling between an inactive GDP-bound form (stabilized by interaction with a guanine nucleotide dissociation inhibitor, Rho-GDI), and an active, membrane-targeted GTP-bound form. The conversion to the active form is induced by the

Figure 1



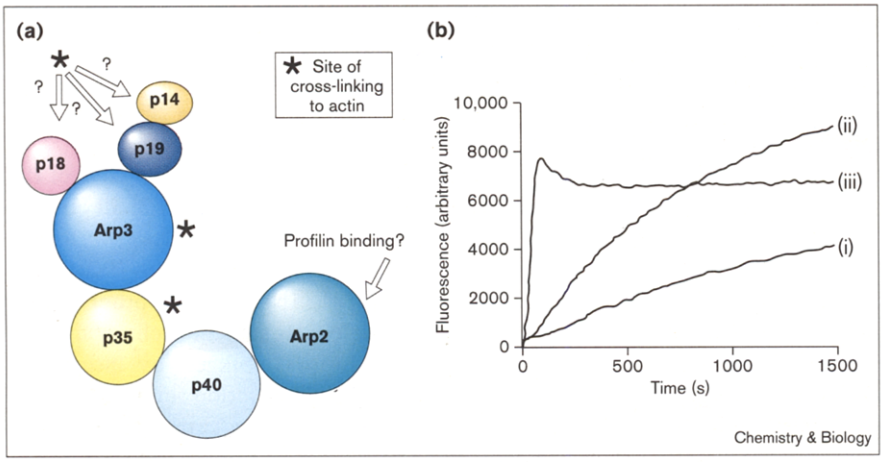
Processes mediated by the polymerization of actin filaments. **(a)** A locomoting keratocyte extends a large lamellipodium in the direction of movement. Actin filaments are visualized by (i) phalloidin staining (cyan), and (ii,iii) in electron microscopy of platinum replicas of the lamellipodium (from [26]). **(b)** Formation of filopodia. Swiss 3T3 cells were transfected with pCMV6-bearing sequences encoding hemagglutinin–Cdc42L61. Filamentous actin distribution was visualized using indirect immunofluorescence (from [27]). **(c)** *Listeria monocytogenes* moving in platelet extracts by continuously polymerizing actin at its surface. The actin meshwork and the bacterium are seen in phase contrast optical microscopy (courtesy of Valerie Laurent).

association of Cdc42 with a guanine nucleotide exchange factor (GEF), thought to be activated by a membrane-bound receptor. Efforts have focused on the identification of effectors of activated Cdc42 that would regulate the actin cytoskeleton.

N-WASP as a target of GTP-bound Cdc42

In 1996, WASP, the product of the gene responsible for the Wiskott–Aldrich syndrome (WAS), was shown to interact with the GTP-bound form of Cdc42 [7–9]. WASP is expressed exclusively in hematopoietic cells. Lymphocytes and platelets of WAS patients present morphological defects that relate to the patients' poor immune response. WASP was shown to be a member of of a larger family of proteins, so far comprising Las17p/Bee1p in budding yeast [10], Scar1/WAVE in *Dictyostelium discoidium* [11], *Caenorhabditis elegans*, *Drosophila*, mouse and humans. WASP has a close homologue, N-WASP, in humans [12], which is ubiquitous and abundant in neural cells. Proteins of the WASP family share a common organization in several domains (Figure 3a). They have a pleckstrin homology (PH) domain, also thought to be a WASP homology domain

Figure 2



Arp2/3 complex structure and function. **(a)** The Arp2/3 subunit composition, showing the seven polypeptides including Arp2 and Arp3 (from [28]). **(b)** Time courses of spontaneous polymerization of actin (2 mM) with (i) no additions or with 30 nM ActA, (ii) in the presence of 30 nM Arp2/3, and (iii) in the presence of 30 nM Arp2/3 complex and 30 nM ActA (adapted from [2]).

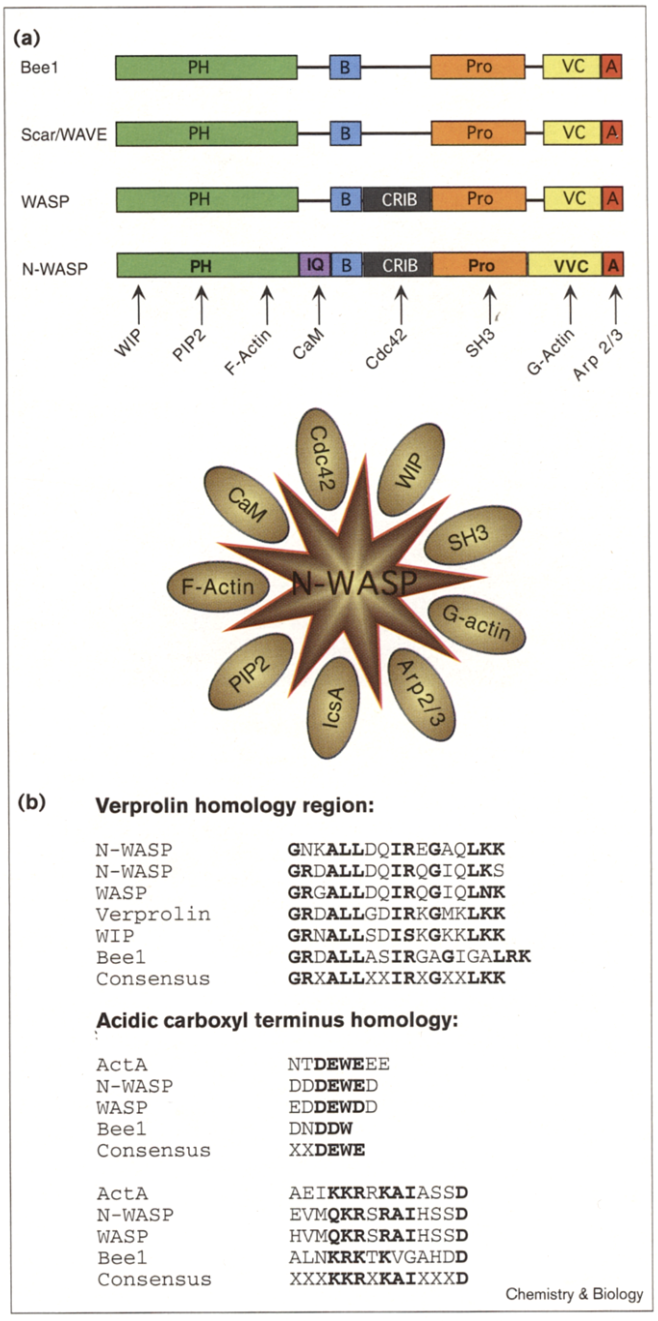
(WH1), the structure of which is probably closer to the recently published structure of enabled-VASP homology 1 domain [13] than the canonical PH structure, a Cdc42–Rac interactive binding (CRIB) domain present only in WASP and N-WASP, and a proline-rich region containing two GRSGPXPPXP repeats (where X is any amino acid; expected to fold in a poly-proline II helix), known to interact with SH3 domains. The highly conserved, carboxy-terminal domain (VCA) consists of a verprolin-homology region (V), a cofilin-homology sequence (C) and an acidic carboxyl terminus (A) that shows some sequence homology to the amino-terminal region of ActA (Figure 3b). The multidomain organization, which is common to signal transduction proteins, suggests that WASP proteins might have a relay function in different signal transduction pathways. *In vivo*, overexpression of N-WASP enhanced Cdc42-induced formation of filopodia [12]. *In vitro*, Cdc42-induced actin assembly was reconstituted in cell-free systems [14] and biochemical fractionation indicated that Arp2/3 was involved in the polymerization of actin induced by Cdc42–GTP [15], but there was no link between Cdc42 and Arp2/3.

Looping the loop: N-WASP mediates the link between Cdc42 and Arp2/3

A definite step forward was made when Machesky and Insall [16] discovered, using a double hybrid screen in yeast, that the carboxy-terminal domain (VCA) of Scar1 interacted with p21-ARC, one of the components of the Arp2/3 complex; they also demonstrated that actin nucleation by Arp2/3 was greatly enhanced, as had first been observed with ActA, by its association with either VCA or the full-length Scar protein [17]. The carboxyl terminus acidic A region (residues 514–559) is sufficient for binding to Arp2/3, but does not enhance the nucleating activity of Arp2/3, indicating that the actin-binding VC region is involved in the activation of Arp2/3. These results strongly suggest that the active complex in the nucleation of actin filaments is not a binary Arp2/3–Scar complex, but a triangular ternary Arp2/3–G-actin–Scar complex, in which each component interacts with the two others. The interaction of Arp2/3 with G-actin is essential for actin nucleation, and it is strengthened in the ternary complex.

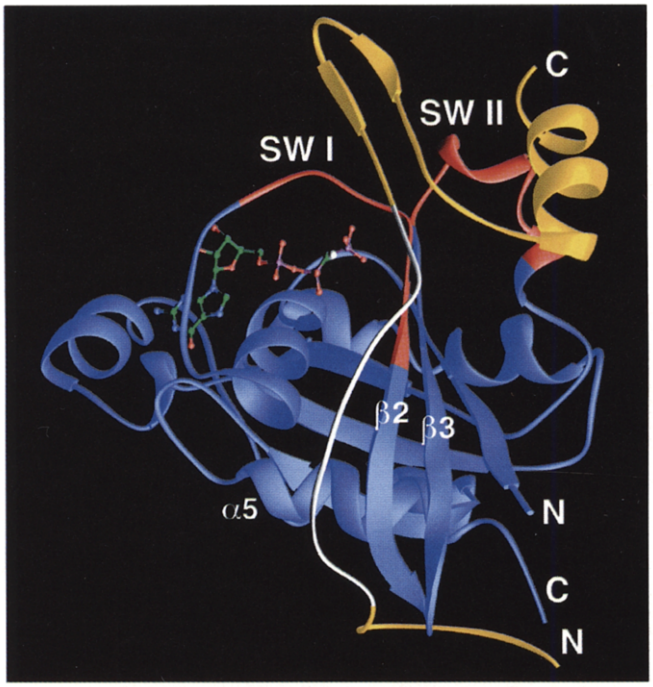
A more complete picture of the connection of Arp2/3 complex to Cdc42 recently emerged from the *in vitro* reconstitution of Cdc42-induced actin assembly from purified components [18]. The ubiquitous protein N-WASP stimulated actin nucleation by Arp2/3 in a Cdc42–GTP-dependent fashion. Although VCA constitutively activates Arp2/3, full-length N-WASP is modestly active in the absence of Cdc42. Hence, binding of Cdc42 to N-WASP induces a structural positioning of the domains, unmasking the carboxy-terminal domain for interaction with G-actin and Arp2/3, and initiating the scaffold that is built at the leading edge to induce filopodium extension. The IcsA surface

Figure 3



N-WASP as a multiple mediator of signalling pathways to actin. **(a)** Domain organization of N-WASP, showing the homology between different members of the WASP family, and the list of ligands known to interact with each domain. The location of IcsA and F-actin-binding sites are not known yet. A, Arp2/3 binding; B, basic cluster (might interact with the A domain in the inactive state); CRIB, Cdc42 binding; IQ, Ca–calmodulin binding; PH, PIP2 and WASP-interacting protein (WIP) binding; Pro, SH3 binding (Nck, Fgr, Fyn and Grb2); VC, G-actin binding. **(b)** Sequence homologies: homology to yeast verprolin in WASP, N-WASP, Bee1 and WIP, and homology in the acidic carboxyl terminus (Arp2/3 binding region) in WASP, N-WASP, Bee1 and the *Listeria* protein ActA.

Figure 4



Structure of the CRIB domain of N-WASP in complex with Cdc42. Ribbon representation of the complex of Cdc42 (in blue) with WASP (230–288) fragment (in yellow). Residues 278–288 are disordered and not represented. Switch 1 (SW I) and 2 (SW II) of Cdc42 are red, and the CRIB motif of WASP is white. Nucleotide analog and Mg^{2+} are ball-and-stick representations. The interaction of the WASP GBD amino terminus with the Cdc42 β2 β3 hairpin and the α5 helix can be seen. (Reproduced from [25] with permission.)

protein of *Shigella* binds N-WASP [19]. We have recently shown (C. Egile, T.P. Loisel, U. Laurent, D. Pantaloni, P.J. Sansonetti and M.-F. Carlier, unpublished observations) that *in vitro* IcsA activates N-WASP in a Cdc42-like fashion and stimulates Arp2/3-mediated actin polymerization. The Arp2/3 complex therefore appears essential in *Shigella* actin-based movement, as well as in that of *Listeria*. WASP-coated microspheres initiate Arp2/3-mediated actin polymerization and move in platelet extracts [20]. In the yeast system, the conserved carboxy-terminal domain of Bee1p also stimulates Arp2/3-mediated actin polymerization [21]. All the evidence indicates that N-WASP might play a general role in Arp2/3 stimulation of actin assembly. The *Listeria* ActA protein, which shares two regions of sequence homology with VCA (Figure 3b), might mimic N-WASP to activate Arp2/3. In conclusion, the stimulation of spontaneous polymerization of G-actin in bulk solution from isolated components of the signalling cascade is the first important step towards the full reconstitution of actin-based movement.

The Scar1 protein, which contrary to N-WASP does not bind Cdc42, appears constitutively active [16], indicating that the domain assembly of members of the WASP family proteins might differ, depending on the presence of the CRIB domain (Figure 3a). It has been postulated [12] that the acidic carboxyl terminus of N-WASP undergoes intramolecular interaction with a cluster of basic residues close to the amino terminus of the CRIB domain, therefore masking the Cdc42, G-actin and Arp2/3 binding sites in inactive N-WASP. Clearly, structural changes linked to ligand binding are at the origin of the functions of Arp2/3 and N-WASP in signalling to actin, and require more extensive investigations.

Thermodynamic and structural analysis of N-WASP–Cdc42 interaction

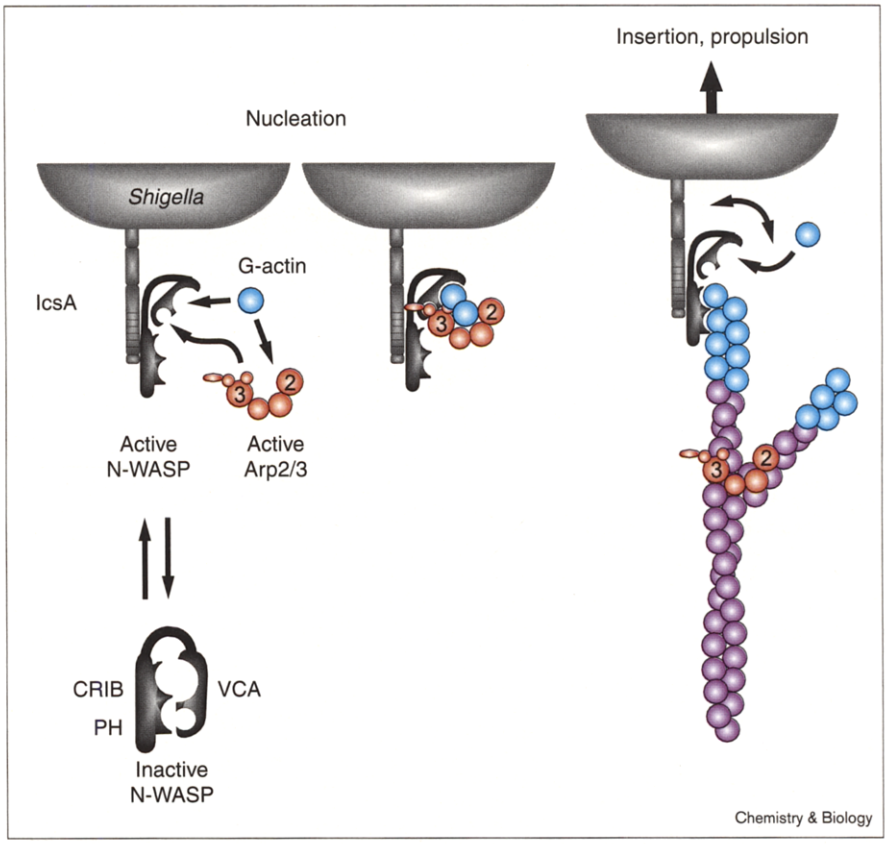
Progress in this direction has been made by analyzing the interaction of peptides containing the CRIB domain of N-WASP with Cdc42–GMPPCP [22]. In initial studies using fluorescence and proton or ^{15}N nuclear magnetic resonance (NMR), a series of N-WASP peptides of different sizes that contained the CRIB domain were assayed for their binding to Cdc42–GMPPNP [23]. The largest fragment (13 kDa) showed secondary structure using circular dichroism (CD) but aggregation problems prevented further NMR studies. These results were recently extended with the complete NMR solution structure of N-WASP fragment 230–288 bound to Cdc42–GMPPCP [22]. The peptide, which contains an antiparallel β hairpin and an α helix (Figure 4), is bound in extended conformation and makes contact with both switches I and II, accounting for its specificity for the GTP-bound form of Cdc42. The buried interface (2930 $Å^2$) is remarkably large, considering the small size of the peptide. For comparison, the buried interface of 1270 $Å^2$ in the Rap1A–Raf complex [24] is closer to the average (1500 $Å^2$) protein–protein interface. Two features of the structure are particularly notable: first, there is a hydrogen bond between Asp38 carboxylate of Cdc42 and His246 or His249 of the CRIB domain. Interaction between these residues had previously been revealed by mutation [25]; second, polar and hydrophobic contacts of highly conserved residues are essential for the complex interaction. The authors further address the question of why WASP binds activated Cdc42 and Rac but not Rho. They conclude that discrimination is presumably governed by the GBD (GTPase-binding domain) contacts to switch 1 but also the residues of the α5 helix. The structure of the CRIB domain in full-length N-WASP is not known, but the extended structure of the bound CRIB domain suggests that a large structural rearrangement of the whole N-WASP must be linked to Cdc42 binding. It will be interesting to examine how binding of the acidic carboxy-terminal region of N-WASP to the basic sequence on the amino-terminal side of CRIB affects the structure of the CRIB region and its interaction with Cdc42.

From actin assembly to movement: N-WASP as a motor?

N-WASP has now come onto the stage as a new player in cell motility. *In vitro* polymerization assays (C. Egile,

Figure 5

Model for N-WASP activation and stimulation of Arp2/3-mediated actin polymerization by the *Shigella* IcsA protein. According to this scheme, the N-WASP molecule is in dynamic equilibrium between two conformations, an inactive folded conformation and an active open conformation induced upon binding ligands like Cdc42 or IcsA, as represented here, or others (SH3 domains?). The activation of N-WASP allows interaction with Arp2/3, G-actin and F-actin, generating actin-based motility of *Shigella* (C. Egile, T.P. Loisel, U. Laurent, D. Pantaloni, P.J. Sansonetti and M.-F. Carlier, unpublished observations).



T.P. Loisel, U. Laurent, D. Pantaloni, P.J. Sansonetti and M.-F. Carlier, unpublished observations) suggest that N-WASP has two different functions in actin assembly. The carboxy-terminal VCA domain binds G-actin and has a profilin-like function, rather than an actin depolymerizing function, as initially thought [12]. In this role, VCA would shuttle G-actin subunits onto barbed ends, therefore preventing their blockage by capping proteins. On the other hand, the amino-terminal domain of N-WASP binds F-actin and stabilizes the filament. Hence N-WASP has the properties expected for a motor of insertional polymerization, maintaining attachment of the filament to the bacterium while facilitating its growth (Figure 5). N-WASP may be the first representative of a new generation of actin-binding proteins that fulfil new functions by combining the properties of known soluble actin-associated regulatory proteins and solid-state biochemistry.

Perspective: regulation of proteins of the N-WASP family

The multipartnership nature of N-WASP raises challenging issues. Does N-WASP perform multiple functions, connecting different signalling pathways to actin? Is Cdc42 the only activator of N-WASP, or does the binding of SH3 domain of adaptors (such as Nck or Grb2) to the proline-rich region of N-WASP lead to the same functional state, resulting in Arp2/3-mediated actin polymerization? What is the function of the PH domain of N-WASP, and of the interaction with the verprolin homologue WASP-interacting protein (WIP)? Finally, how can cellular extensions of different morphologies (filopodia, lamellipodia and phagocytic cup) be generated using Arp2/3 as a common downstream target? As progress in this area is so rapid, these problems may well be solved in the near future.

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