

Physical Mechanisms of Signal Integration by WASP Family Proteins

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Key Words

actin regulation, allostery, Arp2/3 complex, Rho GTPase, signal transduction

Abstract

The proteins of the Wiskott-Aldrich syndrome protein (WASP) family are activators of the ubiquitous actin nucleation factor, the Arp2/3 complex. WASP family proteins contain a C-terminal VCA domain that binds and activates the 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 the Arp2/3 complex, and dimerization/oligomerization, in which multi-VCA complexes are better activators of the 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 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.

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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, the Arp2/3 complex (1, 2). By controlling the degree, rate, and location of filament nucleation by the 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 biology. The central concept that pervades our discussion is that the activity of WASP proteins toward the 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 an active WASP to stimulate the 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.

WASP: Wiskott-Aldrich syndrome protein

Arp2/3 complex: the actin-related protein 2/actin-related protein 3 complex

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.

DOMAIN ORGANIZATION OF WASP AND NEURAL WASP

The WASP family is defined by a conserved C-terminal VCA domain (Figure 1a) named for its three sub-elements, the verprolin (V) homology (also called WASP homology 2 or WH2), central (C) hydrophobic, and acidic (A) regions. The VCA domain strongly stimulates actin nucleation by the 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 the 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 the 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 the 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 the Arp2/3 complex (both are homologous to actin), creating a pseudotrimer/tetramer nucleus for filament growth (7, 9, 10, 14–18). A recent small-angle X-ray scattering study suggests that a high-affinity site for the VCA:actin complex is

VCA domain: the verprolin homology, central hydrophobic, and acidic C-terminal regions of WASP family proteins

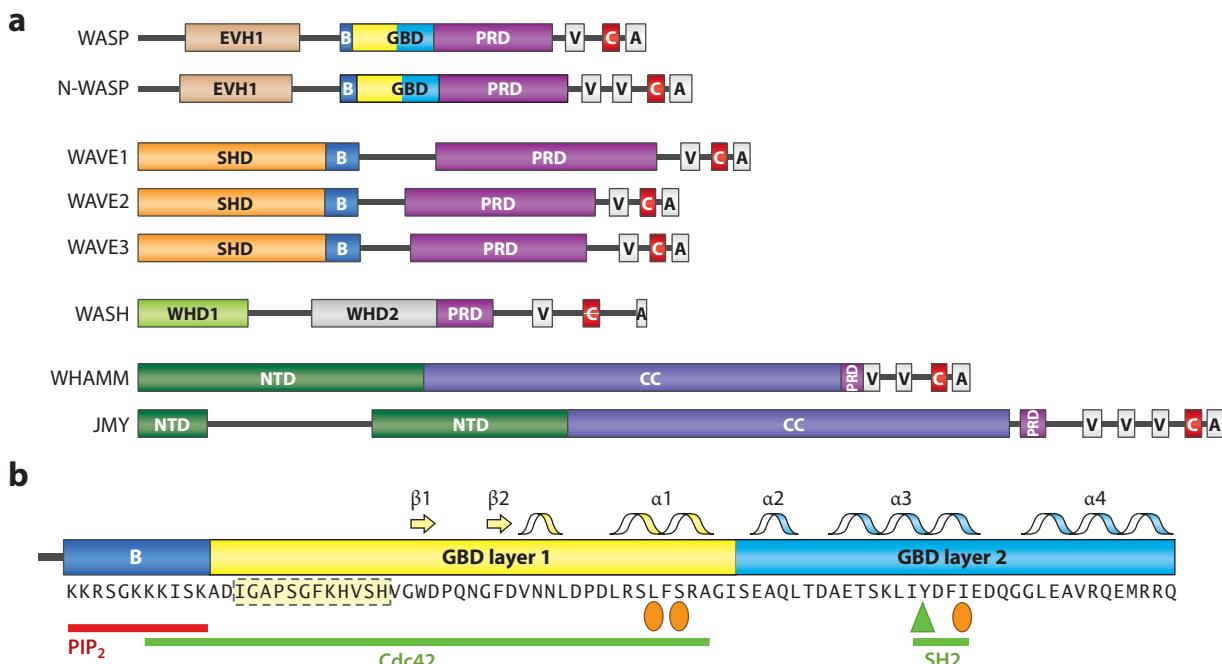


Figure 1

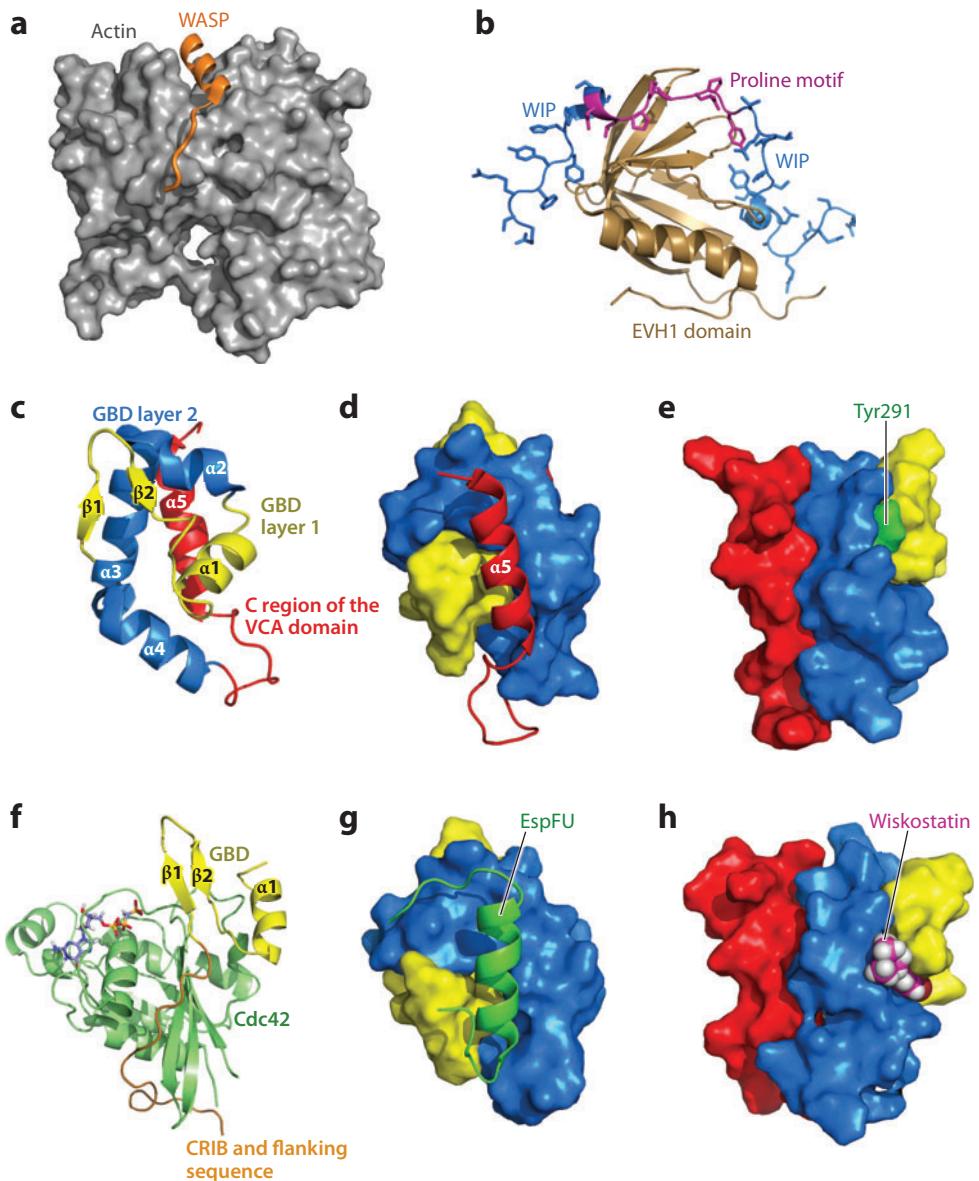
The Wiskott-Aldrich syndrome protein (WASP) family of proteins. (a) Domain structure of human WASP family proteins. Different domains are abbreviated as follows: A, acidic region; B, basic region; C, central hydrophobic region; CC, coiled-coil domain; EVH1, Ena/VASP homology domain 1; GBD, GTPase-binding domain; NTD, N-terminal homology domain; PRD, proline-rich domain; SHD, Scar homology domain; V (also WH2), verprolin homology domain; WHD1, WASH homology domain 1; WHD2, WASH homology domain 2. 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 (National Center for Biotechnology Information reference number NP_878908). (b) WASP has distinct binding sites for different ligands. Human WASP GBD-C-autoinhibited structure is shown (see Figure 2c–e). Bars indicate the binding sites for phosphatidylinositol 4,5-diphosphate (PIP₂), Cdc42, and SH2 domains. Other annotations: dashed box, Cdc42-Rac-interactive-binding or CRIB; orange ovals, X-linked neutropenia WASP mutations; green triangle, green triangle, X-linked neutropenia WASP mutations; green triangle, tyrosine 291.

WIP: WASP-interacting protein

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 the Research

Collaboratory for Structural Bioinformatics, <http://www.rcsb.org>, database entry for Protein Data Bank number 2vcp). As detailed in recent reviews, V/WH2 repeats are also found in many other proteins, and can act to either sequester an 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 the 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 neural Wiskott-Aldrich syndrome protein (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 halfway around the domain. Mutations that disrupt this interface decrease WIP binding, leading *in vivo* to proteolytic degradation of WASP (25) and many forms of WASP (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 this functional interaction (35). As elaborated below, the B region also plays an important role in selective binding of the WASP activator, Cdc42 (36).

PIP₂:
phosphatidylinositol
4,5-diphosphate

Figure 2

Structures of Wiskott-Aldrich syndrome protein (WASP). (a) The structure of WASP verprolin homology/WASP homology 2 (V/WH2) region in complex with actin. Actin is shown as a gray surface, and WASP is shown as an orange ribbon. (b) Structure of the neural Wiskott-Aldrich syndrome protein Ena/VASP homology 1 domain (EVH1) in complex with WASP-interacting protein (WIP) residues 451–485 (only the ordered WIP residues 454–481 are shown). The EVH1 domain is gold, and the proline motif (461DLPPPEPY468) and flanking regions of WIP are magenta and blue, respectively. (c–e) Structure of autoinhibited WASP GTPase-binding domain fused to the C-region of the VCA (GBD-C). (c) Structure of the GBD-C protein. The three layers of this structure are colored yellow (GBD layer 1), blue (GBD layer 2) and red (C region of the VCA domain), respectively. Secondary structure elements are labeled. (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 the Y291 phosphorylation site in green. (f,g) Structures of active WASP complexes. (f) Structure of the Cdc42:WASP GBD complex. Cdc42 is a green ribbon, with GMPPNP in sticks. Ordered GBD residues (231–277) are shown in orange [Cdc42-Rac-interactive-binding (CRIB) motif and flanking sequence] and yellow (layer one as in panel c) ribbon. (g) Structure of the WASP GBD:EspFU 1R complex. GBD is colored as in panel c and shown in surface representation. EspFU 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.

GBD: GTPase-binding domain

PRD: proline-rich domain

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 of 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 the Arp2/3 complex (38, 39).

Between the GBD and the 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 the Arp2/3 complex: allostery, which controls accessibility of the VCA to the Arp2/3 complex, and oligomerization, which controls how strongly the exposed VCA can stimulate the 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 the 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 (α1). 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 α2–α4 and also the C terminus of helix α1 through the open end of the layer two C. A number of C region side chains, which are buried in the layer two-layer three interface (**Figures 2c,d**), also contribute substantially to the activity and affinity of the VCA for the 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 (**Figure 1**) (42, 43).

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 the 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 the 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, which is based on classical Monod-Wyman-Changeux (MWC) formalism (**Figure 3a**) (30, 44, 45). In this model, WASP exists in equilibrium between an inhibited state, where the GBD

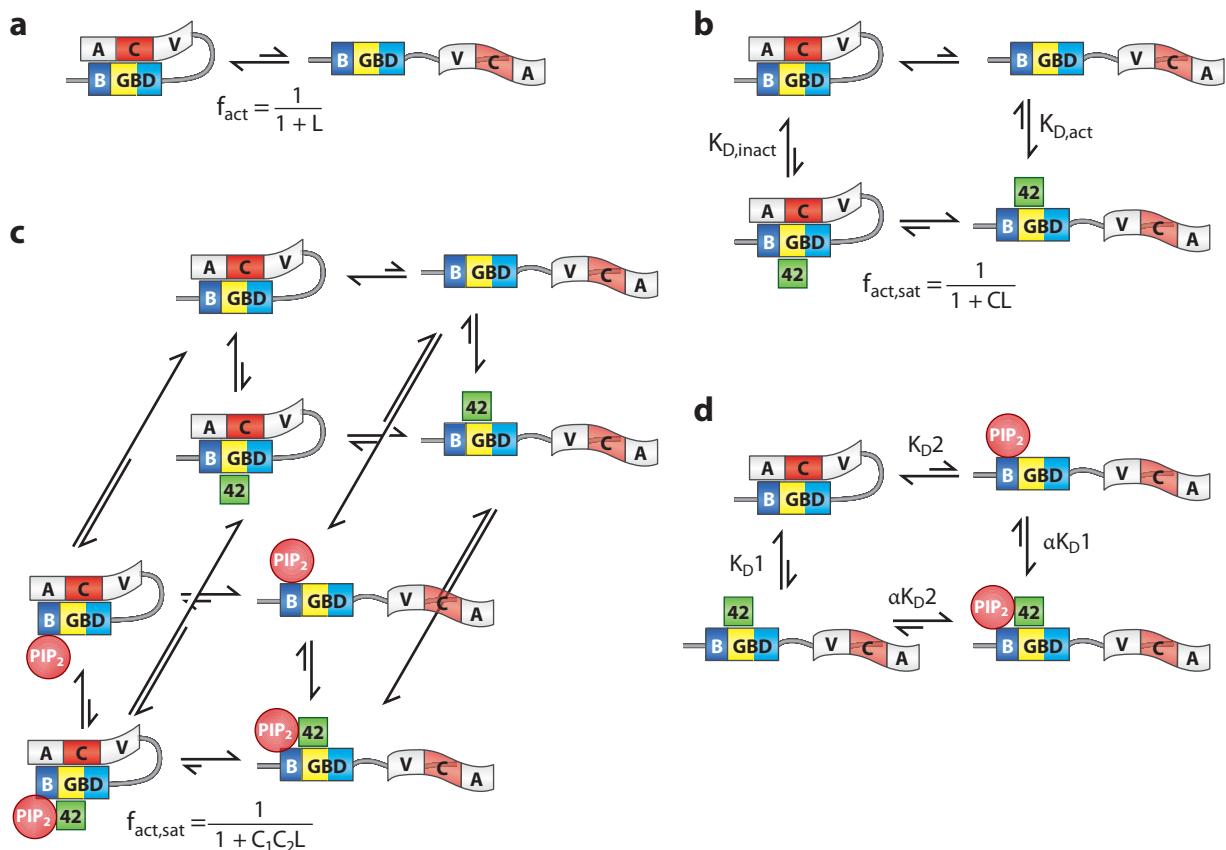


Figure 3

Thermodynamic models of allostery in a Wiskott-Aldrich syndrome protein (WASP). (a) The two-state Monod-Wyman-Changeux (MWC)-based allosteric model for WASP regulation by controlling access to the C-helix through association with the GTPase-binding domain (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 ($f_{act,sat}$) are given below the images. L is defined as $[WASP_{inactive}]/[WASP_{active}]$; C is the ratio of dissociation constants of a ligand for the active and inactive states, $K_{D,act}/K_{D,inact}$. (d) Simplified allosteric model for response to two activators. K_{D1} and K_{D2} are the dissociation constants of the two ligands for free WASP; α is the cooperativity constant between the ligands. Abbreviations: A, acidic region; B, basic region; V, verprolin homology/WH2 region; 42, Cdc42.

is bound to the VCA, and an active state, where the elements are dissociated. Because the GBD and the VCA are both unfolded in isolation, the allosteric equilibrium can be described as the folding equilibrium. Thus, for any given WASP, 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 simply on the basis of their free energies of unfolding (30). These proteins contained various linkers between the GBD and the 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 an effective means of understanding 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 the Arp2/3 complex are buried in the interface (10, 13, 39). Biochemical data show that BGBD fragments compete with the 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 the 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 the VCA) binds to and actively inhibits the 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, because 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 and leading to activation.

In the solution structure of the Cdc42:GBD complex (**Figure 2f**), the CRIB motif extends in a linear fashion along the $\beta 2$ 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 (X. Yang, E. Ugochukwu, J. Elkins, M. Soundararajan, J. Eswaran, A.C.W. Pike, N. Burgess, J.E. Debreczeni, O. Gileadi, S. Knapp, D. Doyle, unpublished, RCSB accession number 2odb) and PAR6 (48), and the interaction is 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 side chains of the conserved HXXH element in the CRIB motif with the side chain 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. The secondary structure elements are nearly identical to those observed in the autoinhibited structure (compare panels c and f in Figure 2). However, packing of these conserved elements against Cdc42 is incompatible with the autoinhibited domain. Extraction of the β -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 side chain, Y291/Y256 (52–56). This modification is needed for processes that include 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 α 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 α 3 in the autoinhibited structure. Thus, SH2 domain binding destabilizes the α 3 helix of phosphorylated WASP/N-WASP, disrupting the GBD-C domain, leading to release of the 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 of WASP and N-WASP with nanomolar affinity, displacing the VCA (65, 66). In the solution structure of a complex of the 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 two interactions with the VCA. Mutagenesis of the contact sites showed that side chains of the

helix contribute appreciably to affinity between the two molecules (65, 66). EspFU residues C-terminal to the helix form an extended arm, which lies across the top of the GBD and contributes an additional \sim 4 kcal·mol $^{-1}$ to the stability of the complex. EspFU has thus developed a high-affinity ligand for WASP/N-WASP that acts by mimicking the VCA and by 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), which both target N-WASP. Both compounds appear to act by stabilizing interactions between the GBD and the VCA.

In the solution structure of a complex between wiskostatin and WASP GBD-C, the small molecule binds in a shallow hydrophobic pocket between layers one and two, at the interface of the β -hairpin and α 3 helix, with the carbazole ring directed inward and the amino alcohol side chain directed toward solvent (**Figure 2b**) (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 the 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. These are the allosteric equilibrium constant in the free protein (L), the affinity of the ligand for the inhibited state of the protein, and the affinity of the ligand for the active state of the protein (44). The ratio of the affinities ($K_{D,\text{active}}/K_{D,\text{inhibited}}$), 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, on the basis of the affinity of Cdc42:GTP for the GBD (as a mimic of the active state) and GBD-C (as a hyperstabilized 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 the GBD-C and the 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 the 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 fivefold 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 the 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 & Pollard (35) reported that glutathione *S*-transferase (GST)-VCA dimers are roughly 100-fold more potent activators of the Arp2/3 complex than VCA monomers. 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, or 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 a distinct class of Arp2/3 activators, the cortactin proteins, which are known to bind the complex simultaneously with a VCA monomer. Importantly, fluorescence binding data showed that VCA dimers bind the 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 laboratories aims to understand this effect in detail.

The increased affinity of VCA dimers for the Arp2/3 complex provides several predictions regarding the biochemical behavior of dimerizing WASP ligands (22). First, at low WASP concentrations, ligands that create WASP dimers (**Figure 4a**) can produce higher Arp2/3 activity than can a VCA monomer (**Figure 4b**). 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 (**Figure 4a**), reducing observed activity (**Figure 4b**). 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 (**Figure 4b**). Finally, monovalent ligands can compete with their divalent counterparts for a binding site on WASP (**Figure 4a**). Thus, addition of a monovalent ligand to a hyperactivated system disrupts WASP dimers and returns the activity to that of a monomeric VCA or less (**Figure 4c**), a process termed back titration. These effects have been explicitly researched 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):(Arp2/3) complexes have been directly observed by analytical ultracentrifugation and gel filtration chromatography, respectively (66,

Hyperactivation: activity of a WASP protein above that of an equal concentration of isolated VCA, usually owing to oligomerization or clustering

Back titration: the reduced activity of a WASP protein that has been hyperactivated by a dimerizing ligand upon addition of an analogous monomeric ligand

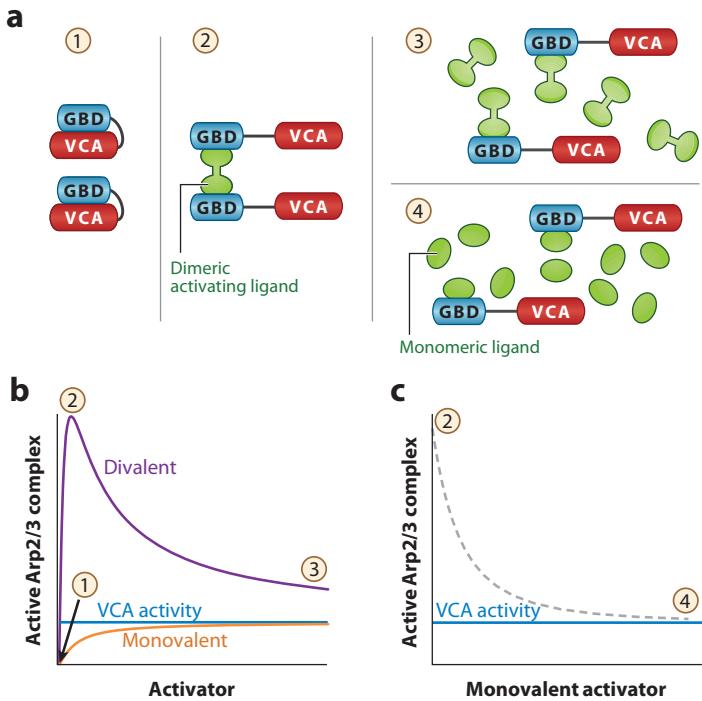


Figure 4

Simulated titrations of mono- and divalent Wiskott-Aldrich syndrome protein (WASP) activators. (a) Cartoons of the WASP:activator mixtures. WASP is shown as a blue GTPase-binding domain (GBD) fused to a red VCA. Inactive WASP (1) is bound and activated by either a dimeric activating ligand [green dumbbells in (2) and (3)] or a monomeric ligand [green ovals in (4)]. (b) Computationally modeled titration of a divalent activator into autoinhibited WASP (purple line). Allosteric activation of WASP proteins occurs concomitantly with activator binding. Activity is modeled by the concentration of WASP bound to the Arp2/3 complex. In the absence of activator, only inactive WASP is present, and activity is low (1). As divalent activator is added, active dimeric WASP species are formed (2), and activation exceeds that of free VCA (blue line). When high concentrations of divalent activator are added, competition for WASP proteins results in disassembly of the dimers, and activity approaches that of free VCA (3). Monovalent activator titration (orange line) monotonically increases WASP activity to that of free VCA. (c) Computationally modeled titration of monovalent activator into a maximal activity mixture of WASP and divalent activator (dashed gray line). The initial conditions are identical to those at (2) in panel b. As monovalent activator is added, dimers are disrupted, and activity drops to that of free VCA (blue line). Mixtures indicated by circled numbers in panels b and c are those in the subpanels of a with the same number. Figure adapted with permission from Cell Press, *Molecular Cell* (22), copyright © 2008.

BAR domain: Bin, amphiphysin, and Rvs161/167 domain

72). Finally, increasing EspFU repeat number correlates with increased actin assembly in cells (22, 66, 71, 72). These data indicate that EspFU 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 the distinct proline-rich domain 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-1, and SNX9 proteins. A final major category of WASP family SH3 ligands are the nonreceptor 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/syndapton (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; 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 the Arp2/3 complex (S.B. Padrick, unpublished), shows similar peaked activation through SH3-proline interactions by GST fusions with facioigenital dysplasia protein (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, although 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 vesicle 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 the Arp2/3 complex.

Peaked titration: the activity of a WASP protein that rises and then falls as a dimerizing ligand is added

INTEGRATION OF MULTIPLE SIGNALS BY WASP PROTEINS

The combination of allostery and oligomerization leads to a hierarchical mechanism of WASP regulation (**Figure 5**) (22). Within this mechanism, allostery controls availability of the VCA to the Arp2/3 complex, and dimerization controls affinity for the Arp2/3 complex.

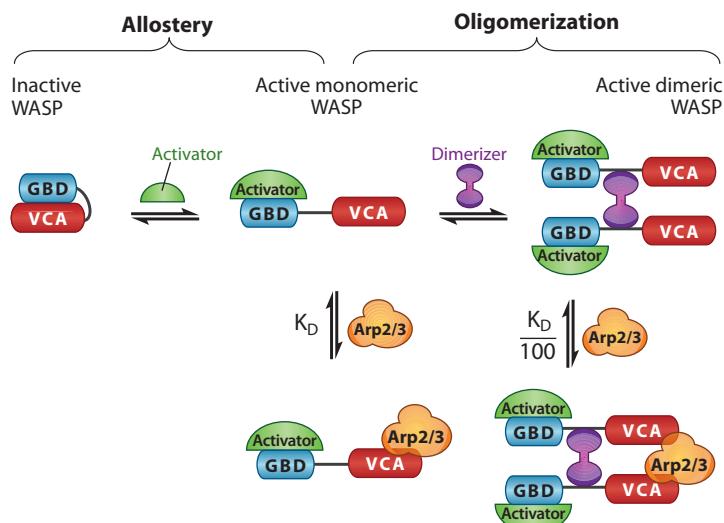


Figure 5

The hierarchical model of Wiskott-Aldrich syndrome protein (WASP) regulation. WASP proteins are regulated hierarchically. An inner layer of allosteroy controls access to the VCA (verprolin homology, central hydrophobic, and acidic regions), and an outer layer of dimerization/oligomerization controls the affinity of the VCA for the Arp2/3 complex. The two layers are thermodynamically coupled, so that binding of the Arp2/3 complex to a VCA dimer will also shift the allosteric equilibrium toward the active state. Figure adapted with permission from Cell Press, *Molecular Cell* (22), copyright 2008. Abbreviations: Arp2/3, the actin-related protein 2/actin-related protein 3 complex; K_D , dissociation constant; GBD, GTPase-binding domain.

L parameter: in the MWC-based model of two-state allosteric equilibrium, the population ratio of inactive to active states of the free protein

C parameter: in the MWC-based model of two-state allosteric equilibrium, the ratio of dissociation constants of a ligand for active and inactive states ($K_{D,\text{act}}/K_{D,\text{inact}}$)

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, because 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 (Figures 3c,d). The first posits that WASP acts in a binary fashion, where it is completely inactive in free form and completely active when bound to any single ligand (32). Because 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 whereby 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 WASP allosteric equilibrium

quantitatively (30, 45), giving a multiligand 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 $L\prod C_i$, 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 $C \ll 1$) 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. Although 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), thus destabilizing its contacts with A or the Arp2/3 complex. They also engage the oligomerizing mechanism (see above). Individually, Cdc42 and PIP₂ can each activate weakly inhibited systems (e.g., the *trans* complex of BGBD + the 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 (**Figure 3d**) (32). In the MWC formalism, the B region shifts the autoinhibitory equilibrium toward the inhibited state such that $L \gg 1$ (30). Thus $C_{\text{Cdc}42} * L$ 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 N-WASP (29, 32) (at saturation $C_{\text{Cdc}42} C_{\text{PIP}_2} L < 1$). Even though 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 two-layer three 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). Because 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 (91) showed that a wide variety of switch-like behaviors can be readily created. 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 owing 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., Reference 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 Reference 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), and others (e.g., Ygr136, Rvs167, and Bzz1) bound multiple motifs. Three of the motifs bound only a single SH3 domain, whereas 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 combination to control WASP protein assembly and activity.

Localization of WASP proteins to membranes can similarly cooperate with dimerizing ligands. Oligomerization can allow WASP proteins to bind membranes in multivalent fashion, increasing affinity. Thus, assembly and membrane binding of complexes can 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 6a**). 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 a high-affinity, trivalent 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

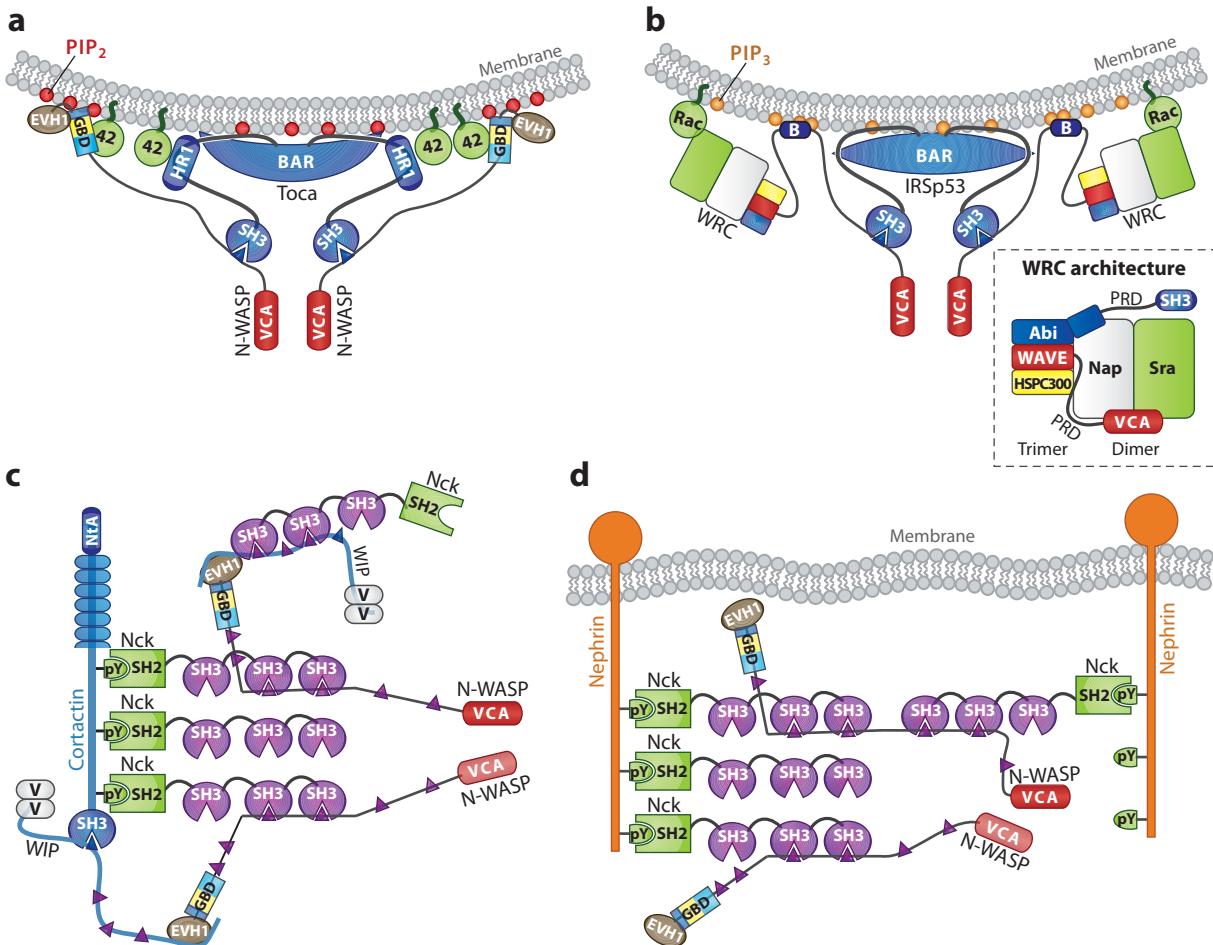


Figure 6

Cooperative activation of the N-WASP/WAVE regulatory complex (WRC) by higher-order complex formation. Multiprotein complexes bring two or more VCAs (verprolin homology, central hydrophobic, and acidic regions) together. (a) Schematic illustration of the complex formed by N-WASP, Toca (*dark blue*, with the BAR, SH3 and HRI domains labeled), Cdc42 (42), and PIP₂ (*red circles*) at membranes. (b) Schematic illustration of the complex formed by the WRC, IRSp53 (*blue*, with the BAR and SH3 domains labeled), Rac, and PIP₃ (*orange circles*) at membranes. Inset shows organization of the WRC. (c) Schematic illustration of the complex formed by phosphorylated Cortactin, Nck, N-WASP, and WIP. (d) Schematic illustration of the complex formed by phosphorylated nephrin, Nck (*purple ovals* and *green square*) and N-WASP (domain colors as in Figure 1). Abbreviations: Abi, Abl interactor; B, basic region; BAR, Bin/amphiphysin/Rvs homology domain; EVH1, Ena/VASP homology 1; GBD, GTPase-binding domain; HRI, protein kinase C-related kinase homology region 1; IRSp53, insulin receptor tyrosine kinase substrate p53; Nap1, Nck-associated protein; N-WASP, neural Wiskott-Aldrich syndrome protein; PIP₃, phosphatidylinositol 3,4,5-triphosphate; PRD, proline-rich domain; pY, phosphotyrosine; Rac, GTP bound Rac GTPase; SH3, Src-homology domain 3; Sra1, specifically Rac-associated protein; Toca, transducer of Cdc42-dependent actin assembly family protein; V, verprolin homology region; WAVE, WASP family verprolin-homologous protein; WIP, WASP interactin protein.

can be brought together to allow potent recruitment and activation of the Arp2/3 complex. As these interactions all contribute thermodynamically to a single assembly, the

individual contacts will reinforce one another. Furthermore, the multivalency of N-WASP (PRD) and Toca (SH3) could also provide mechanisms of higher-order oligomerization,

leading to spatial clustering of all components and sustaining transiently high local activation levels. As discussed below, such assemblies are abundant in WASP 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 further enhance WASP activity by increasing the likelihood that two active WASP molecules will be spatially 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 EspFU

Two-repeat fragments of EspFU hyperactivate WASP by engaging both allostery and dimerization. However, EspFU 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 EspFU repeat by WASP is small ($P \ll 1$), the fraction of EspFU 2R molecules with two bound WASP molecules is equal to P^2 . However, as the number of repeats (n) in EspFU 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 EspFU.

EspFU and its relative EspF from enteropathogenic *E. coli* 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 EspFU to IRSp53 (92) and IRTKS (98). These interactions, which can provide additional clustering of EspF/EspFU-bound WASP molecules (98), are necessary for bacterially induced actin assembly in cells (92, 97, 98). Moreover, EspF and EspFU 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 can enhance activation of the 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 $(n-1) \times P^2$ (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 the 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 the 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 the N-WASP-Arp2/3 complex (29, 86). The surface of BAR-induced membrane tubules is coated with SH3 domains at high density. These structures are known to recruit N-WASPs (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 in the presence of added membranes (29) (discussed above). The less-autoinhibited N-WASP (without WIP) was activated by FBP17 alone, but FBP17 activation was enhanced by a phospholipid 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 6c**) (81). Cortactin is a weak activator of the 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 a 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 the Arp2/3 complex. The further enhancement seen when WIP was also added 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 6d**) (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 of 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 multicompartment 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

WAVE: WASP family verprolin homologous protein (also known as Scar)

WAVE regulatory complex (WRC): a complex comprising WAVE/Scar, Abi, HSPC300, Nap1/Hem2, and Sra1/Pir121/CYFIP

PIP₃: phosphatidylinositol 3,4,5-triphosphate

complexes to understand their energetics, stoichiometry, structural organization, and dynamics as well as the relation of these properties to activity represents an exciting and challenging area for future investigation.

OTHER WASP FAMILY MEMBERS

In addition to WASP and N-WASP, the WAVE, WASH, WHAMM, and JMY proteins have VCA domains capable of activating the Arp2/3 complex. Many of the regulatory principles discussed above for WASP and N-WASP appear to hold for these proteins as well.

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 (Figure 1) (15, 109, 110). A variety of data indicate the WAVEs function downstream of the Rac GTPase is to stimulate actin assembly through the 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 6b, inset), consisting of WAVE, Abi (Abl interactor), HSPC300 (hematopoietic 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, although the molecular details are different, the basic principles of WAVE and WASP regulation are highly analogous. Although the basal activity of the WRC toward the 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 an 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 μM). 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 Reference 3). VCA phosphorylation increases affinity for the Arp2/3 complex but paradoxically decreases activity (124, 125). WAVE2 can be phosphorylated on the conserved Tyr150 by Abl upon cell stimulation, but its 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., IRS_p53 (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 a cooperative fashion to control activity toward the 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 6b**). Takenawa and colleagues (123) have shown that the combination of Rac, IRS_p53, and PIP₃ activates the WRC better than any of the individual effectors alone. 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 a multivalent 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 a 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 the Arp2/3 complex.

Other Family Members

Several vertebrate WASP family members have been discovered only recently: WASH (129),

WHAMM (130), and JMY (**Figure 1**) (131). All of these proteins have a C-terminal VCA domain, which in isolation can activate the 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 the Arp2/3 complex and thus how functionally analogous they are 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 of 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 WASP proteins and their assemblies. Taunton and coworkers (17) 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. 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 the Arp2/3 complex is also necessary for rapid exchange of N-WASP into and out of membrane complexes created by vaccinia viruses (134). N-WASP exchange is necessary for

efficient actin-based movement of the virus. Such negative feedback between the actin–Arp2/3 complex and the 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 needs 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 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 the 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 the Arp2/3 complex? What are the physical properties of the large, multivalent 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

1. WASP proteins are regulated by two processes, allostery and dimerization.
2. Allostery controls access of the VCA to the Arp2/3 complex and is understood structurally and energetically.
3. Dimerization controls affinity of WASP protein for the 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 further enhance WASP activity and sensitize it toward allosteric activators.
6. The five-component WAVE assembly is regulated analogously to WASP.

FUTURE ISSUES

1. Where are the two VCA-binding sites on the Arp2/3 complex, what are the functions of each VCA, and which elements of the 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, and how do these control VCA activity and respond to signals? What signals control them?

DISCLOSURE STATEMENT

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