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Physical Mechanisms of Signal Integration by WASP Family Proteins

Shae B. Padrick and Michael K. Rosen

Howard Hughes Medical Institute and Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75390

Shae B. Padrick: Shae.Padrick@UTSouthwestern.edu; Michael K. Rosen: Michael.Rosen@UTSouthwestern.edu

Abstract

The proteins of the Wiskott-Aldrich Syndrome protein (WASP) family are activators of the ubiquitous actin nucleation factor, Arp2/3 complex. WASP family proteins contain a C-terminal VCA domain that binds and activates Arp2/3 complex in response to numerous inputs, including Rho family GTPases, phosphoinositide lipids, SH3 domain containing proteins, kinases and phosphatases. In the archetypal members of the family, WASP and N-WASP, these signals are integrated through two levels of regulation, an allosteric autoinhibitory interaction, in which the VCA is sequestered from Arp2/3 complex, and dimerization/oligomerization, in which multi-VCA complexes are better activators of Arp2/3 complex than monomers. Here we review the structural, biochemical and biophysical details of these mechanisms and illustrate how they work together to control WASP protein activity in response to multiple inputs. These regulatory principles, derived from studies of WASP and N-WASP, are likely to apply broadly across the family.

Keywords

Actin regulation; allostery; signal transduction; Arp2/3 complex; Rho GTPase

Introduction

Cells are constantly bathed in a multitude of signals from the environment. Proper cellular function requires integration of these signals to yield complex processes and behaviors such as differentiation, division and movement. Often signal integration is the specific response of individual molecules to multiple simultaneous physical interactions. The mechanisms of this processing are a fascinating and important area of research in biochemistry, biophysics and structural biology.

Dynamic rearrangements of the actin cytoskeleton underlie many cellular processes, including division, endocytosis and movement. Control over actin dynamics is achieved through the integration of numerous signals, which together guide the assembly, disassembly, architecture and movement of the actin filament network in a spatially and temporally defined manner.

Members of the Wiskott-Aldrich Syndrome Protein (WASP) family are central hubs in the signaling networks that control actin. These proteins integrate a huge range of inputs. In response, they activate the ubiquitous actin nucleating machine, Arp2/3 complex (1,2). By

controlling the degree, rate and location of filament nucleation by Arp2/3 complex, WASP proteins shape the structure and dynamics of filament networks throughout biology.

Several excellent reviews have recently discussed the cellular functions of WASP proteins, highlighting genetic and cell biological findings (2–6). Here we focus on complementary structural, biophysical and biochemical findings, with an eye toward illustrating how these data inform on biology. The central concept that pervades our discussion is that the activity of WASP proteins toward Arp2/3 complex is controlled at two levels. First, an allosteric process involving transitions between inactive and active conformations controls the accessibility of the Arp2/3-stimulatory element of WASP proteins. Second, oligomerization modulates that ability of active WASP to stimulate Arp2/3 complex, with dimers or higher order oligomers having much greater potency than monomers. These two mechanisms function synergistically to allow WASP family members to integrate diverse signals and control Arp2/3 activity with precision. This view provides a unifying lens through which WASP biology can be understood in quantitative terms.

Domain Organization of WASP and N-WASP

The WASP family is defined by a conserved C-terminal VCA domain named for its three sub-elements, the Verprolin homology (also called WASP homology 2), Central hydrophobic and Acidic regions. The VCA domain strongly stimulates actin nucleation by Arp2/3 complex (1,7,8). The three elements have distinct functions during this process. The C-terminal A region contributes substantial binding affinity toward Arp2/3 complex (9–12). There is also a connection between the A region and activity; different WASP family VCAs have quite different maximal activities, which correlate with A region sequence (11). The C region also contributes both to binding affinity and to an affinity-independent aspect of activation (10,13), as well as to autoinhibitory regulation (see below). The C region appears to contact Arp2/3 complex using the hydrophobic face of an amphipathic helix (13).

The V region binds to G-actin and delivers it to the Arp2 and/or Arp3 subunits of Arp2/3 complex (which both are homologous to actin), creating a pseudo-trimer/tetramer nucleus for filament growth (7,9,10,14–18). A recent SAXS study suggests that a high-affinity site for the VCA:actin complex is positioned to deliver actin to the Arp2 subunit (18); it is also possible that a second VCA binding to a different site (see below) could deliver actin to Arp3. V region peptides form an amphipathic helix that inserts into the hydrophobic groove between actin subunits 1 and 3, followed by an extended segment that traverses up the actin face toward the nucleotide binding cleft (Figure 2a) (16,19) (see also RSCB database entry PDB#2vcp). As detailed in recent reviews, V/WH2 repeats are also found in many other proteins, and can act to either sequester actin monomer or, when in tandem arrays, nucleate filaments directly (20,21).

An important caveat to this straightforward characterization of the V, C and A functions is that nearly all experiments to date do not take into account the recent discovery of a second VCA binding site on Arp2/3 complex (22). This finding requires new experiments (and reconsideration of previous data) to analyze these functions in the context of the individual sites. In general, the VCAs could have quite different actions at the two sites, perhaps even promoting temporally distinct steps in the nucleation process. Understanding both the thermodynamic and kinetic aspects of Arp2/3 activation through the two VCA binding sites represents an important avenue for future research.

N-terminal elements of WASP family proteins control localization, association with ligands and biochemical activity of the VCA. These elements are divergent among different family members, affording the proteins distinct molecular details of regulation. However as argued

below, the physical regulatory principles are likely similar across the family. These principles have emerged from studies of the archetypal members, WASP and N-WASP.

The N-terminal Ena/VASP homology 1 (EVH1) domain of WASP and N-WASP (Figure 1) binds to a conserved proline motif in members of the WIP family (23). This interaction stabilizes and localizes WASP and N-WASP in cells (23–25). Structural studies show that the WIP proline motif contacts the canonical EVH1 ligand binding site through a type II polyproline helix (Figure 2b) (26). The flanking sequences extend linearly around the EVH1 domain in opposite directions, wrapping over half way around the domain. Mutations that disrupt this interface decrease WIP binding, leading in vivo to proteolytic degradation of WASP (25) and many forms of WAS (27). WIP binding also negatively regulates the N-WASP VCA (28,29).

The EVH1 domain is followed by a hydrophilic, low-complexity sequence, without an ascribed function for either WASP or N-WASP. Following this is a sequence of basic residues known as the basic (B) region (Figure 1). In both proteins the B region stabilizes autoinhibitory interactions (see below) (30–32). In N-WASP it is clear that this element also binds PIP₂ and mediates many PIP₂ effects on activity (31–34) (see below). The element probably plays a similar role in WASP, but additional regions appear necessary for the functional interaction (35). As elaborated below, the B region also plays an important role in selective binding of the WASP activator, Cdc42 (36).

After the B region, WASP and N-WASP contain an ~85 residue element termed the GTPase binding domain (GBD, Figure 1). The first 20 residues of this element contain a Cdc42-Rac Interactive Binding (CRIB) motif (37), which mediates nucleotide-dependent binding of numerous effectors to the Cdc42 and Rac GTPases. The GBD in WASP and N-WASP binds the VCA intramolecularly and plays a central role in controlling activity toward Arp2/3 complex (38,39).

Between the GBD and VCA, WASP and N-WASP contain a 100–125 residue proline rich domain (PRD, Figure 1). The PRD contains approximately six canonical binding sites for SH3 domains, and three profilin binding sites (40). This segment mediates interactions with SH3 domains of a large and diverse range of ligands. As WIP also contains a PRD, WASP:WIP complexes will have even more SH3 binding sites.

The five functional elements of WASP allow binding to multiple ligands simultaneously. A central concept developed in the following sections is that the ligands can act through two distinct regulatory mechanisms to control the biochemical activity of WASP toward Arp2/3 complex: allostery, which controls accessibility of the VCA to Arp2/3 complex, and oligomerization, which controls how strongly the exposed VCA can stimulate Arp2/3 complex. When multiple ligands engage one or both of these mechanisms simultaneously, they act cooperatively to control WASP activity, thus integrating multiple signals.

Allosteric Regulation of WASP and N-WASP

The idea of autoinhibition in WASP, mediated by intramolecular binding of the GBD to VCA, has guided thinking in the field since its discovery in 1998 (38). A large body of work has revealed structural and thermodynamic mechanisms of autoinhibition and its relief by upstream signals. We discuss these data in the context of single activators here, and show how autoinhibition contributes to integrative behavior below.

The WASP Autoinhibitory Mechanism

Physical studies have shown that isolated GBD and VCA peptides are largely unfolded in solution (39,41). Upon binding, the C region of the VCA and most of the GBD, except for the CRIB motif fold together to make a small domain (39) (Figures 2c,d). From a functional standpoint, the structure can be considered in three layers. The first, consists of a short β -hairpin and an α -helix ($\alpha 1$). This layer represents the minimal high affinity Cdc42 binding element. The second layer is formed by three additional helices organized into a planar C shape. Layer three consists of the C helix region of the VCA. This element lies behind layer two with its hydrophobic face contacting predominantly helices $\alpha 2$ – $\alpha 4$, and also the C-terminus of helix $\alpha 1$ through the open end of the layer two “C”. A number of C region sidechains that are buried in the layer 2-layer 3 interface (Figures 2c,d) also contribute substantially to activity and affinity of the VCA for Arp2/3 complex (10,13). Thus, occlusion of these residues in the GBD:VCA complex is an important mechanistic component of autoinhibition in WASP and N-WASP. Mutations of the GBD, including L270P, I294T and S272P disrupt autoinhibition and cause the immunodeficiency disease, X-linked neutropenia (42,43) (Figure 1).

A variety of data suggest that additional elements in WASP contribute to binding of the GBD to the VCA, and consequently to autoinhibition. Partial deletion of the A region increases N-WASP activity in vitro (12), and complete removal (including several residues typically classified as being in the C region) greatly decreases the affinity of GBD-containing constructs for the VCA in both WASP and N-WASP (31,39). Similarly, partial truncation of the B region in WASP and N-WASP decreased autoinhibition (30–32), concomitant with decreased unfolding stability of BGBD-VCA WASP (30). The potential for favorable electrostatic contacts between the A and B regions, has led to suggestions that these elements may interact to stabilize BGBD-VCA. Finally, the EVH1 domain can bind weakly to the VCA, and GBD constructs containing the EVH1 domain bind the VCA more strongly than those that do not (31). Truncation of the EVH1 has also been reported to cause a small increase in the basal activity of N-WASP (31), although this has not been universally observed (12). The structural basis for the interaction of the EVH1 domain with the VCA and the contribution of the EVH1 domain to autoinhibition, remain unknown.

The thermodynamics of autoinhibition in WASP have been analyzed through a quantitative two-state allosteric model based on the classical Monod-Wyman-Changeux (MWC) formalism (30,44,45) (Figure 3a) In this model, WASP exists in equilibrium between an inhibited state where the GBD is bound to the VCA, and an active state where the elements are dissociated. Since the GBD and VCA are both unfolded in isolation, the allosteric equilibrium can be described as the folding equilibrium. Thus, for any given WASP protein, the free energy of the allosteric transition is equal to the stability of the GBD-VCA domain against unfolding. As the latter can be directly measured by standard denaturation assays, the model provides a ready means of quantifying the allosteric equilibrium in WASP. This model quantitatively predicted the activity of many WASP proteins based simply on their free energies of unfolding (30). These proteins contained various linkers between GBD and VCA, different portions of the B region, and different destabilizing mutations in the GBD. As described below, this thermodynamic view of WASP as existing in equilibrium between a folded, inhibited state and an unfolded, active state is also effective means to understand activation by effectors.

The combined structural, biochemical and biophysical data have led to a straightforward mechanism of autoinhibition in WASP. The structure of the GBD-C construct shows that residues of the C region needed for activation of and affinity toward Arp2/3 complex are buried in the interface (10,13,39). Biochemical data show that BGBD fragments compete with Arp2/3 complex for VCA binding, in *cis* (30) and *trans* (31,35). Finally, the activity of

a large range of GBD-VCA and BGBD-VCA proteins toward Arp2/3 complex correlates quantitatively with the allosteric equilibrium of the free proteins (30). Thus, autoinhibition is due to sequestration of the C region by the GBD. Additional functional regions outside of the GBD enhance autoinhibition by further stabilizing the GBD-C fold (e.g. interactions between the B and A regions in BGBD-VCA proteins).

A distinct inhibitory mechanism has been proposed in which the closed protein (BGBD bound to VCA) binds to and actively inhibits Arp2/3 complex through contacts of the B and A regions (32). It is difficult to reconcile this second model with the data described above and we favor the first model for its consistency with the more extensive and quantitative body of evidence. Importantly, both models lead to essentially identical thermodynamic descriptions of the system, since both invoke inhibited states that are stabilized by multiple interactions, and an active state involving the free VCA. Thus, they lead to nearly identical descriptions of signal integration, and we will not distinguish them in our discussions of this issue below.

Mechanism of Allosteric Activation and Inhibition

Physical studies have revealed diverse structural mechanisms by which the allosteric equilibrium in WASP/N-WASP can be controlled by ligands. The unfolded nature of the GBD in active WASP plays an important role in enabling this diversity by allowing a wide range of conformations to be recognized. This property also affords novel means of WASP inhibition through conformational stabilization. These ideas can be incorporated into thermodynamic descriptions of WASP regulation.

Structural basis of Cdc42 activation—Cdc42 is the best characterized activator of WASP and N-WASP. GTP-bound Cdc42 binds the WASP GBD, destabilizing its interactions with the VCA, leading to activation.

In the solution structure of the Cdc42:GBD complex (Figure 2f), the CRIB motif extends in linear fashion along the β 2 strand and contiguous Switch I region of the GTPase, forming an irregular β -strand (41). This interaction is very similar to that seen in complexes of Cdc42 with the CRIB domains from PAK1 (46, 47), PAK6 (unpublished, RCSB accession number 2odb) and PAR6 (48), and more distantly related to the complex with the longer CRIB motif of ACK (49). Contacts to switch I enable CRIB effectors to distinguish the GTP and GDP nucleotide states of Cdc42 and Rac (41, 49). Hydrogen bonds between the sidechains of the conserved HXXH element in the CRIB motif with the sidechain of D38 in Cdc42 (and Rac) appear to select against Rho which contains a Glu at this position (41). WASP sequences between the CRIB motif and B region contribute to selectivity against Rac (50). Contacts of the B region to the β 2– β 3 turn and α 5 also make significant contributions to the interactions between WASP and Cdc42 (36). Neutralizing mutations to the B region of WASP can decrease the association rate and affinity for Cdc42 by over two orders of magnitude. This electrostatic steering effect also contributes to the specificity of WASP for Cdc42 over the closely related GTPase, TC10.

The CRIB motif is unstructured in the autoinhibited GBD-VCA protein (39). Thus, its interactions with Cdc42 do not drive WASP activation directly. In the Cdc42:WASP complex, WASP residues following the CRIB motif form a β -hairpin and α -helix, which make hydrophobic contacts to the switch I and switch II regions of Cdc42 (41). These interactions also likely contribute to nucleotide switch sensitivity. These secondary elements are nearly identical to those observed in the autoinhibited structure (compare Figures 2c and 2f). However, packing of these conserved elements against Cdc42 is incompatible with the autoinhibited domain. Extraction of the beta hairpin and α 1 from the autoinhibited domain by Cdc42 thus greatly destabilizes the domain, releasing the VCA. This structural model is

consistent with a 400-fold increase in hydrogen exchange rates in the core of the GBD upon binding to Cdc42 (45). A highly analogous regulatory mechanism is also found for Pak, where Cdc42 binding disrupts a similar autoinhibitory domain, leading to activation of the adjacent kinase domain (51).

Structural basis of activation by Src family SH2 domains—In response to various stimuli, the GBD of WASP/N-WASP becomes phosphorylated by Src-family kinases on a conserved tyrosine sidechain, Y291/Y256 (52–56). This modification is needed for processes including T cell activation (52), neuronal differentiation (53) and intracellular movement of the pathogen *Shigella flexneri* (54), and provides a mechanism for WASP activation by SH2 domains (57,58).

In the autoinhibited GBD-C structure, Y291 (on $\alpha 3$) is > 90% buried in the interface between layers one and two, with only the phenolic hydroxyl group exposed (Figure 2e). The sequence surrounding Y291 comprises a consensus binding motif for the SH2 domains of Src-family kinases (59). Structural studies have shown that Src family SH2 domains bind their ligands in extended conformation (60), which is incompatible with the helical conformation of $\alpha 3$ in the autoinhibited structure. Thus, SH2 domain binding destabilizes the $\alpha 3$ helix of phosphorylated WASP/N-WASP, disrupting the GBD-C domain, leading to release of VCA (57, 58).

Structural basis of activation by EspF_U—Enterohemorrhagic *E. coli* (EHEC) is a food-borne human pathogen that adheres to the surface of intestinal epithelial cells and injects a protein named EspF_U into the host cytoplasm (61,62). EspF_U binds and locally activates N-WASP, leading to formation of actin-rich “pedestals” beneath the bound bacterium (63).

EspF_U contains 2–6 nearly exact repeats of a 27-residue hydrophobic segment and a 20 residue proline rich segment (63,64). The hydrophobic segment binds the GBD WASP and N-WASP with nanomolar affinity, displacing the VCA (65,66). In the solution structure of a complex between WASP GBD bound to a single EspF_U repeat, the GBD adopts a conformation that is essentially identical to that observed in the autoinhibited GBD-VCA structure (Figure 2g) (65). The hydrophobic EspF_U segment forms an amphipathic helix which closely mimics the GBD layer 2 interactions with the VCA. Mutagenesis of the contact sites showed that sidechains of the helix contribute appreciably to affinity between the two molecules (65,66). EspF_U residues C-terminal to the helix form an extended arm which lies across the top of the GBD and contributes an additional $\sim 4 \text{ kcal} \cdot \text{mol}^{-1}$ to stability of complex based. EspF_U has thus developed a high-affinity ligand for WASP/N-WASP that acts by mimicking the VCA and competitively displacing it from its binding site on the GBD.

Structural basis of inhibition by wiskostatin—Chemical screens for small molecule inhibitors of actin assembly by the PIP₂/Cdc42/N-WASP/Arp2/3 system have yielded a cyclic peptide, termed 187-1 (67), and a modified carbazole compound, named wiskostatin (68), that both target N-WASP. Both compounds appear to act by stabilizing interactions between the GBD and VCA.

In the solution structure of complex between wiskostatin and WASP GBD-C the small molecule binds in a shallow hydrophobic pocket between layers 1 and 2, at the interface of the β -hairpin and $\alpha 3$ helix, with the carbazole ring directed inward and the amino alcohol sidechain directed toward solvent (Figure 2h) (68). Wiskostatin makes no direct contacts to the VCA or the GBD-VCA interface. However, NMR studies showed that wiskostatin can stabilize the autoinhibited conformation of the isolated GBD (68). Such stabilization of the

VCA-bound conformation should increase affinity of the GBD for the VCA, accounting for increased autoinhibition.

One important caveat regarding wiskostatin is that in both published (69) and unpublished (C. Wülfing, personal communication) accounts, the effects of the compound in cells do not seem to be specific to inhibition of WASP/N-WASP. Thus, wiskostatin may not be a useful reagent to probe the function of these proteins in cells. Nevertheless, its discovery and effects *in vitro* demonstrate the basic principle that allosteric equilibria can be targeted by small molecules for inhibitory effect (70).

Thermodynamics of activation and inhibition—In MWC formalism, activity of an allosteric protein in the presence of a given concentration of regulatory ligand can be described by three parameters: the allosteric equilibrium constant in the free protein (L) and the affinity of the ligand for the inhibited and active states of the protein (44). The ratio of these affinities ($K_{D,active}/K_{D,inhibited}$), defined as C , provides the driving force to change the allosteric equilibrium (Figure 3b). Activators have higher affinity for the active state ($C < 1$) and inhibitors have higher affinity for the inactive state ($C > 1$). As described above, in the allosteric model for WASP, L is equal to the folding stability of the GBD-VCA domain and can be measured directly (45). For WASP activation, C can also be measured directly, based on the affinity of Cdc42:GTP for the GBD (as a mimic of the active state) and GBD-C (as a hyper-stabilized mimic of the fully inhibited state) (30,45). These measurements have provided $C \sim 2.5 \times 10^{-3}$, indicating that at saturation Cdc42:GTP will shift the allosteric equilibrium by $1/C \sim 400$ -fold. Using this parameter, the model was able to quantitatively predict the effect of Cdc42 on hydrogen exchange rates in cores of GBD-C and GBD-VCA, the affinities of Cdc42 for WASP proteins with a large range of stabilities, and the effect of Cdc42 on the activity of WASP toward Arp2/3 complex (30,45).

One surprising finding of this work was that the nucleotide state of Cdc42 controls not only affinity for WASP, as had been previously shown for the GBD, but also the degree to which the GTPase can drive the allosteric equilibrium (30). That is, Cdc42:GTP not only has a higher affinity for WASP than does Cdc42:GDP, but also a 5-fold smaller value of C . Thus for a given degree of saturation, Cdc42:GTP causes a larger shift in the allosteric equilibrium of WASP upon binding than does Cdc42:GDP. This effect was postulated to increase the fidelity of WASP signaling to the nucleotide switch in Cdc42.

Activation by Oligomerization

Autoinhibition and its relief by upstream signals can explain many aspects of WASP/N-WASP function. However, several observations could not be explained by this mechanism. This conundrum was resolved through discovery of an additional regulatory mechanism in which dimerization of active WASP proteins greatly increases their potency toward Arp2/3 complex (22). Below we explain this effect and show how it clarifies the actions of several WASP activators.

The VCA dimerization effect

In 2000 Higgs and Pollard reported that GST-VCA dimers are roughly 100-fold more potent activators of Arp2/3 complex than VCA monomers (35). This observation, which suggested a potentially important aspect of regulation independent of autoinhibitory control, was recently pursued systematically (22). These efforts led to the realization that enhanced potency results generally from VCA dimerization. Thus, several VCAs dimerized through GST, covalently or using the FKBP:mTOR:rapamycin system, gave similarly high activity. Surprisingly, light scattering and sedimentation velocity ultracentrifugation experiments showed that a single Arp2/3 complex binds to a VCA dimer, suggesting the presence of a

previously unrecognized VCA binding site on the assembly. Competition experiments suggested that this site is the same as that occupied by the distinct class of Arp2/3 activators, the cortactin proteins, which are known to bind the complex simultaneously with VCA monomer. Importantly, fluorescence binding data showed that VCA dimers bind Arp2/3 complex with ~100-fold higher affinity than do monomers. Higher affinity provides a potential explanation for the enhanced actin polymerization kinetics produced by VCA dimers, and current research in several labs aims to understand this effect in detail.

The increased affinity of VCA dimers for Arp2/3 complex makes several predictions regarding the biochemical behavior of dimerizing WASP ligands (22). First, at low WASP concentrations, ligands that create WASP dimers can produce higher Arp2/3 activity than can a VCA monomer. Such ligand-mediated stimulation of WASP beyond the level of the free VCA is termed “hyperactivation.” Next, at high concentrations a dimerizing ligand competes for its own binding site and breaks up the WASP dimer, reducing observed activity. Thus, titrations of WASP with dimerizing ligands do not produce a monotonic increase in Arp2/3 activity, but rather show a peaked response as WASP₂ species are populated and then decay. Finally, monovalent ligands can compete with their divalent counterparts for a binding site on WASP. Thus, addition of a monovalent ligand to a hyperactivated system will disrupt WASP dimers and return the activity to that of a monomeric VCA or less, a process termed “back-titration.” These effects have been explicitly searched for in three disparate systems, EspF_U, SH3 ligands, and membrane phosphoinositides.

Oligomerization by EspF_U

The strongest evidence that dimerization plays a role in WASP/N-WASP regulation derives from studies of EspF_U and its function during pathogenesis of EHEC. EspF_U contains multiple repeat elements, each of which is capable of driving allosteric activation (65). However, constructs with two repeats (2R) or more are better activators of both WASP and N-WASP (22,66,71,72). Consistent with the dimerization model, 2R produces a peaked activity profile when added to N-WASP in Arp2/3-mediated actin assembly assays, with strong hyperactivation at the maximum (22). 1R can block hyperactivation by 2R, resulting in back-titration behavior. 2R:(N-WASP)₂ and 2R:(N-WASP)₂:(Arp2/3) complexes have been directly observed by analytical ultracentrifugation and gel filtration chromatography, respectively (66,72). Finally, increasing EspF_U repeat number correlates with increased actin assembly in cells (22,66,71,72). These data indicate that EspF_U activates WASP/N-WASP by engaging both the allosteric and dimerization mechanisms.

Oligomerization by SH3 ligands

WASP family proteins bind dozens of SH3-domain containing ligands through the numerous proline motifs in the PRD, and in that of the constitutively associated WIP (23). These ligands can largely be grouped into three categories as recently detailed (6). Most contain multiple SH3 domains, either by the nature of their primary sequence or through homo-oligomerization. In the first category are the multi-SH3 signaling proteins, including Nck and Grb2. The second category, oligomerized SH3 ligands, includes many proteins that contain the dimeric BAR domain, such as PACSIN, Toca proteins, and SNX9. A final major category of WASP family SH3 ligands are the non-receptor tyrosine kinases, which typically contain only a single SH3 domain. Activation of WASP proteins has been studied for about half of the known SH3 ligands. Of these, Nck, Grb2, WISH, Abi, Cortactin, Abp1 and a number of BAR proteins have been shown to directly enhance WASP activity in vitro (22,29,73–84).

Members of the dimeric BAR domain family coordinate membrane dynamics and actin assembly in a variety of processes (6,85). They act by simultaneously binding membrane through the BAR domain and WASP proteins through an adjacent SH3 domain, potentially dimerizing the WASP proteins. Several BAR proteins directly activate WASP proteins: FBP17 (29,83), SNX9 (84), PACSIN2/syndapin (22), EndophilinA (86) and Nwk (79). When examined with an eye toward dimerization, this activation shows the hallmarks of the dimerization mechanism. PACSIN2 and CIP4 can hyperactivate an N-WASP mutant in which autoinhibition is disrupted (22, and S.B. Padrick, unpublished). In addition, hyperactivation by PACSIN is lost at high concentrations or when the free PACSIN SH3 domain is added, fulfilling the peaked titration and back-titration predictions of the dimerization mechanism, respectively. Analogous behavior is seen for GST-Nck (22), suggesting that dimerization-mediated N-WASP activation is likely to be widespread among SH3 proteins. Interestingly, cortactin, which also appears to have two binding sites on Arp2/3 complex (S.B. Padrick, unpublished), shows similar peaked activation through SH3-proline interactions by GST-Fgd1 (87).

Activation by high density PIP₂

N-WASP is both allosterically activated and oligomerized by the phosphoinositide PIP₂ (31,32). The N-WASP basic region is a disordered cluster of arginine and lysine residues that likely engages multiple headgroups simultaneously. Thus, the affinity of N-WASP for liposomes depends on their fractional PIP₂ content (i.e. the PIP₂ surface density), as shown in a detailed analysis (34). However, in that study while strong activation was observed at high PIP₂ density, intermediate densities that were still capable of binding N-WASP did not activate well (34), suggesting that density itself is a parameter of activation. Interpreted in light of the dimerization model, at high density, VCA domains from two different N-WASP molecules could have access to the same Arp2/3 complex. This will result in high level activation through effective dimerization.

In more recent work, high density PIP₂ liposomes were found to hyperactivate an N-WASP mutant lacking GBD-VCA contacts (22). Activation declined at the highest liposome concentrations. Similar effects were observed previously in PIP₂-mediated activation of WASP purified from tissue (35). This behavior is similar to the peaked titrations seen with dimerizing ligands, where N-WASP dimers were disrupted by excess ligand. Here, effective N-WASP dimers were disrupted by increased vesicle surface area and consequent decreased N-WASP density. Hyperactivation, but not vesicle binding of N-WASP, was lost when PIP₂ density on individual vesicles was decreased, holding total PIP₂ in solution constant (22). This is analogous to the back titration phenomenon with dimeric ligands. Thus, activation of WASP proteins by phosphoinositides, and likely membrane recruitment in general, shows the hallmarks of dimerization-mediated activation. We note that a similar effect was also suggested in recent studies of actin assembly by the intracellular pathogen, *Listeria monocytogenes* (88). During infection, *Listeria* displays its own Arp2/3 activator, the ActA protein, on its surface to usurp the actin cytoskeleton. ActA is arrayed at a density equivalent to a GST-mediated dimer, suggesting that the bacterium uses the density-mediated dimerization effect to more potently activate Arp2/3 complex.

Integration of Multiple Signals by WASP Proteins

The combination of allostery and oligomerization leads to a hierarchical mechanism of WASP regulation (Figure 4) (22). Within this mechanism, allostery controls availability of the VCA to Arp2/3 complex and dimerization controls affinity for Arp2/3 complex. Maximal activation of the WASP-Arp2/3 pathway will occur when both branches are simultaneously engaged. We note that the two branches are mechanistically distinct, but thermodynamically coupled, such that binding of Arp2/3 complex to a VCA dimer will also

shift the allosteric equilibrium toward the active state. Here we show how cooperativity between multiple inputs arises in each branch, and how the branches function cooperatively together. These two layers of regulation thus allow WASP proteins to act as powerful signal integration devices in the cell.

Allosteric activation of WASP by two ligands

The autoinhibited state of WASP is stabilized by multiple inhibitory interactions (e. g. GBD:C and B:A contacts (see above)) that act together to repress the activity of the VCA. Activators such as Cdc42, PIP₂ and SH2 domains target distinct sequences in the autoinhibited fold. However, since the ligands all act on the same allosteric equilibrium, their binding and effects on that equilibrium are thermodynamically coupled. Thus, two ligands acting together will provide greater engagement and activation of WASP than either acting alone.

Two related formalisms have been developed to describe this underlying concept quantitatively. The first posits that WASP acts in binary fashion, being completely inactive in free form, and completely active when bound to any single ligand (Figures 3c,d) (32). Since activating ligands bind more tightly to the active form, when one ligand activates WASP, the second can bind with higher affinity. The degree of thermodynamic coupling between the two ligands can be expressed as a ‘cooperativity factor’ by which the affinity of a second activator is enhanced by a first (Figure 3d) (32). As described below, this formalism nicely explains cooperative binding to and synergistic activation of WASP/N-WASP by the Cdc42-PIP₂ and Cdc42-SH2 activator pairs (32, 58).

The second formalism extends the first by including the allosteric equilibrium in WASP quantitatively (30,45), giving a multi-ligand version of the MWC allosteric model described in previous sections (Figure 3c). In this model, the autoinhibitory equilibrium in the presence of multiple saturating ligands is given by L/C_i , where L is the equilibrium constant in free WASP ([inactive]/[active]), and C_i is the ratio of affinity for the active and inactive states of WASP for each ligand, respectively. Thus, the cooperative effects of multiple ligands are reflected in their multiplicative action on the regulatory equilibrium in WASP. This model allows inhibitory ligands such as WIP to be accounted for, in addition to activators; activation of a WASP:WIP complex (where $C_{WIP} > 1$, favoring the inhibited state of WASP) can be described as requiring an activator with a strong binding preference for the active state (smaller $C < 1$) or cooperation between multiple activators (each with their own $C < 1$). In general, this model explains how the balance of L and C parameters tunes the system to respond to one or more activators.

Synergy between Cdc42 and PIP₂—The minimal autoinhibited GBD-VCA WASP construct is further stabilized by additional inhibitory contacts such as those of the B region. While B region inhibition may derive from B-A contacts or from B-Arp2/3-A contacts (see above), the effects on binding and activation are the same. Activation by Cdc42 is understood structurally (39). PIP₂ vesicles engage the allosteric mechanism by interacting with the B region (31) and thus destabilizing its contacts with A or Arp2/3 complex, and also oligomerizing mechanisms (see above). Individually, Cdc42 and PIP₂ can each activate weakly inhibited systems (e.g. the trans complex of BGBD + VCA). But combinations of the two are required to activate more strongly inhibited constructs (32,35,89). Importantly, Cdc42 and PIP₂ activate WASP and N-WASP by binding to distinct regions of the BGBD element (Figure 1). Thus, their binding is coupled through the autoinhibitory equilibrium. Systematic measurement of N-WASP activation by the two ligands found a binding cooperativity factor of 350 (32) (Figure 3d). In the MWC formalism, the B region shifts the autoinhibitory equilibrium toward the inhibited state such that $L \gg 1$ (30). Thus $C_{Cdc42} * L$

remains greater than one, and the GTPase alone cannot strongly activate. PIP₂ has a similarly small effect. However, the combination of the two together can activate (29,32) (at saturation $C_{Cdc42}C_{PIP_2}L < 1$). While this analysis only considers the allosteric part of the interaction, it explains the requirement for multiple allosteric effectors in activation of the endogenous WASP:WIP complex, which has additional autoinhibitory contacts.

Synergy between Cdc42 and WASP phosphorylation—Phosphorylation of WASP/N-WASP on the conserved Y291/Y256 (see above) exemplifies signal integration through the allosteric equilibrium in several respects. Tyrosine 291 is >90% buried by layer 2-layer 3 contacts in the autoinhibited GBD-C structure, and thus is a poor substrate for kinases and phosphatases (Figure 2e) (57). Destabilization of the GBD-C domain by Cdc42 accelerates phosphorylation and dephosphorylation by 40-fold and 30-fold, respectively (58). The fact that Y291/pY291 are only accessible in the open state couples allosteric effectors to covalent modifications. This results in WASP having a covalently encoded memory of recent activation.

Phosphorylation of Y291/Y256 has two biochemical effects (57). First, phosphorylated GBD-VCA is destabilized, enhancing the basal activity of WASP (i.e. decreasing L in the MWC formalism). Second, phosphorylation creates a binding site for SH2 domains, which serve as allosteric activators of WASP (discussed above). Since SH2 and Cdc42 bind different regions of the GBD (Figure 1) they can bind simultaneously and thus act cooperatively on the allosteric equilibrium. A cooperativity factor of 50 was measured for the two ligands acting on phosphorylated WASP (58). Thus, the location of Y291/256 in the autoinhibited fold enables WASP/N-WASP to integrate signals in both the phosphorylation event itself, and in the functional consequences of phosphorylation.

Engineering WASP—WASP has proven to be an excellent platform for illustrating the principles of allostery and its evolution through protein design (34,90,91). By appending additional interaction modules to the GBD-VCA core, Lim and colleagues showed that a wide variety of switch-like behaviors can be readily created (91). These include “OR gates”, where only a single ligand can activate, “AND gates,” where multiple ligands are required for activation, and even antagonistic functionality, where multiple ligands oppose each other’s actions, depending on the balance between autoinhibitory stability and cooperativity in effector binding. When multiple identical inhibitory modules are appended, ultrasensitivity akin to that observed in hemoglobin can be created (90). These data suggest that evolutionary shuffling of modular elements in signaling proteins provides a mechanism to readily create allosteric systems with complex behaviors.

Integration of multiple oligomerizing signals

When multiple dimeric/oligomeric ligands contact distinct regions of WASP simultaneously, their binding will occur cooperatively. That is, when one ligand dimerizes WASP, a second will bind tightly due to an avidity effect. Thus, ligands acting in combination will produce WASP oligomers at lower concentrations than any individual ligand acting alone. This will provide an additional mechanism of integrating signals to enhance WASP activity that is distinct from the allosteric mechanisms discussed above.

SH3 interactions are a likely source of such cooperativity, but similar principles will hold for any combination of oligomerizing factors. WASP proteins and WASP:WIP complexes (23) have numerous proline motifs that bind SH3 domains. The specificity of an SH3 dimer for the different motifs, will determine its propensity to engage a single WASP molecule in bivalent fashion or to dimerize two WASP molecules. Only the latter binding mode will strongly affect WASP activity (see above). Thus, the activation strength of a given SH3

dimer will be dictated significantly by its binding specificity. An analogous but more complex argument can be made for SH3 ligands with multiple, different SH3 domains (e.g. Nck, which has three SH3 domains). This reasoning is consistent with the wide variability in the effects of SH3 dimers on WASP activity (e.g. (74)). SH3 specificity will also affect cooperativity between different ligands. For example, two dimeric SH3 ligands, each highly specific for a single proline motif in WASP, will strongly reinforce each other's binding and produce an avidity enhanced multi-WASP complex. But ligands with overlapping specificities will compete for the same proline motifs, and will not act cooperatively. SH3 ligand specificity for the different proline motifs in the mammalian WASP proteins has not been extensively explored (although see (92) for a recent example). However, such an analysis was done for the proline motifs in the *Saccharomyces cerevisiae* WASP protein, Las17p/Bee1 (93). There, the five proline motifs in the PRD were systematically mapped against all SH3 proteins in the yeast genome. Some SH3 domains interacted strongly with only a single motif (e.g. Bbc1, Sho1 and Myo3), while others (e.g. Ygr136, Rvs167 and Bzz1) bound multiple motifs. Three of the motifs bound only a single SH3 domain, while two bound many. Clearly there is potential for cooperative, neutral and competitive interactions in this system. This type of analysis will be instrumental in predicting the effects of SH3 proteins acting individually and in combinations to control WASP protein assembly and activity.

Localization of WASP proteins to membranes can similarly cooperate with dimerizing ligands. Oligomerization will allow WASP proteins to bind membranes in multi-valent fashion, increasing affinity. Thus, assembly and membrane binding of complexes will be synergistic. For example, association of N-WASP with PIP₂ vesicles is relatively weak (34). However, N-WASP dimers created by GST-Nck will have two basic regions, and thus a greater affinity for PIP₂ vesicles, consistent with strongly cooperative activation of N-WASP by GST-Nck and PIP₂ (80).

Combining allosteric activation and oligomerization

Few WASP activators are purely allosteric or purely oligomerizing factors. At high density, membrane associated Cdc42 will be both an allosteric activator and a clustering effector. This is consistent with its high activity relative to soluble Cdc42 (35). Likewise PIP₂ both destabilizes BGBD-VCA interactions (31) and can be an oligomerizer at high density (22,34,80). Thus, the actions of ligands functioning individually and in combination should be considered in terms of both aspects of regulation.

IQGAP1 is a well studied signaling protein that can activate N-WASP. IQGAP1 functions through allostery, as it can activate a BGBD:VCA complex assembled in trans (94). However, full-length IQGAP1 is oligomerized through an N-terminal domain, and its activation of N-WASP showed a peaked titration similar to that of PACSIN2 (22,95). Thus, IQGAP1 likely acts through both altering the allosteric equilibrium in N-WASP and creating high potency N-WASP oligomers.

The Toca proteins (Toca-1, CIP4, and FBP17) provide a more complex example (Figure 5a). Toca proteins have BAR, SH3 and Cdc42-binding HR1 domains (29, 96). When active Cdc42 and PIP₂ are both present in membranes, N-WASP, Toca, Cdc42 and PIP₂ will assemble a multiply reinforced complex. Toca will bind membranes through cooperative BAR-lipid and HR1-Cdc42 interactions. N-WASP can then simultaneously associate in high-affinity, tri-valent fashion with the Toca SH3 domain, PIP₂ and Cdc42, through its PRD, B region and GBD, respectively. This will provide high-level engagement of N-WASP by Cdc42, and consequently potent allosteric activation. Finally, as the Toca protein is a dimer, two N-WASP molecules can be brought together, to allow potent recruitment and activation of Arp2/3 complex. As these interactions all contribute thermodynamically to a

single assembly, the individual contacts will reinforce one another. Further, the multi-valency of N-WASP (PRD) and Toca (SH3) could also provide mechanisms of higher-order oligomerization, leading to spatial clustering of all components, sustaining transiently high local activation levels. As discussed below, such assemblies are abundant in WASP protein biology, and likely play an important role in governing the specificity and dynamics of actin regulatory signaling.

Higher Order Oligomerization Further Stimulates WASP Activation

Allostery and dimerization are the core elements of the hierarchical model of WASP regulation. However, a probabilistic argument suggests that higher-order oligomerization may enhance WASP activity further by increasing the likelihood that two active WASP molecules will be spatial proximal (22). This effect may explain the frequent appearance of WASP proteins in large assemblies *in vivo*. Below we explain two related versions of higher order oligomerization that lead to WASP activation, and illustrate how this effect could play a role in a number of signaling systems.

Clustering of EspF_U

Two-repeat fragments of EspF_U hyperactivate WASP by engaging both allostery and dimerization. However, EspF_U proteins with >2 repeats produce even greater activation (22,62,66,72). Statistical considerations provide a likely explanation for this effect. When the fractional saturation of each EspF_U repeat by WASP is small ($P \ll 1$), the fraction of EspF_U 2R molecules with two bound WASP molecules is equal to P^2 . However, as the number of repeats (n) in EspF_U increases, there are more ways that any two repeats can bind WASP. So the probability of creating a WASP dimer increases. For a fixed total repeat concentration, the fraction of complexes containing two or more WASP molecules scales with $(n-1)$ (22). Thus, higher order multimerization of WASP further enhances actin polymerization by EspF_U.

EspF_U and its relative EspF from enteropathogenic *E. coli* (EPEC) have mechanisms to undergo oligomerization *in vivo*. Each repeat of these proteins contains both a WASP-binding element and a proline-rich element. The latter has been shown to bind the SH3 domain of dimeric BAR proteins, EspF to Snx9 (97) and EspF_U to IRSp53 (92) and IRTKS (98). These interactions, which can provide additional clustering of EspF/EspF_U-bound WASP molecules (98) are necessary for bacterially-induced actin assembly in cells (92,97,98). Moreover, EspF and EspF_U are both recruited to the transmembrane protein Tir, which is itself clustered through extracellular interactions with the intimin protein expressed on the bacterial surface (63,92,98). Thus, actin is assembled by this system through N-WASP molecules that are clustered at multiple levels, consistent with the idea that creation of large WASP arrays will enhance activation of Arp2/3 complex.

Clustering by endogenous effectors

WASP proteins oligomerized by natural ligands can be viewed analogously (22). In a WASP cluster of size n , if the probability of any molecule being in the active state is P (dictated by the allosteric equilibrium), then the probability of any two molecules being simultaneously active is approximately ' $(n-1)*P^2$ ' (for low values of P). For example, a cluster containing ten WASP proteins is nine times more likely to have an active, VCA dimer than a cluster containing only two WASP proteins. Thus, oligomerization, or arraying WASP proteins in close proximity on a membrane, lowers the degree of WASP allosteric activation needed for high-level stimulation of Arp2/3 complex. Geometric considerations should limit this effect in very large clusters. But the long linkers connecting the VCA to

other elements, and the ability of some clusters to rearrange, suggest that it should remain significant out to large sizes (see below).

This effect is likely significant to the actions of BAR proteins, which bind membranes in close-packed, high density arrays, causing formation of tubular structures (83,99,100). Many BAR proteins contain SH3 domains that recruit WASP/N-WASP and Arp2/3 complex to these arrays, causing actin polymerization (85,101). Recent studies have shown a high degree of cooperativity between membrane binding of BAR proteins and actin polymerization through N-WASP-Arp2/3 complex (29,86). The surface of BAR-induced membrane tubules will be coated with SH3 domains at high density. These structures are known to recruit N-WASP (83,102). Given the geometry of the BAR domain array on membranes (100), and the spacing between VCA elements in known hyperactive N-WASP dimers, clusters of more than ten N-WASP molecules should be able to access a single Arp2/3 complex (22). Thus, the dimerization effect likely contributes to actin assembly by BAR domain arrays.

The idea that SH3 clusters will result in larger, better Arp2/3 activating WASP complexes, is consistent with in vitro biochemical data. Dimeric SNX9 was shown to activate N-WASP in solution, but SNX9 aggregates were more potent (84). Addition of PIP₂ lipids produced even more potent SNX9 dependent activation. Similarly, Toca-1 and FBP17 activated the N-WASP:WIP complex, but only in the context of membrane surfaces for the BAR domains to array upon (29) (discussed above). The less autoinhibited N-WASP (without WIP) was activated by FBP17 alone, but it too was enhanced by a phospholipid binding surface. Finally, Endophilin A showed a similar enhancement of N-WASP stimulatory activity in the presence of acidic lipids (86). Thus, activation of N-WASP by many BAR proteins appears to be generally enhanced by assembly onto liposomes.

A distinct assembly containing cortactin, Nck, WIP and N-WASP has been elegantly dissected biochemically (Figure 5c) (81). Cortactin is a weak activator of Arp2/3 complex that can also act as a molecular scaffold. Cortactin is phosphorylated on three tyrosine sites near its C-terminus upon cell stimulation, and these sites bind the Nck SH2 domain (81). The SH3 domains in the p-cortactin: Nck complex (one from cortactin and three from each Nck) can bind the proline motifs in WIP (103) and N-WASP (80) to form a large assembly. The high multivalency in this system (three pTyr on p-cortactin; numerous proline motifs in N-WASP and WIP; and three SH3 domains in Nck) suggests very cooperative assembly of the components, consistent with surface plasmon resonance data (81). Moreover, the size of the complex, although not measured in this work, is expected to be large. A key finding in this study was that phosphorylation of cortactin greatly increased the Arp2/3-mediated actin assembly of mixtures of cortactin, Nck and N-WASP. Thus, assembling Nck:N-WASP complexes on p-cortactin increases activity toward Arp2/3 complex. The further enhancement seen when WIP was added also, suggests an advantage to creating even higher-order assemblies. In the future it will be interesting to examine correlations between complex stoichiometry, size and activity in this system.

A conceptually related system controls the assembly of actin rich podocytes in the kidney (Figure 5d) (104, 105). These cellular structures require phosphorylation of multiple tyrosine residues on the intracellular tail of the cell surface receptor, nephrin (104, 105). Analogous to p-cortactin, these phosphotyrosines each recruit the SH2 domain of Nck, allowing activated nephrin to display an array of Nck SH3 domains. This results in actin assembly, likely through recruitment N-WASP:WIP complexes. Interestingly, strong actin assembly by this system in cells requires multiplicity, either in the number of pTyr sites on nephrin or the number of SH3 domains in Nck (104), consistent with the idea that larger clusters of N-WASP have higher activity and are functionally important. This effect is similar to the

antibody dependent clustering to Nck leading to N-WASP dependent actin polymerization (106).

Finally, there are additional multi-component complexes that probably contain multiple WASP proteins, but have not been characterized in detail. These include the WASP:WIP:Nck:SLAP:SLP76:VASP assembly formed in T cells upon T cell receptor ligation (24,107), and the Las17p:Vrp:Myo3:Myo5:Bzz1:Bbc1p:Hsp70p complex that can be isolated from budding yeast (108). Physical characterization of these complexes to understand their energetics, stoichiometry, structural organization and dynamics, and the relation of these properties to activity represents an exciting and challenging area for future investigation.

Other WASP Family Members

Regulation of WAVE

Shortly after the discovery of WASP and N-WASP the WAVE/Scar (WASP family Verprolin-homologous protein/Suppressor of cyclic AMP Receptor) proteins were identified in Dictyostelium (109) and vertebrates (15,109,110) (Figure 1). A variety of data indicate the WAVEs function downstream of the Rac GTPase to stimulate actin assembly through Arp2/3 complex (3,4). WAVE proteins contain a C-terminal VCA domain, but unlike WASP, isolated WAVEs are not autoinhibited (8). Instead, WAVEs are regulated by constitutive incorporation into a heteropentameric complex (Figure 5b, inset) consisting of WAVE, Abi (Abl interactor), HSPC300 (haematopoietic stem progenitor cell 300), Nap/Hem (Nck associated protein/hematopoietic protein) and Sra1/Pir121/CYFIP (specifically Rac associated protein/p53-inducible mRNA 121/cytoplasmic FMRP interacting protein), which we term the WAVE Regulatory Complex (WRC) (111). The complex appears to be organized as a trimer held together by contacts between HSPC300 and N-terminal elements of WAVE and Abi, and an Sra:Nap dimer (22,112–116). Contacts between Nap and Abi link the dimer and trimer (112). Homologs of WRC components all incorporate into similar complexes (111,112,116,117). Since its initial biochemical purification (111), genetic studies have shown that the WRC is conserved in plants, slime mold, flies, worms and vertebrates (3,4). As reviewed elsewhere, cell biological and genetic data have established the functional importance of the WRC in cellular processes including spreading, motility and cell-cell adhesion (3,4).

Recent studies have shown that while the molecular details are different, the basic principles of WAVE and WASP regulation are highly analogous. Although the basal activity of the WRC toward Arp2/3 complex has been controversial (111,115,118,119), recent, definitive studies have shown that both the human and fly complexes are strongly inhibited (114,120). Inhibition involves binding of the Sra:Nap dimer to a constitutively active trimer (114). This behavior suggests analogy to GBD-VCA contacts in WASP, although the molecular mechanism of inhibition awaits further studies. Highly purified inactive WRC can be activated in a nucleotide dependent fashion by Rac, which is known to bind Sra1 (121), similar to WASP activation by Cdc42 (111,114). Rac may function with other factors, as activation requires relatively high concentrations of the GTPase (5–10 μ M). For example, PIP₃ binds a conserved basic region near the center of WAVE (122). This interaction is needed for proper WAVE localization, and also contributes to in vitro activation by Rac, although the mechanism of this effect is not known (122,123). WAVE is phosphorylated at numerous tyrosine, serine and threonine sites in its N-terminal, PRD and VCA elements (reviewed in (3)). VCA phosphorylation increases affinity for Arp2/3 complex, but paradoxically decreases activity (124,125). WAVE2 can be phosphorylated on the conserved Tyr150 by Abl upon cell stimulation, but effects on WRC activity have not been examined (113). It is not yet known whether any of the phosphorylation events occur

cooperatively with Rac binding, as in the Cdc42-WASP system (57). Finally, the large PRD in WAVE, and an analogous region in Abi endow the WRC with numerous SH3 binding sites. Many SH3 proteins bind these elements, including multi-SH3 adaptors (e.g. Nck (111) and Grb2 (126)) and dimeric BAR proteins (e.g. IRSp53 (123), Wrp (127) and Tuba (128)). Thus, the WRC appears to be regulated by the same hierarchical process as WASP, with collections of allosteric and dimerizing/oligomerizing ligands acting in cooperative fashion to control activity toward Arp2/3 complex. Both WASP and WAVE have similar responses to the same classes of signals, suggesting that there is an evolutionary driving force for WASP proteins to stimulate actin assembly in response to phosphoinositide, SH3 and Rho GTPase cues.

Recent reports illustrate how many of these interactions could work together to cluster and activate the WRC at membranes (Figure 5b). Takenawa and colleagues have shown that the combination of Rac, IRSp53 and PIP₃ activates the WRC better than any of the individual effectors alone (123). This system is highly analogous to the activation of N-WASP by Cdc42, Toca-1 and PIP₂ (see above). In both cases the GTPase, BAR protein and phospholipid recognize different elements of the WRC/N-WASP, and likely can bind it simultaneously. When the ligands are present together at a membrane, they should act in multi-valent fashion to strongly recruit the WRC/N-WASP. The ligands become coupled through WRC/N-WASP, strengthening membrane association of the BAR protein, and causing clustering of all components. Clustering should be further enhanced by the dimeric nature of the BAR protein. In the case of the WRC, these cooperative interactions should effectively overcome the modest affinity of Rac, enabling the WRC to be engaged and allosterically activated to a high degree. Thus, through the combination of all three ligands, both the allosteric and oligomerization mechanisms can be engaged, leading to strong, localized activation of Arp2/3 complex.

Other family members

Several vertebrate WASP family members have been discovered only recently: WASH (129), WHAMM (130), and JMY (131) (Figure 1). All these proteins all have a C-terminal VCA domain, which in isolation can activate Arp2/3 complex, and an adjacent PRD that can bind SH3 domains. Their more N-terminal elements are distinct and their molecular mechanisms of regulation are not yet known. Current work shows that WASH is incorporated into a multiprotein complex of a size and complexity similar to the WRC (D. Jia, T. Gomez, D. Billadeau, unpublished), as is WHAMM (K. Campellone and M. Welch, personal communication). However, it is not known whether these assemblies are active toward Arp2/3 complex, and thus how functionally analogous they will be to the WRC. WASH activity has also been linked to the GTPase Rho (132), suggesting analogies to WASP and WAVE, although it is unclear if Rho acts directly or indirectly. Characterization, reconstitution and assay these complexes is still needed to understand their mechanisms of regulation, and their relationships to WASP and/or WAVE.

Actin Feedback

This review focuses on upstream signals that act on WASP proteins. However, there is increasing evidence that the downstream actin network also feeds back into the WAS proteins and their assemblies. Taunton and coworkers have shown that the V region of membrane associated N-WASP, after delivering actin to the Arp2/3 branch, can remain attached to the filament barbed end (17). This tethers the actin network to membranes, and may enhance membrane recruitment and/or clustering of WASP proteins. This idea is consistent with recent data showing Arp2/3 complex-dependent polarization of N-WASP on rocketing vesicles (133). Interactions with the actin network should further stabilize the multiprotein assemblies that recruit and activate WASP at membranes. Paradoxically, the

interaction of the VCA with Arp2/3 complex is also necessary for rapid exchange of N-WASP into and out of membrane complexes created by vaccinia virus (134). N-WASP exchange is necessary for efficient actin-based movement of the virus. Such negative feedback between actin-Arp2/3 complex and VCA may also account for the rapid membrane dissociation of the WRC observed in mobile waves of actin assembly during cell spreading and motility (135). As more complete biochemical descriptions of WASP activation emerge, the feedback from the actin network will need to be included to advance beyond bulk biochemical descriptions into descriptions of cellular processes.

Concluding Remarks

The hierarchical model provides a unifying regulatory framework for the WASP protein family. The allosteric arm quantitatively explains autoinhibition and its relief by individual or combined ligands that act on the folding equilibrium of the GBD-VCA element. The dimerization/oligomerization arm explains how formation of assemblies of WASP proteins by multivalent ligands and membrane association enhances stimulation of Arp2/3 complex. Together, the two arms explain how WASP proteins are endowed with the ability to integrate a large number of diverse signals through interactions of multiple domains to precisely control actin assembly in cells. The model also raises many new questions. How do two WASP molecules engage and activate Arp2/3 complex? What are the physical properties of the large, multi-valent assemblies that contain WASP proteins; how do these affect and respond through feedback to downstream actin dynamics? Can other more recently discovered WASP family members also be described by the hierarchical model, and if so, what signals control the two arms and allow them to work together? These questions and others that extend from them will drive research in this exciting area for years to come.

Summary Points List

1. WASP proteins are regulated by 2 processes, allostery and dimerization.
2. Allostery controls access of the VCA to Arp2/3 complex and is understood structurally and energetically.
3. Dimerization controls affinity of WASP protein for Arp2/3 complex, and explains aspects of WASP activation by a variety of ligands.
4. Allostery and dimerization work together to integrate multiple, simultaneous, diverse signaling inputs.
5. Probabilistic considerations suggest that higher-order assemblies will enhance WASP activity further and sensitize it toward allosteric activators.
6. The five-component WAVE assembly is regulated analogously to WASP.

Future Issues List

1. Where are the two VCA binding sites on Arp2/3 complex, what are the functions of each VCA, which elements of VCA are needed at each site? What is the kinetic pathway of nucleation?
2. What are the quantitative principles that underlie coupling between membrane binding, oligomerization and allosteric activation of WASP proteins?
3. What are the physical properties (stoichiometries, stabilities, dynamics) of multi-WASP assemblies, and how do these lead to enhanced actin assembly activity?

4. How does interaction with the actin network modulate the kinetic and thermodynamic properties of WASP assemblies?
5. How is the activity of the WAVE Regulatory Complex controlled structurally and energetically?
6. How are the recently discovered WASP family members WASH, WHAMM and JMY regulated? What are the components of their regulatory complexes, how do these control VCA activity and respond to signals? What signals control them?

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Literature Cited

1. Pollard TD. Regulation of actin filament assembly by Arp2/3 complex and formins. *Annu Rev Biophys Biomol Struct* 2007;36:451–477. [PubMed: 17477841]
2. Chhabra ES, Higgs HN. The many faces of actin: matching assembly factors with cellular structures. *Nat Cell Biol* 2007;9:1110–1121. [PubMed: 17909522]
3. Pollitt AY, Insall RH. WASP and SCAR/WAVE proteins: the drivers of actin assembly. *J Cell Sci* 2009;122:2575–2578. [PubMed: 19625501]
4. Kurisu S, Takenawa T. The WASP and WAVE family proteins. *Genome Biol* 2009;10:226. [PubMed: 19589182]
5. Stradal TE, Scita G. Protein complexes regulating Arp2/3-mediated actin assembly. *Curr Opin Cell Biol* 2006;18:4–10. [PubMed: 16343889]
6. Takenawa T, Suetsugu S. The WASP-WAVE protein network: connecting the membrane to the cytoskeleton. *Nat Rev Mol Cell Biol* 2007;8:37–48. [PubMed: 17183359]
7. Machesky LM, Insall RH. Scar1 and the related Wiskott-Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex. *Curr Biol* 1998;8:1347–1356. [PubMed: 9889097]
8. Machesky LM, Mullins RD, Higgs HN, Kaiser DA, Blanchoin L, et al. Scar, a WASp-related protein, activates nucleation of actin filaments by the Arp2/3 complex. *Proc Natl Acad Sci U S A* 1999;96:3739–3744. [PubMed: 10097107]
9. Hufner K, Higgs HN, Pollard TD, Jacobi C, Aeppelbacher M, Linder S. The verprolin-like central (vc) region of Wiskott-Aldrich syndrome protein induces Arp2/3 complex-dependent actin nucleation. *J Biol Chem* 2001;276:35761–35767. [PubMed: 11459849]
10. Marchand JB, Kaiser DA, Pollard TD, Higgs HN. Interaction of WASP/Scar proteins with actin and vertebrate Arp2/3 complex. *Nat Cell Biol* 2000;3:76–82. [PubMed: 11146629]
11. Zalevsky J, Lempert L, Kranitz H, Mullins RD. Different WASP family proteins stimulate different Arp2/3 complex-dependent actin-nucleating activities. *Curr Biol* 2001;11:1903–1913. [PubMed: 11747816]
12. Suetsugu S, Miki H, Takenawa T. 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* 2001;276:33175–33180. [PubMed: 11432863]
13. Panchal SC, Kaiser DA, Torres E, Pollard TD, Rosen MK. A conserved amphipathic helix in WASP/Scar proteins is essential for activation of Arp2/3 complex. *Nat Struct Biol* 2003;10:591–598. [PubMed: 12872157]

14. Miki H, Miura K, Takenawa T. N-WASP, a novel actin-depolymerizing protein, regulates the cortical cytoskeletal rearrangement in a PIP2-dependent manner downstream of tyrosine kinases. *Embo J* 1996;15:5326–5335. [PubMed: 8895577]
15. Miki H, Suetsugu S, Takenawa T. WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac. *EMBO J* 1998;17:6932–6941. [PubMed: 9843499]
16. Chereau D, Kerff F, Graceffa P, Grabarek Z, Langsetmo K, Dominguez R. Actin-bound structures of Wiskott-Aldrich syndrome protein (WASP)-homology domain 2 and the implications for filament assembly. *Proc Natl Acad Sci U S A* 2005;102:16644–16649. [PubMed: 16275905]
17. Co C, Wong DT, Gierke S, Chang V, Taunton J. Mechanism of actin network attachment to moving membranes: barbed end capture by N-WASP WH2 domains. *Cell* 2007;128:901–913. [PubMed: 17350575]
18. Boczkowska M, Rebowski G, Petoukhov MV, Hayes DB, Svergun DI, Dominguez R. X-ray scattering study of activated Arp2/3 complex with bound actin-WCA. *Structure* 2008;16:695–704. [PubMed: 18462674]
19. Hertzog M, van Heijenoort C, Didry D, Gaudier M, Coutant J, et al. The beta-thymosin/WH2 domain; structural basis for the switch from inhibition to promotion of actin assembly. *Cell* 2004;117:611–623. [PubMed: 15163409]
20. Qualmann B, Kessels MM. New players in actin polymerization--WH2-domain-containing actin nucleators. *Trends Cell Biol* 2009;19:276–285. [PubMed: 19406642]
21. Chesarone MA, Goode BL. Actin nucleation and elongation factors: mechanisms and interplay. *Curr Opin Cell Biol* 2009;21:28–37. [PubMed: 19168341]
22. Padrick SB, Cheng HC, Ismail AM, Panchal SC, Doolittle LK, et al. Hierarchical regulation of WASP/WAVE proteins. *Mol Cell* 2008;32:426–438. [PubMed: 18995840]
23. Ramesh N, Geha R. Recent advances in the biology of WASP and WIP. *Immunol Res* 2009;44:99–111. [PubMed: 19018480]
24. Sasahara Y, Rachid R, Byrne MJ, de la Fuente MA, Abraham RT, et al. Mechanism of recruitment of WASP to the immunological synapse and of its activation following TCR ligation. *Mol Cell* 2002;10:1269–1281. [PubMed: 12504004]
25. Konno A, Kirby M, Anderson SA, Schwartzberg PL, Candotti F. The expression of Wiskott-Aldrich syndrome protein (WASP) is dependent on WASP-interacting protein (WIP). *Int Immunol* 2007;19:185–192. [PubMed: 17205972]
26. Peterson FC, Deng Q, Zettl M, Prehoda KE, Lim WA, et al. Multiple WASP-interacting protein recognition motifs are required for a functional interaction with N-WASP. *J Biol Chem* 2007;282:8446–8453. [PubMed: 17229736]
27. Jin Y, Mazza C, Christie JR, Giliani S, Fiorini M, et al. Mutations of the Wiskott-Aldrich Syndrome Protein (WASP): hotspots, effect on transcription, and translation and phenotype/genotype correlation. *Blood* 2004;104:4010–4019. [PubMed: 15284122]
28. Martinez-Quiles N, Rohatgi R, Anton IM, Medina M, Saville SP, et al. WIP regulates N-WASP-mediated actin polymerization and filopodium formation. *Nat Cell Biol* 2001;3:484–491. [PubMed: 11331876]
29. Takano K, Toyooka K, Suetsugu S. EFC/F-BAR proteins and the N-WASP-WIP complex induce membrane curvature-dependent actin polymerization. *EMBO J* 2008;27:2817–2828. [PubMed: 18923421]
30. Leung DW, Rosen MK. The nucleotide switch in Cdc42 modulates coupling between the GTPase-binding and allosteric equilibria of Wiskott-Aldrich syndrome protein. *Proc Natl Acad Sci U S A* 2005;102:5685–5690. [PubMed: 15821030]
31. Rohatgi R, Ho HY, Kirschner MW. Mechanism of N-WASP activation by CDC42 and phosphatidylinositol 4, 5-bisphosphate. *J Cell Biol* 2000;150:1299–1310. [PubMed: 10995436]
32. Prehoda KE, Scott JA, Mullins RD, Lim WA. Integration of multiple signals through cooperative regulation of the N-WASP-Arp2/3 complex. *Science* 2000;290:801–806. [PubMed: 11052943]
33. Suetsugu S, Miki H, Yamaguchi H, Takenawa T. Requirement of the basic region of N-WASP/WAVE2 for actin-based motility. *Biochem Biophys Res Commun* 2001;282:739–744. [PubMed: 11401525]

34. Papayannopoulos V, Co C, Prehoda KE, Snapper S, Taunton J, Lim WA. A polybasic motif allows N-WASP to act as a sensor of PIP(2) density. *Mol Cell* 2005;17:181–191. [PubMed: 15664188]
35. Higgs HN, Pollard TD. Activation by Cdc42 and PIP(2) of Wiskott-Aldrich syndrome protein (WASP) stimulates actin nucleation by Arp2/3 complex. *J Cell Biol* 2000;150:1311–1320. [PubMed: 10995437]
36. Hemsath L, Dvorsky R, Fiegen D, Carlier MF, Ahmadian MR. An electrostatic steering mechanism of Cdc42 recognition by Wiskott-Aldrich syndrome proteins. *Mol Cell* 2005;20:313–324. [PubMed: 16246732]
37. Burbelo PD, Drechsel D, Hall A. A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. *J Biol Chem* 1995;270:29071–29074. [PubMed: 7493928]
38. Miki H, Sasaki T, Takai Y, Takenawa T. Induction of filopodium formation by a WASP-related actin-depolymerizing protein N-WASP. *Nature* 1998;391:93–96. [PubMed: 9422512]
39. Kim AS, Kakalis LT, Abdul-Manan N, Liu GA, Rosen MK. Autoinhibition and activation mechanisms of the Wiskott-Aldrich syndrome protein. *Nature* 2000;404:151–158. [PubMed: 10724160]
40. Li SS. Specificity and versatility of SH3 and other proline-recognition domains: structural basis and implications for cellular signal transduction. *Biochem J* 2005;390:641–653. [PubMed: 16134966]
41. Abdul-Manan N, Aghazadeh B, Liu GA, Majumdar A, Ouerfelli O, et al. Structure of Cdc42 in complex with the GTPase-binding domain of the 'Wiskott-Aldrich syndrome' protein. *Nature* 1999;399:379–383. [PubMed: 10360578]
42. Devriendt K, Kim AS, Mathijs G, Frints SG, Schwartz M, et al. Constitutively activating mutation in WASP causes X-linked severe congenital neutropenia. *Nat Genet* 2001;27:313–317. [PubMed: 11242115]
43. Ancliff PJ, Blundell MP, Cory GO, Calle Y, Worth A, et al. Two novel activating mutations in the Wiskott-Aldrich syndrome protein result in congenital neutropenia. *Blood* 2006;108:2182–2189. [PubMed: 16804117]
44. Monod J, Wyman J, Changeux JP. On the Nature of Allosteric Transitions: A Plausible Model. *J Mol Biol* 1965;12:88–118. [PubMed: 14343300]
45. Buck M, Xu W, Rosen MK. A two-state allosteric model for autoinhibition rationalizes WASP signal integration and targeting. *J Mol Biol* 2004;338:271–285. [PubMed: 15066431]
46. Morreale A, Venkatesan M, Mott HR, Owen D, Nietlispach D, et al. Structure of Cdc42 bound to the GTPase binding domain of PAK. *Nat Struct Biol* 2000;7:384–388. [PubMed: 10802735]
47. Gizachew D, Guo W, Chohan KK, Sutcliffe MJ, Oswald RE. Structure of the complex of Cdc42Hs with a peptide derived from P-21 activated kinase. *Biochemistry* 2000;39:3963–3971. [PubMed: 10747784]
48. Garrard SM, Capaldo CT, Gao L, Rosen MK, Macara IG, Tomchick DR. Structure of Cdc42 in a complex with the GTPase-binding domain of the cell polarity protein, Par6. *EMBO J* 2003;22:1125–1133. [PubMed: 12606577]
49. Mott HR, Owen D, Nietlispach D, Lowe PN, Manser E, et al. Structure of the small G protein Cdc42 bound to the GTPase-binding domain of ACK. *Nature* 1999;399:384–388. [PubMed: 10360579]
50. Owen D, Mott HR, Laue ED, Lowe PN. Residues in Cdc42 that specify binding to individual CRIB effector proteins. *Biochemistry* 2000;39:1243–1250. [PubMed: 10684602]
51. Lei M, Lu W, Meng W, Parrini MC, Eck MJ, et al. Structure of PAK1 in an autoinhibited conformation reveals a multistage activation switch. *Cell* 2000;102:387–397. [PubMed: 10975528]
52. Badour K, Zhang J, Shi F, Leng Y, Collins M, Siminovitch KA. Fyn and PTP-PEST-mediated regulation of Wiskott-Aldrich syndrome protein (WASP) tyrosine phosphorylation is required for coupling T cell antigen receptor engagement to WASP effector function and T cell activation. *J Exp Med* 2004;199:99–112. [PubMed: 14707117]

53. Suetsugu S, Hattori M, Miki H, Tezuka T, Yamamoto T, et al. Sustained activation of N-WASP through phosphorylation is essential for neurite extension. *Dev Cell* 2002;3:645–658. [PubMed: 12431372]
54. Burton EA, Oliver TN, Pendergast AM. Abl kinases regulate actin comet tail elongation via an N-WASP-dependent pathway. *Mol Cell Biol* 2005;25:8834–8843. [PubMed: 16199863]
55. Wu X, Suetsugu S, Cooper LA, Takenawa T, Guan JL. Focal adhesion kinase regulation of N-WASP subcellular localization and function. *J Biol Chem* 2004;279:9565–9576. [PubMed: 14676198]
56. Yokoyama N, Loughheed J, Miller WT. Phosphorylation of WASP by the Cdc42-associated kinase ACK1: dual hydroxyamino acid specificity in a tyrosine kinase. *J Biol Chem* 2005;280:42219–42226. [PubMed: 16257963]
57. Torres E, Rosen MK. Contingent phosphorylation/dephosphorylation provides a mechanism of molecular memory in WASP. *Mol Cell* 2003;11:1215–1227. [PubMed: 12769846]
58. Torres E, Rosen MK. Protein-tyrosine kinase and GTPase signals cooperate to phosphorylate and activate Wiskott-Aldrich syndrome protein (WASP)/neuronal WASP. *J Biol Chem* 2006;281:3513–3520. [PubMed: 16293614]
59. Yaffe MB, Leparo GG, Lai J, Obata T, Volinia S, Cantley LC. A motif-based profile scanning approach for genome-wide prediction of signaling pathways. *Nat Biotechnol* 2001;19:348–353. [PubMed: 11283593]
60. Waksman G, Shoelson SE, Pant N, Cowburn D, Kuriyan J. Binding of a high affinity phosphotyrosyl peptide to the Src SH2 domain: crystal structures of the complexed and peptide-free forms. *Cell* 1993;72:779–790. [PubMed: 7680960]
61. Campellone KG, Robbins D, Leong JM. EspFU is a translocated EHEC effector that interacts with Tir and N-WASP and promotes Nck-independent actin assembly. *Dev Cell* 2004;7:217–228. [PubMed: 15296718]
62. Garmendia J, Phillips AD, Carlier MF, Chong Y, Schuller S, et al. TccP is an enterohaemorrhagic *Escherichia coli* O157:H7 type III effector protein that couples Tir to the actin-cytoskeleton. *Cell Microbiol* 2004;6:1167–1183. [PubMed: 15527496]
63. Hayward RD, Leong JM, Koronakis V, Campellone KG. Exploiting pathogenic *Escherichia coli* to model transmembrane receptor signalling. *Nat Rev Microbiol* 2006;4:358–370. [PubMed: 16582930]
64. Garmendia J, Ren Z, Tennant S, Midolli Viera MA, Chong Y, et al. Distribution of tccP in clinical enterohemorrhagic and enteropathogenic *Escherichia coli* isolates. *J Clin Microbiol* 2005;43:5715–5720. [PubMed: 16272509]
65. Cheng HC, Skehan BM, Campellone KG, Leong JM, Rosen MK. Structural mechanism of WASP activation by the enterohaemorrhagic *E. coli* effector EspF(U). *Nature* 2008;454:1009–1013. [PubMed: 18650809]
66. Sallee NA, Rivera GM, Dueber JE, Vasilescu D, Mullins RD, et al. The pathogen protein EspF(U) hijacks actin polymerization using mimicry and multivalency. *Nature* 2008;454:1005–1008. [PubMed: 18650806]
67. Peterson JR, Lokey RS, Mitchison TJ, Kirschner MW. A chemical inhibitor of N-WASP reveals a new mechanism for targeting protein interactions. *Proc Natl Acad Sci U S A* 2001;98:10624–10629. [PubMed: 11553809]
68. Peterson JR, Bickford LC, Morgan D, Kim AS, Ouerfelli O, et al. Chemical inhibition of N-WASP by stabilization of a native autoinhibited conformation. *Nat Struct Mol Biol* 2004;11:747–755. [PubMed: 15235593]
69. Bompard G, Rabeharivelo G, Morin N. Inhibition of cytokinesis by wiskostatin does not rely on N-WASP/Arp2/3 complex pathway. *BMC Cell Biol* 2008;9:42. [PubMed: 18667055]
70. Hardy JA, Wells JA. Searching for new allosteric sites in enzymes. *Curr Opin Struct Biol* 2004;14:706–715. [PubMed: 15582395]
71. Garmendia J, Carlier MF, Egile C, Didry D, Frankel G. Characterization of TccP-mediated N-WASP activation during enterohaemorrhagic *Escherichia coli* infection. *Cell Microbiol* 2006;8:1444–1455. [PubMed: 16922863]

72. Campellone KG, Cheng HC, Robbins D, Siripala AD, McGhie EJ, et al. Repetitive N-WASP-binding elements of the enterohemorrhagic *Escherichia coli* effector EspF(U) synergistically activate actin assembly. *PLoS Pathog* 2008;4:e1000191.
73. Carlier MF, Nioche P, Broutin-L'Hermite I, Boujemaa R, Le Clainche C, et al. 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* 2000;275:21946–21952. [PubMed: 10781580]
74. Fukuoka M, Suetsugu S, Miki H, Fukami K, Endo T, Takenawa T. A novel neural Wiskott-Aldrich syndrome protein (N-WASP) binding protein, WISH, induces Arp2/3 complex activation independent of Cdc42. *J Cell Biol* 2001;152:471–482. [PubMed: 11157975]
75. Innocenti M, Gerboth S, Rottner K, Lai FP, Hertzog M, et al. Abi1 regulates the activity of N-WASP and WAVE in distinct actin-based processes. *Nat Cell Biol* 2005;7:969–976. [PubMed: 16155590]
76. Kowalski JR, Egile C, Gil S, Snapper SB, Li R, Thomas SM. Cortactin regulates cell migration through activation of N-WASP. *J Cell Sci* 2005;118:79–87. [PubMed: 15585574]
77. Martinez-Quiles N, Ho HY, Kirschner MW, Ramesh N, Geha RS. Erk/Src phosphorylation of cortactin acts as a switch on-switch off mechanism that controls its ability to activate N-WASP. *Mol Cell Biol* 2004;24:5269–5280. [PubMed: 15169891]
78. Pinyol R, Haeckel A, Ritter A, Qualmann B, Kessels MM. Regulation of N-WASP and the Arp2/3 complex by Abp1 controls neuronal morphology. *PLoS One* 2007;2:e400. [PubMed: 17476322]
79. Rodal AA, Motola-Barnes RN, Littleton JT. Nervous wreck and Cdc42 cooperate to regulate endocytic actin assembly during synaptic growth. *J Neurosci* 2008;28:8316–8325. [PubMed: 18701694]
80. Rohatgi R, Nollau P, Ho HY, Kirschner MW, Mayer BJ. Nck and phosphatidylinositol 4,5-bisphosphate synergistically activate actin polymerization through the N-WASP-Arp2/3 pathway. *J Biol Chem* 2001;276:26448–26452. [PubMed: 11340081]
81. Tehrani S, Tomasevic N, Weed S, Sakowicz R, Cooper JA. Src phosphorylation of cortactin enhances actin assembly. *Proc Natl Acad Sci U S A* 2007;104:11933–11938. [PubMed: 17606906]
82. Tomasevic N, Jia Z, Russell A, Fujii T, Hartman JJ, et al. Differential regulation of WASP and N-WASP by Cdc42, Rac1, Nck, and PI(4,5)P2. *Biochemistry* 2007;46:3494–3502. [PubMed: 17302440]
83. Tsujita K, Suetsugu S, Sasaki N, Furutani M, Oikawa T, Takenawa T. Coordination between the actin cytoskeleton and membrane deformation by a novel membrane tubulation domain of PCH proteins is involved in endocytosis. *J Cell Biol* 2006;172:269–279. [PubMed: 16418535]
84. Yarar D, Waterman-Storer CM, Schmid SL. SNX9 couples actin assembly to phosphoinositide signals and is required for membrane remodeling during endocytosis. *Dev Cell* 2007;13:43–56. [PubMed: 17609109]
85. Frost A, Unger VM, De Camilli P. The BAR domain superfamily: membrane-molding macromolecules. *Cell* 2009;137:191–196. [PubMed: 19379681]
86. Otsuki M, Itoh T, Takenawa T. Neural Wiskott-Aldrich syndrome protein is recruited to rafts and associates with endophilin A in response to epidermal growth factor. *J Biol Chem* 2003;278:6461–6469. [PubMed: 12477732]
87. Kim K, Hou P, Gorski JL, Cooper JA. Effect of Fgd1 on cortactin in Arp2/3 complex-mediated actin assembly. *Biochemistry* 2004;43:2422–2427. [PubMed: 14992579]
88. Footer MJ, Lyo JK, Theriot JA. Close packing of *Listeria monocytogenes* ActA, a natively unfolded protein, enhances F-actin assembly without dimerization. *J Biol Chem* 2008;283:23852–23862. [PubMed: 18577520]
89. Rohatgi R, Ma L, Miki H, Lopez M, Kirchhausen T, et al. The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. *Cell* 1999;97:221–231. [PubMed: 10219243]
90. Dueber JE, Mirsky EA, Lim WA. Engineering synthetic signaling proteins with ultrasensitive input/output control. *Nat Biotechnol* 2007;25:660–662. [PubMed: 17515908]
91. Dueber JE, Yeh BJ, Chak K, Lim WA. Reprogramming control of an allosteric signaling switch through modular recombination. *Science* 2003;301:1904–1908. [PubMed: 14512628]

92. Weiss SM, Ladwein M, Schmidt D, Ehinger J, Lommel S, et al. IRSp53 links the enterohemorrhagic *E. coli* effectors Tir and EspFU for actin pedestal formation. *Cell Host Microbe* 2009;5:244–258. [PubMed: 19286134]
93. Tong AH, Drees B, Nardelli G, Bader GD, Brannetti B, et al. A combined experimental and computational strategy to define protein interaction networks for peptide recognition modules. *Science* 2002;295:321–324. [PubMed: 11743162]
94. Le Clainche C, Schlaepfer D, Ferrari A, Klingauf M, Grohmanova K, et al. IQGAP1 stimulates actin assembly through the N-WASP-Arp2/3 pathway. *J Biol Chem* 2007;282:426–435. [PubMed: 17085436]
95. Bensenor LB, Kan HM, Wang N, Wallrabe H, Davidson LA, et al. IQGAP1 regulates cell motility by linking growth factor signaling to actin assembly. *J Cell Sci* 2007;120:658–669. [PubMed: 17264147]
96. Ho HY, Rohatgi R, Lebensohn AM, Le M, Li J, et al. Toca-1 mediates Cdc42-dependent actin nucleation by activating the N-WASP-WIP complex. *Cell* 2004;118:203–216. [PubMed: 15260990]
97. Alto NM, Weflen AW, Rardin MJ, Yarar D, Lazar CS, et al. The type III effector EspF coordinates membrane trafficking by the spatiotemporal activation of two eukaryotic signaling pathways. *J Cell Biol* 2007;178:1265–1278. [PubMed: 17893247]
98. Vingadassalom D, Kazlauskas A, Skehan B, Cheng HC, Magoun L, et al. Insulin receptor tyrosine kinase substrate links the *E. coli* O157:H7 actin assembly effectors Tir and EspF(U) during pedestal formation. *Proc Natl Acad Sci U S A* 2009;106:6754–6759. [PubMed: 19366662]
99. Itoh T, Erdmann KS, Roux A, Habermann B, Werner H, De Camilli P. Dynamin and the actin cytoskeleton cooperatively regulate plasma membrane invagination by BAR and F-BAR proteins. *Dev Cell* 2005;9:791–804. [PubMed: 16326391]
100. Frost A, Perera R, Roux A, Spasov K, Destaing O, et al. Structural basis of membrane invagination by F-BAR domains. *Cell* 2008;132:807–817. [PubMed: 18329367]
101. Dawson JC, Legg JA, Machesky LM. Bar domain proteins: a role in tubulation, scission and actin assembly in clathrin-mediated endocytosis. *Trends Cell Biol* 2006;16:493–498. [PubMed: 16949824]
102. Shin N, Ahn N, Chang-Ileto B, Park J, Takei K, et al. SNX9 regulates tubular invagination of the plasma membrane through interaction with actin cytoskeleton and dynamin 2. *J Cell Sci* 2008;121:1252–1263. [PubMed: 18388313]
103. Kinley AW, Weed SA, Weaver AM, Karginov AV, Bissonette E, et al. Cortactin interacts with WIP in regulating Arp2/3 activation and membrane protrusion. *Curr Biol* 2003;13:384–393. [PubMed: 12620186]
104. Blasutig IM, New LA, Thanabalasuriar A, Dayarathna TK, Goudreault M, et al. Phosphorylated YDXV motifs and Nck SH2/SH3 adaptors act cooperatively to induce actin reorganization. *Mol Cell Biol* 2008;28:2035–2046. [PubMed: 18212058]
105. Jones N, Blasutig IM, Eremina V, Ruston JM, Bladt F, et al. Nck adaptor proteins link nephrin to the actin cytoskeleton of kidney podocytes. *Nature* 2006;440:818–823. [PubMed: 16525419]
106. Rivera GM, Briceno CA, Takeshima F, Snapper SB, Mayer BJ. Inducible clustering of membrane-targeted SH3 domains of the adaptor protein Nck triggers localized actin polymerization. *Curr Biol* 2004;14:11–22. [PubMed: 14711409]
107. Krause M, Sechi AS, Konradt M, Monner D, Gertler FB, Wehland J. Fyn-binding protein (Fyb)/SLP-76-associated protein (SLAP), Ena/vasodilator-stimulated phosphoprotein (VASP) proteins and the Arp2/3 complex link T cell receptor (TCR) signaling to the actin cytoskeleton. *J Cell Biol* 2000;149:181–194. [PubMed: 10747096]
108. Soulard A, Friant S, Fitterer C, Orange C, Kaneva G, et al. The WASP/Las17p-interacting protein Bzz1p functions with Myo5p in an early stage of endocytosis. *Protoplasma* 2005;226:89–101. [PubMed: 16231105]
109. Bear JE, Rawls JF, Saxe CL 3rd. SCAR, a WASP-related protein, isolated as a suppressor of receptor defects in late Dictyostelium development. *J Cell Biol* 1998;142:1325–1335. [PubMed: 9732292]

110. Suetsugu S, Miki H, Takenawa T. Identification of two human WAVE/SCAR homologues as general actin regulatory molecules which associate with the Arp2/3 complex. *Biochem Biophys Res Commun* 1999;260:296–302. [PubMed: 10381382]
111. Eden S, Rohatgi R, Podtelejnikov AV, Mann M, Kirschner MW. Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck. *Nature* 2002;418:790–793. [PubMed: 12181570]
112. Gautreau A, Ho HY, Li J, Steen H, Gygi SP, Kirschner MW. Purification and architecture of the ubiquitous Wave complex. *Proc Natl Acad Sci U S A* 2004;101:4379–4383. [PubMed: 15070726]
113. Leng Y, Zhang J, Badour K, Arpaia E, Freeman S, et al. Abelson-interactor-1 promotes WAVE2 membrane translocation and Abelson-mediated tyrosine phosphorylation required for WAVE2 activation. *Proc Natl Acad Sci U S A* 2005;102:1098–1103. [PubMed: 15657136]
114. Ismail AM, Padrick SB, Chen B, Umetani J, Rosen MK. The WAVE regulatory complex is inhibited. *Nat Struct Mol Biol* 2009;16:561–563. [PubMed: 19363480]
115. Innocenti M, Zucconi A, Disanza A, Frittoli E, Areces LB, et al. Abi1 is essential for the formation and activation of a WAVE2 signalling complex. *Nat Cell Biol* 2004;6:319–327. [PubMed: 15048123]
116. Stovold CF, Millard TH, Machesky LM. Inclusion of Scar/WAVE3 in a similar complex to Scar/WAVE1 and 2. *BMC Cell Biol* 2005;6:11. [PubMed: 15752430]
117. Weiner OD, Rentel MC, Ott A, Brown GE, Jedrychowski M, et al. Hem-1 complexes are essential for Rac activation, actin polymerization, and myosin regulation during neutrophil chemotaxis. *PLoS Biol* 2006;4:e38. [PubMed: 16417406]
118. Kim Y, Sung JY, Ceglia I, Lee KW, Ahn JH, et al. Phosphorylation of WAVE1 regulates actin polymerization and dendritic spine morphology. *Nature* 2006;442:814–817. [PubMed: 16862120]
119. Steffen A, Rottner K, Ehinger J, Innocenti M, Scita G, et al. Sra-1 and Nap1 link Rac to actin assembly driving lamellipodia formation. *EMBO J* 2004;23:749–759. [PubMed: 14765121]
120. Derivery E, Lombard B, Loew D, Gautreau A. The Wave complex is intrinsically inactive. *Cell Motil Cytoskeleton*. 2009
121. Kobayashi K, Kuroda S, Fukata M, Nakamura T, Nagase T, et al. p140Sra-1 (specifically Rac1-associated protein) is a novel specific target for Rac1 small GTPase. *J Biol Chem* 1998;273:291–295. [PubMed: 9417078]
122. Oikawa T, Yamaguchi H, Itoh T, Kato M, Ijuin T, et al. PtdIns(3,4,5)P3 binding is necessary for WAVE2-induced formation of lamellipodia. *Nat Cell Biol* 2004;6:420–426. [PubMed: 15107862]
123. Suetsugu S, Kurisu S, Oikawa T, Yamazaki D, Oda A, Takenawa T. Optimization of WAVE2 complex-induced actin polymerization by membrane-bound IRSp53, PIP(3), and Rac. *J Cell Biol* 2006;173:571–585. [PubMed: 16702231]
124. Nakanishi O, Suetsugu S, Yamazaki D, Takenawa T. Effect of WAVE2 phosphorylation on activation of the Arp2/3 complex. *J Biochem* 2007;141:319–325. [PubMed: 17202194]
125. Pocha SM, Cory GO. WAVE2 is regulated by multiple phosphorylation events within its VCA domain. *Cell Motil Cytoskeleton* 2009;66:36–47. [PubMed: 19012317]
126. Miki H, Fukuda M, Nishida E, Takenawa T. Phosphorylation of WAVE downstream of mitogen-activated protein kinase signaling. *J Biol Chem* 1999;274:27605–27609. [PubMed: 10488099]
127. Soderling SH, Binns KL, Wayman GA, Davee SM, Ong SH, et al. The WRP component of the WAVE-1 complex attenuates Rac-mediated signalling. *Nat Cell Biol* 2002;4:970–975. [PubMed: 12447388]
128. Salazar MA, Kwiatkowski AV, Pellegrini L, Cestra G, Butler MH, et al. Tuba, a novel protein containing bin/amphiphysin/Rvs and Dbl homology domains, links dynamin to regulation of the actin cytoskeleton. *J Biol Chem* 2003;278:49031–49043. [PubMed: 14506234]
129. Linardopoulou EV, Parghi SS, Friedman C, Osborn GE, Parkhurst SM, Trask BJ. Human subtelomeric WASH genes encode a new subclass of the WASP family. *PLoS Genet* 2007;3:e237. [PubMed: 18159949]

130. Campellone KG, Webb NJ, Znameroski EA, Welch MD. WHAMM is an Arp2/3 complex activator that binds microtubules and functions in ER to Golgi transport. *Cell* 2008;134:148–161. [PubMed: 18614018]
131. Zuchero JB, Coutts AS, Quinlan ME, Thangue NB, Mullins RD. p53-cofactor JMY is a multifunctional actin nucleation factor. *Nat Cell Biol* 2009;11:451–459. [PubMed: 19287377]
132. Liu R, Abreu-Blanco MT, Barry KC, Linardopoulou EV, Osborn GE, Parkhurst SM. Wash functions downstream of Rho and links linear and branched actin nucleation factors. *Development* 2009;136:2849–2860. [PubMed: 19633175]
133. Delatour V, Helfer E, Didry D, Le KH, Gaucher JF, et al. Arp2/3 controls the motile behavior of N-WASP-functionalized GUVs and modulates N-WASP surface distribution by mediating transient links with actin filaments. *Biophys J* 2008;94:4890–4905. [PubMed: 18326652]
134. Weisswange I, Newsome TP, Schleich S, Way M. The rate of N-WASP exchange limits the extent of ARP2/3-complex-dependent actin-based motility. *Nature* 2009;458:87–91. [PubMed: 19262673]
135. Weiner OD, Marganski WA, Wu LF, Altschuler SJ, Kirschner MW. An actin-based wave generator organizes cell motility. *PLoS Biol* 2007;5:e221. [PubMed: 17696648]

Terms/Definitions List Acronyms list

Hyperactivation	Activity of a WASP protein above that of an equal concentration of isolated VCA; usually due to oligomerization or clustering.
Peaked titration	Activity of a WASP protein that rises and then falls as a dimerizing ligand is added.
Back titration	Reduced activity of a WASP protein that has been hyperactivated by a dimerizing ligand upon addition of analogous monomeric ligand.
L parameter	In MWC-based model of 2-state allosteric equilibrium, the population ratio of inactive to active states of the free protein.
C parameter	In MWC-based model of 2-state allosteric equilibrium, ratio of dissociation constants of a ligand for active and inactive states ($K_{D,act}/K_{D,inact}$)
Arp2/3 complex	Actin related protein 2/actin related protein 3 complex
BAR domain	Bin, Amphiphysin and Rvs161/167 domain
GBD	GTPase binding domain
PIP₂/PIP₃	phosphatidylinositol 4,5-diphosphate/phosphatidylinositol 3,4,5-triphosphate
PRD	Proline rich domain
VCA	VCA, verprolin homology region, central hydrophobic and acidic C-terminal region of WASP family proteins
WASP	Wiskott-Aldrich Syndrome Protein
WAVE/Scar	WASP family Verprolin-homologous protein/Suppressor of cyclic AMP Receptor
WIP	WASP interacting protein
WRC	WAVE regulatory complex, consisting of WAVE/Scar, Abi, HSPC300, Nap1/Hem2 and Sra1/Pir121/CYFIP

Figure 1. The WASP family of proteins

(A) Domain structure of human WASP family proteins. Different domains are abbreviated as EVH1, Ena/VASP Homology domain 1; B, Basic region; GBD, GTPase Binding Domain; PRD, Proline Rich Domain; V, Verprolin homology domain (also WH2); C, Central hydrophobic region; A, Acidic region; SHD, Scar homology domain; WHD1, WASH homology domain 1; WHD2, WASH homology domain 2; NTD, WHAMM/JMY N-terminal homology domain; CC, Coiled Coil domain. In JMY, the NTD has an insertion of > 100 amino acids when compared to WHAMM. There are multiple closely related WASH proteins in humans, shown here is WASH1 (NCBI ref# NP_878908). (B) WASP has distinct binding sites for different ligands. Human WASP B-GBD sequence is shown with domains colored as in Figure 1. Secondary structure for the WASP GBD-C autoinhibited structure is shown (Figure 2c–e). Green bars indicate the binding sites for PIP₂, Cdc42 and SH2 domain. Other annotations: dashed box, CRIB; Orange ovals, X-Linked neutropenia WASP mutations; green triangle, Tyrosine 291.

Figure 2. Structures of WASP

(A) The structure of WASP V/WH2-region in complex with actin. Actin is shown as a gray surface, WASP is shown as an orange ribbon. (B) Structure of the N-WASP EVH1:WIP(451–485) complex (structured WIP residues 454–481 shown). EVH1 domain is gold, proline motif (⁴⁶¹DLPPPEPY⁴⁶⁸) and flanking regions of WIP are purple and blue, respectively. (C–E) Structure of autoinhibited WASP (GBD-C). (C) Structure of the GBD-C protein. The three layers of structure are colored yellow, blue (GBD) and red (C region of VCA), respectively. (D) Structure of the GBD-C protein, rotated 180° from panel C. GBD and C elements are shown in surface and ribbon representations, respectively. (E) Surface representation of GBD-C protein, rotated ~90° from panel C, showing Y291 phosphorylation site in green. (F–G) Structures of active WASP complexes. (F) Structure of the Cdc42:WASP GBD complex. Cdc42 is green ribbon, with GMPPNP in sticks. Ordered GBD residues (231–277) are shown in gold (CRIB motif and flanking sequence) or yellow (Layer 1 as in panel C) ribbon. (G) Structure of the WASP GBD:EspF_U 1R complex. GBD is colored as in panel C and shown in surface representation. EspF_U is shown as a green ribbon. (H) Structure of the WASP GBD-C:Wiskostatin complex, orientation and color as in panel E, with wiskostatin shown as van der Waals spheres.

Figure 3. Thermodynamic models of allostery in WASP

(A) Two-state MWC-based allosteric model for WASP regulation by control of access to the C-helix through association with the GBD. (B) Two-state allosteric model with one activator. (C) Two-state allosteric model with two activators. Equations describing the fraction of WASP in the active state, either free (f_{act}) or in the presence of saturating ligand ($f_{\text{act,sat}}$) are given below the images. L is defined as $[\text{WASP}_{\text{inactive}}]/[\text{WASP}_{\text{active}}]$; C is the ratio of dissociation constants of a ligand for the active and inactive states, $K_{\text{D,act}}/K_{\text{D,inact}}$. (D) Simplified allosteric model for response to two activators. $K_{\text{D}1}$ and $K_{\text{D}2}$ are the dissociation constants of the two ligands for free WASP, α is the cooperativity constant between the ligands.

Figure 4. The hierarchical model of WASP regulation

WASP proteins are regulated hierarchically. An inner layer of allostery controls access of the VCA, and outer layer of dimerization/oligomerization controls affinity of the VCA for Arp2/3 complex. The two layers are thermodynamically coupled, so that binding of Arp2/3 complex to a VCA dimer will also shift the allosteric equilibrium toward the active state. Figure adapted by permission from Cell Press: Molecular Cell (22), copyright 2008.