Activation by Cdc42 and PIP$_2$ of Wiskott-Aldrich Syndrome protein (WASp) Stimulates Actin Nucleation by Arp2/3 Complex

Henry N. Higgs and Thomas D. Pollard

The Salk Institute for Biological Studies, La Jolla, California 92037

Abstract. We purified native WASp (Wiskott-Aldrich Syndrome protein) from bovine thymus and studied its ability to stimulate actin nucleation by Arp2/3 complex. WASp alone is inactive in the presence or absence of 0.5 μM GTP-Cdc42. Phosphatidylinositol 4,5 bisphosphate (PIP$_2$) micelles allowed WASp to activate actin nucleation by Arp2/3 complex, and this was further enhanced twofold by GTP-Cdc42. Filaments nucleated by Arp2/3 complex and WASp in the presence of PIP$_2$ and Cdc42 concentrated around lipid micelles and vesicles, providing that Cdc42 was GTP-bound and prenylated. Thus, the high concentration of WASp in neutrophils (9 μM) is dependent on interactions with both acidic lipids and GTP-Cdc42 to activate actin nucleation by Arp2/3 complex. The results also suggest that membrane binding increases the local concentrations of Cdc42 and WASp, favoring their interaction.

Key words: prenylation • membrane • GBD • neutrophil • thymus

Introduction

Arp2/3 complex (Machesky et al., 1994) initiates the barbed end growth of actin filaments by enhancing their nucleation (Mullins et al., 1998). Filaments initiated by Arp2/3 complex grow from the sides of other actin filaments to form branches in a process termed dendritic nucleation. Dendritic nucleation occurs at the leading edge of motile cells, and Arp2/3 complex localizes to branch points (Bailly et al., 1999; Svitkina and Borisy, 1999). The importance of Arp2/3 complex for actin dynamics in general is supported by work in diverse organisms including the following: mammalian tissue culture cells (Machesky et al., 1997; Welch et al., 1997a; Machesky and Insall, 1998) and lymphocytes (Weiner et al., 1999); extracts from brain (Ma et al., 1998b) and Acanthamoeba (Mullins and Pollard, 1999); and genetics in both budding (Winter et al., 1999b) and fission yeast (Balasubramanian et al., 1996; McCollum et al., 1996; Morrell et al., 1999).

Highly purified Arp2/3 complex alone does not enhance actin nucleation unless activated by members of the WASp/Scar protein family (Higgs et al., 1999; Machesky et al., 1999; Rohatgi et al., 1999a; Winter et al., 1999a; Yarar et al., 1999) or bacterial proteins (Welch et al., 1998). Activation by WASp/Scar proteins requires only their COOH-terminal 70–100 amino acids, termed the WA region (see Fig. 1 A). WA contains an actin-binding WH2 motif, an Arp2/3 complex–binding COOH-terminal acidic motif, and a connecting region in between, which might contribute to activation (Marchand et al., 2000).

Mammals have genes for at least five WASp/Scar proteins (for review see Higgs and Pollard, 1999). Wiskott-Aldrich Syndrome protein (WASp) appears to be restricted to hematopoietic cells and mutations to the WASp gene can cause Wiskott-Aldrich Syndrome, causing defects in platelets and lymphocytes (Ochs, 1998). N-WASP is closely related in sequence to WASp and is more widely expressed (Miki et al., 1996). Less is known about the three Scar isoforms, also called WAVE (Suetsugu et al., 1999).

WA regions of WASp/Scar proteins constitutively activate Arp2/3 complex. The NH$_2$-terminal 85% of these proteins contain sequences capable of interacting with many other proteins including the following: the Rho family GTPase, Cdc42; Src family tyrosine kinases; Tec family tyrosine kinases; the adaptor proteins, Nck and Grb2; and calmodulin (for review see Higgs and Pollard, 1999). An attractive model for WASp regulation (Miki et al., 1998; Kim et al., 2000) is that the NH$_2$-terminal region autoinhibits the COOH-terminal WA region, and binding of regulatory proteins with COOH-terminal acidic motifs can result in activation of the WA region.

Abbreviations used in this paper: CF-PE, carboxyfluorescein-labeled PE; GBD, GTPase binding domain; GST, glutathione-S-transferase; HSS, high speed supernatant; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PIP$_2$, phosphatidylinositol 4,5 bisphosphate; PS, phosphatidylserine; PMN, polymorphonuclear leukocytes; WA, WASp/Scar protein COOH-terminal 70–100 amino acids; WASp, Wiskott-Aldrich Syndrome protein.
proteins to NH₂-terminal sequences relieves this inhibition, resulting in Arp2/3 complex activation and actin nucleation. The lipid second messenger, phosphatidylinositol-4,5-bisphosphate (PIP₂), also might bind WASp and N-WASP and might assist in activation (Rohatgi et al., 1999).

Several lines of evidence support the model that GTP-bound Cdc42 (GTP-Cdc42) activates WASp or N-WASP by relief of autoinhibition. GTP-Cdc42 strongly stimulates actin nucleation in resting cell extracts (Zigmund et al., 1997; Ma et al., 1998a; Mullins and Pollard, 1999). Nuclear magnetic resonance structures show that GTP-Cdc42 binds a specific region (GTPase binding domain or GBD) on WASp and N-WASP (Abdul-Manan et al., 1999). WASp or N-WASP displayed considerable Arp2/3 complex activation in vitro, an effect which is enhanced by PIP₂ (Rohatgi et al., 1999).

However, results obtained using recombinant full-length WASp or N-WASP to activate Arp2/3 complex in vitro are inconsistent. First, in all published experiments, WASp or N-WASP displayed considerable Arp2/3 complex activation in the absence of Cdc42, ranging from full constitutive activity of WASp (Yarar et al., 1999) to partial activity of N-WASP (Egile et al., 1999; Rohatgi et al., 1999). Second, Rohatgi et al. (1999) found that both GTP- and GDP-Cdc42 activated N-WASP, whereas Egile et al. (1999) showed that only GTP-Cdc42 was activated. Finally, lipid modification of Cdc42 varied in these studies as Rohatgi et al. (1999) used insect cell–expressed, prenylated Cdc42, whereas Egile et al. (1999) used Escherichia coli–expressed, unprenylated Cdc42. Both forms stimulated N-WASP. Attention to prenylation is significant since studies with cell extracts suggest that prenylated Cdc42 is required to activate actin polymerization (Zigmund et al., 1997; Ma et al., 1998a).

These inconsistencies leave open many questions regarding the mechanism of WASp or N-WASP activation by Cdc42. In principle, autoinhibition should be very tight to prevent activation of Arp2/3 complex in the absence of Cdc42, as observed in cell extracts. Also, the requirement for GTP-bound Cdc42 should be absolute since experiments in both whole cells and cell extracts suggest that GDP-Cdc42 is inactive (Nobes and Hall, 1995; Zigmund et al., 1997). For these reasons, we purified native WASp for the first time and studied its ability to activate Arp2/3 complex in the presence and absence of Cdc42 and PIP₂. We find that the regulation of WASp by PIP₂ and GTP-Cdc42 is robust, with WASp being inactive in their absence. PIP₂ alone stimulates WASp, whereas GTP-Cdc42 alone does not. However, GTP-Cdc42 does augment PIP₂ stimulation. This augmentation requires that Cdc42 be both GTP-bound and prenylated. The synergy between PIP₂ and GTP-Cdc42 causes WASP-mediated actin nucleation on lipid surfaces.

Materials and Methods

Protein Preparation

We purified Arp2/3 complex from bovine thymus following the method described in Higgs et al. (1999). Rabbit skeletal muscle actin was purified from acetone powder (Spudich and Watt, 1971), gel filtered (MacLean-Fletcher and Pollard, 1980), and labeled with pyrenylsodiumacetamide (Pollard and Cooper, 1984).

GST WASp-WA and WASp-WA were prepared as previously described (Higgs et al., 1999). WASp152-309 was produced by expression in PET15b (Novagen), purification by nickel-NTA affinity chromatography (QIAGEN), and cleavage of the 6xHis tag with thrombin. Human Cdc42 was expressed in SF9 cells as a glutathione-S-transferase (GST) fusion protein in ActHGLT (PharMingen) using the baculovirus expression system, and was purified from the membrane fraction by glutathione-Sepharose affinity chromatography with subsequent cleavage of the GST moiety (Heyworth et al., 1993). Human Cdc42 was expressed in E. coli as a GST fusion protein in pGEX-2T, and purified from the nonmembrane fraction by glutathione-Sepharose affinity chromatography and cleavage of the GST (Heyworth et al., 1993). Cdc42 was charged with GTP or GDP by incubating 50 μM Cdc42 with 2.7 mM nucleotide in 10 mM Tris-HCl, pH 7.5, 1 mM DTT, 3 mM diethylenetri-nitriro-pentaacetic acid, 0.5 mM MgCl₂, for 10 min at 30°C. MgCl₂ was added to 4 mM, the mixture was kept on ice and used within 2 h. This nucleotide exchange procedure was used because the high concentration of EDTA used in other procedures (Heyworth et al., 1993) accelerated actin polymerization from monomers in the pyrene-actin assay.

Antibody Production

The WASp 209-226 peptide was synthesized with an added cysteine residue at its NH₂-terminus on an Applied Biosystems 432A peptide synthesizer and coupled to keyhole limpet hemocyanin following instructions from the Pierce Chemical Co. The material was used to raise polyclonal antiserum in New Zealand white rabbits. AB1 was affinity-purified from serum using 209-226 peptide immobilized on Sulfolink gel (Pierce Chemical Co.).

WASP Concentration in Human Polymorphonuclear Leukocytes (PMN)

Human PMNs were isolated from freshly drawn blood from healthy donors (The General Clinical Research Center, Scripps Research Institute) in ACD (Higgs et al., 1999). The cell number was determined using a hemocytometer. Cells (final concentration, 2.3 × 10⁵ cells/μl) were added to boiling SDS-PAGE sample buffer and were boiled for 5 min. Protein concentration was 1 mg/ml by Bradford assay on a parallel sample diluted into 0.1% Thesit (Roche Biochemicals). The sample in SDS-PAGE buffer was diluted to various concentrations in the absence or presence of various concentrations of WASp152-309 in SDS-PAGE buffer. Samples were separated by SDS-PAGE and analyzed by Western blotting against AB1 with chemiluminescence detection. The integrated densities of the WASp and WASp152-309 bands were determined using NIH Image 1.60/ppc. Significance of linearity for WASp between 1 and 10 μg PMN homogenate, and for WASp152-309 between 2.5 and 20 ng in a background of 5 μM PMN homogenate (44 pg protein/PMN determined by Bradford assay). WASp was determined to be 10.2 ng in 5 μg PMN homogenate from the Pierce Chemical Co. This material was used to raise polyclonal antiserum in New Zealand white rabbits. AB1 was affinity-purified from serum using 209-226 peptide immobilized on Sulfolink gel (Pierce Chemical Co.).

WASP Purification

All steps were carried out at 4°C or on ice. All chromatographic supplies were purchased from Amersham Pharmacia Biotech. Six frozen bovine calf thymus (PolFroce) were broken into small bits with a hammer, and homogenized in a blender followed by a Polytron (Branson) in 1 ml/g of EB (40 mM Tris-HCl, pH 8.0, 5 mM EGTA, 1 mM MgCl₂, 1 mM DTT, 1 mM PMSF, 20 μg/ml leupeptin, 6 μg/ml pepstatin A). The homogenate was centrifuged at 11,000 g for 15 min, and the resulting supernatant was centrifuged at 100,000 g for 1 h to obtain a high speed supernatant (HSS).

The conductivity of the HSS was adjusted to the equivalent of QA (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EGTA, 1 mM MgCl₂, 1 mM DTT, 2 μg/ml leupeptin, 2 μg/ml pepstatin A) with solid NaCl. The HSS was loaded onto 300 ml of Q Sepharose Fast Flow, and WASp was eluted with 3,000 ml of linear gradient to QB (QA + 200 mM NaCl). Fractions containing full-length WASp (by Western blot) were pooled and solid ammonium sulfate (GIBCO BRL) was added to 40% saturation (243 g/liter)
with vigorous mixing. After 15 min, the sample was centrifuged at 10,000 rpm for 15 min, and the pellet was redissolved in PB (20 mM Tris-HCl, pH 8.0, 1 mM EGTA, 1 mM MgCl₂, 1 mM DTT). The conductivity was adjusted to the equivalent of PA (PB + 1,000 mM NaCl), and the sample was loaded onto 50 ml of phenyl-Sepharose high performance equilibrated with PA. WASp was eluted with a linear gradient to PB. WASp-containing fractions were pooled, dialyzed against SA (10 mM Pipes, pH 6.8, 50 mM NaCl, 1 mM EGTA, 1 mM MgCl₂, 1 mM DTT), and loaded onto a MonoS 10/10 column equilibrated in SA. WASp was eluted with a linear gradient from SA to SB (SA + 500 mM NaCl). WASp-containing fractions were pooled and loaded onto a 1.6 x 60-cm Superdex200 column equilibrated in QA. WASp-containing fractions were pooled and concentrated by loading onto a MonoQ5/5 column equilibrated in QA and eluting with a linear gradient to QB. Sedimentation equilibrium ultracentrifugation of 40 nM bovine WASp in 10 mM imidazole, pH 7.0, 50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, and 1 mM DTT at 23°C was carried out in a Beckman Optima XL-I. WASp sedimentation was monitored at 225 nm. Distributions of WASp at equilibrium were fitted using the nonlinear least squares method of Winnonlin (Johnson et al., 1981).

**Results**

**Characterization of Antibodies Against WASp**

Antibodies against a peptide consisting of residues 209–226 of human WASp, a region with no significant homology to N-WASP or Scar proteins, recognized a 65-kD band in lysates of human neutrophils and bovine thymus (Fig. 1 B). Although the calculated molecular mass of WASp is 1313

**Fluorescence Microscopy**

Nucleation reactions were initiated as described above with 4 μM rhodamine-phalloidin (Sigma Chemical Co.). After incubation at 23°C for 20 min, reactions were diluted either 625-fold (michelie experiments) or 25-fold (vesicle experiments) into microscopy buffer (50 mM KCl, 1 mM MgCl₂, 100 mM DTT; 20 μg/ml catalase, 100 μg/ml glucose oxidase, 3 mg/ml glucose, 0.5% methylcellulose, 10 mM imidazole, pH 7.0). Diluted sample (2 μl) was applied to coverslips coated with 0.1% nitrocellulose, and fluorescence was viewed with an Olympus X-70 microscope (Blanchoin et al., 2000).

**Phospholipid Vesicles**

All phospholipids were suspended in chloroform and purchased from Avanti Polar Lipids including the following: egg phosphatidylcholine (PC); egg phosphatidylethanolamine (PE); brain phosphatidylserine (PS); brain phosphatidylinositol (PI); egg phosphatidic acid (PA); brain PIβ, carboxyfluorescein-labeled PE (CF-PE); and cholesterol. For microscopy, lipids were mixed in the appropriate quantities, and the chloroform was removed under vacuum. The lipid film was resuspended in 10 mM imidazole, pH 7.0, 50 mM KCl, 1 mM MgCl₂ by vortexing, and subjected to five freeze/thaw cycles in dry ice/ethanol and a 37°C water bath. For vesicle pelleting assays, the same procedure was followed except: 0.05% di-bodipy-PC (Molecular Probes) was added to the lipids; 170 mM sucrose was added to the resuspension buffer; and the vesicles were extruded 10 times through 0.1-μm pore size polycarbonate filters (Lipex). Pelleting assays were conducted by mixing 2 μM WASp152-309 with unilamellar vesicles (500 μl total lipid) for 5 min at 23°C, and then centrifuging in a TLA-100 rotor for 15 min at 90,000 rpm. For all lipid mixtures, >99% of the lipid was in the pellet as judged by the depletion of di-bodipy-PC fluorescence from the supernatant. Pellets and supernatants were analyzed by SDS-PAGE and Coomassie staining.

**Fluorescence Anisotropy**

Nucleation reactions were initiated as described above with 4 μM rhodamine-labeled WASp WA and varying concentrations of binding partners were incubated in 10 mM imidazole, pH 7.0, 50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, and 0.025% Thesit (Roche Biochemicals) for 10 min at 23°C. Fluorescence anisotropy was measured in an AlphaScan spectrophotometer (Photon Technologies International) at 552 nm excitation and 574 nm emission.

**Fluorescence Spectroscopy**

A detailed procedure is described in Higgs et al. (1999). Reactions contained 4 μM Mg-ATP actin (5% pyrene-labeled), 10 mM imidazole, pH 7.0, 50 mM KCl, 1 mM EGTA, and 1 mM MgCl₂ in G buffer-Mg (2 mM Tris-HCl, pH 8.0, 0.5 mM DTT, 0.2 mM ATP, 0.1 mM MgCl₂, 0.02% w/v vol NaN₃). When used, PIP2 was added from a 2 mM stock in water. Pyrene fluorescence data (excitation at 365 nm and emission at 407 nm) were collected on a PFI AlphaScan spectrofluorometer (Photon Technology International) at a rate of 1 point/s. The concentration of filament ends (Ends) was determined at the point on the polymerization curve at which 80% of the actin had polymerized because few filaments form after Ends.
53,041 D, migration of the band at 65 kD agrees with previous studies (Zhu et al., 1996). WASp from thymus migrated as a doublet, which might be due to limited proteolysis or to posttranslational modification. AB1 also reacted with several lower molecular mass bands, the most prominent in thymus at 40 kD, which varied in intensity between preparations. Several of these bands may be proteolytic fragments of full-length WASp since they were reduced by stringent regimes of protease inhibitors and they increased in lysates over time as the full-length WASp band decreased.

Most of the full-length WASp in these lysates partitioned into the 100,000-g supernatant, with minor amounts in the 4,000-g pellet and 100,000-g pellet (Fig. 1 B). The concentration of WASp in PMN cytoplasm was determined to be 9 ± 2 μM (Fig. 1 C), a value similar to the concentration of Arp2/3 complex in PMN (Higgs et al., 1999).

### Purification and Physical Properties of WASp

We purified WASp from the 100,000-g supernatant of bovine calf thymus by anion exchange chromatography, ammonium sulfate precipitation, phenyl-Sepharose chromatography, and cation exchange chromatography, assessing purification steps by Western blotting. Although the yield of WASp was consistent among three preparations (8, 10, and 14 μg), the purity varied. After SDS-PAGE, one preparation yielded only one visible band by Coomassie staining (Fig. 1 D, inset). The other two preparations contained minor contaminating bands, none of which reacted with AB1. Purified WASp from all preparations had a Stokes’ radius of 5.0 nm on Superdex 200 gel filtration chromatography (Fig. 1 D), which is considerably larger than that expected for a globular protein of 55 kD. By sedimentation equilibrium analytical ultracentrifugation, 40 nM native WASp had a molecular mass of 122 kD (not shown). The data were noisy owing to the low concentration of WASp, but rule out a monomer. Larger quantities will be required to determine if WASp is a dimer or a trimer.

We also attempted to purify the WASp expressed in insect cells using the baculovirus infection system. Despite good expression, we were unable to recover purified WASp using the native WASp purification procedure. A majority of recombinant WASp pelleted at low speed in the extract, and remained insoluble upon detergent and salt treatment. Furthermore, the WASp expressed in insect cells eluted heterogeneously from all columns, in contrast to the native WASp, which eluted as a single peak from each column. One possibility is that the WASp expressed in insect cells was in heterogeneous aggregates that caused it to fractionate poorly.

### Effect of WASp, Cdc42, and PIP2 on Actin Nucleation through Arp2/3 Complex

We tested the ability of full-length WASp to activate actin nucleation through Arp2/3 complex using fluorescence of pyrene-actin to follow the polymerization time course. WASp alone (4 nM) or with 10 nM Arp2/3 complex had no effect on the time course of actin polymerization (Fig. 2 A). The addition of 500 nM GTPγS-Cdc42 to WASp and Arp2/3 complex did not alter the polymerization time course. However, addition of PIP2 micelles to WASp and Arp2/3 complex accelerated polymerization and produced 12-fold more filaments (Fig. 2, A and B, and Table I). The addition of GTPγS-Cdc42 doubled the effect of PIP2 alone. This synergy between PIP2 and Cdc42 required GTPγS and recombinant Cdc42 from insect cells, which contained the prenyl modification (prenylated Cdc42). The Cdc42 expressed in Escherichia coli (unprenylated Cdc42) was inactive, as was prenylated GDPβS-Cdc42 (Table I). The effects of PIP2 and prenylated GTPγS-Cdc42 required Arp2/3 complex and WASp. Neither PIP2 nor prenylated GTPγS-Cdc42 changed the ability of WASp WA, the constitutively active COOH-terminal 70 residues of WASp, to activate Arp2/3 complex (not shown).

The degree to which WASp activated Arp2/3 complex...
Table I. Effect of WASp Activators on Actin Nucleation by WASp and Arp2/3 Complex

| Conditions                      | Concentration ends | Relative concentration ends |
|--------------------------------|--------------------|-----------------------------|
| Actin                          | 0.13               | 1                           |
| Actin, Arp2/3 complex          | 0.13               | 1                           |
| Actin, Arp2/3 complex, WASp    | 0.15               | 1.2                         |
| Actin, Arp2/3 complex, WASp, PIP2 | 1.57             | 12.1                        |
| Actin, Arp2/3 complex, WASp, prenylated GTPγS-Cdc42 | 0.15 | 1.2 |
| Actin, Arp2/3 complex, WASp, PIP2, prenylated GTPγS-Cdc42 | 2.99 | 23.0 |
| Actin, Arp2/3 complex, WASp, PIP2, unprenylated GDPβS-Cdc42 | 1.55 | 12.1 |
| Actin, Arp2/3 complex, WASp, PIP2, unprenylated GTPγS-Cdc42 | 1.08 | 8.3 |
| Actin, Arp2/3 complex, WASp-WA | 7.50               | 57.7                        |

Reagent concentrations: 4 μM actin, 10 nM Arp2/3 complex, 4 nM WASp, 20 μM PIP2, 0.5 μM Cdc42, and 1 μM WASp-WA. Concentration ends were determined at 80% polymerization (Higgs et al., 1999).

in the absence of prenylated GTPγS-Cdc42 and PIP2 varied slightly among the three WASp preparations. Two of the preparations displayed no activation ability, whereas the third stimulated a twofold increase in filaments nucleated in the absence of activators. However, all three preparations responded in near identical fashion to activation by PIP2 and prenylated GTPγS-Cdc42.

Native WASp was remarkably active in the presence of PIP2 and prenylated GTPγS-Cdc42. At 4 nM, WASp generated 3-nM filaments (Table I). At the same concentration, WASp-WA had no detectable effect on polymerization (Fig. 2 C). To investigate whether the multimeric state of full-length WASp contributes to this high activity, we tested dimeric GST WASp-WA. Dimeric GST-WA was tested dimeric GST WASp-WA. Dimeric GST-WA was almost 100-fold more efficient at activating Arp2/3 complex than monomeric WA, resulting in activation similar to activated native WASp (Fig. 2 C).

**Actin Networks Generated by WASp and Arp2/3 Complex**

We next examined the effect of WASp on Arp2/3 complex–mediated actin networks by fluorescence microscopy (Blanchin et al., 2000). In the presence of Arp2/3 complex, WASp alone (from the partially active preparation) induced a greater number of shorter filaments than Arp2/3 complex alone (Fig. 3 C and Table II), but created few branches. The addition of prenylated GTPγS-Cdc42 did not change filament length or the degree of branching (Fig. 3 E and Table II). The addition of PIP2 to WASp and Arp2/3 complex dramatically increased the number of branches and reduced the mean filament length (Fig. 3 D and Table II) because of the more complete activation of Arp2/3 complex by the limiting quantity of WASp (Higgs et al., 1999; Blanchin et al., 2000). PIP2 and prenylated GTPγS-Cdc42 with WASp and Arp2/3 complex produced highly fluorescent halos of actin filaments (Fig. 3 F). These halos, which were much larger (diameter of 7.2 ± 3.1 μm, n = 25) than the 10-nm diam PIP2 micelles, did not represent the majority of the polymerized actin, but were clearly sites of intense nucleation. Unprenylated GTPγS-Cdc42 did not generate actin filament halos with PIP2, WASp, and Arp2/3 complex (not shown). Because of the intense fluorescence of the halos, individual filaments were hard to resolve. Those that could be resolved were not heavily branched, and the branches were oriented randomly with respect to the halo (out of six discernible branches, three branched toward the halo and three away from it). The halos were unstable and rapidly shed filaments upon prolonged exposure to the halogen lamp, sometimes resulting in exploding halos. The shed filaments were short (<2 μm) and highly branched. Filaments distant from the halos appeared to exist in two populations: long unbranched filaments and short branched filaments (Table II). These results suggest that aggregates consisting of PIP2 micelles and prenylated GTPγS-Cdc42 serve as activation centers for WASp. These effects depended on both GTPγS and prenylation of Cdc42.

Multilamellar vesicles containing 10% PIP2 also stimulated the formation of filament-rich halos, providing prenylated GTPγS-Cdc42 was also present (Fig. 3 H). These halos of actin filaments, similar in size (8.1 ± 3.8–μm diam, n = 52) to those forming around micelles, had a vesicle at their core, revealed using vesicles containing 5% fluorescein-PE (Fig. 3 H, inset). Only ~10% of the fluorescent vesicles were surrounded by actin, possibly because of nonuniform distribution of prenylated GTPγS-Cdc42 on the vesicles. In contrast to the micelle experiments, most of the fluorescent actin filaments were associated with vesicles. Few filaments were >20 μm from the filament-encircled vesicles. Filaments dissociated from the halos over time (not shown), suggesting that filaments not associated with vesicles were probably nucleated from the vesicle and then dissociated. Actin filaments also polymerized around PS-containing vesicles in the presence of GTPγS-Cdc42-BV, WASp, and Arp2/3 complex (Fig. 3 G). Although the mean sizes of these actin-containing structures were comparable (7.4 ± 3.7 μm, n = 11), they were 40-fold less abundant than the actin-surrounded PIP2 vesicles and did not represent the majority of polymerized actin. PIP2-containing vesicles with WASp and Arp2/3 complex without Cdc42 produced many short, highly branched filaments (not shown), indicating increased actin nucleation. However, these actin filaments did not cluster around vesicles. In summary, both prenylated GTPγS-Cdc42 and PIP2 were required to produce actin filament–rich foci around micelles and vesicles.
Figure 3. Fluorescence micrographs of the products of actin polymerization reactions. Polymerization reactions contained 4 μM monomeric MgATP actin, with or without 100 nM of Arp2/3 complex, 4 nM WASp, 0.5 μM prenylated GTPγS-Cdc42, 22 μM PIP$_2$ micelles, or 100 μM of phospholipid vesicles. After polymerization for 20 min at 23°C in KMEI containing 4 μM rhodamine-phalloidin, samples were diluted 625-fold (A–F) or 25-fold (G and H) into motility buffer, mounted on nitrocellulose-coated coverslips, and viewed with filters for rhodamine. (A) Arp2/3 complex, prenylated GTPγS-Cdc42, PIP$_2$ micelles, (B) WASp, prenylated GTPγS-Cdc42, PIP$_2$ micelles, (C) Arp2/3 complex, WASp, (D) Arp2/3 complex, WASp, PIP$_2$ micelles, (E) Arp2/3 complex, WASp, prenylated GTPγS-Cdc42, (F) Arp2/3 complex, WASp, prenylated GTPγS-Cdc42, PIP$_2$ micelles, (G) Arp2/3 complex, WASp, prenylated GTPγS-Cdc42, multilamellar vesicles containing 50% cholesterol/14.5% PC/14.5% PE/20% PS/1% CF-PE, or (H) Arp2/3 complex, WASp, prenylated GTPγS-Cdc42, multilamellar vesicles containing 50% cholesterol/19.5% PC/19.5% PE/10% PIP$_2$/1% CF-PE. Inset in G shows corresponding fluorescein image of G, showing the vesicle at the interior of the actin halo (left) and a vesicle not surrounded by actin.

Table II. Quantitative Analysis of Polymerization Products

| Conditions                                      | Filament length (μm ± SD) | No filaments (unbranched/branched) | Percent branched |
|-------------------------------------------------|---------------------------|------------------------------------|-----------------|
| Arp2/3 complex, prenylated GTPγS-Cdc42, PIP$_2$ | 7.0 ± 6.5                 | 105/1                               | 1               |
| WASp, prenylated GTPγS-Cdc42, PIP$_2$           | 6.6 ± 6.2                 | 140/1                               | 1               |
| Arp2/3 complex, WASp                            | 2.6 ± 1.9                 | 125/2                               | 2               |
| Arp2/3 complex, PIP$_2$                        | 3.2 ± 2.6                 | 101/37                              | 27              |
| Arp2/3 complex, WASp, prenylated GTPγS-Cdc42   | 2.7 ± 1.8                 | 87/1                                | 1               |
| Arp2/3 complex, WASp, prenylated GTPγS-Cdc42, PIP$_2$ | 7.2 ± 4.1                 | 127/92                              | 42              |
Our finding that native WASp does not activate Arp2/3 complex unless stimulated by PIP2 and prenylated GTPgS-Cdc42 supports an autoinhibition mechanism for WASp (Miki et al., 1998; Kim et al., 2000). We tested the possibility that NH2-terminal WASp motifs bind the WA region and prevent it from activating Arp2/3 complex using a construct of WASp (termed WASp152-309) expressed in E. coli. This construct begins immediately after the predicted EVH1 domain and ends just before the polyproline region (Fig. 1A). Thus, WASp152-309 contains the entire WASp GBD as well as residues essential for binding WASp-WA (Kim et al., 2000).

The addition of WASp152-309 to nucleation reactions containing 0.25 μM WASp-WA and 10 nM Arp2/3 complex inhibited filament production, with half-maximal inhibition at 3 μM (Fig. 4A). Importantly, WASp152-309 did not inhibit nucleation by Scar1-WA and Arp2/3 complex, as predicted, because residues of WASp-WA that bind WASp GBD (Kim et al., 2000) are completely different from the corresponding part of Scar1 WA. In a fluorescence anisotropy assay, WASp152-309 bound WASp-WA with a dissociation equilibrium constant of 0.42 μM (Fig. 4B). WASp152-309 also inhibited binding of Arp2/3 complex to rhodamine WASp-WA (Fig. 4C), suggesting that WASp152-309 inhibits nucleation by interfering with WASp-WA binding to Arp2/3 complex.

Both prenylated GTPgS-Cdc42 (Fig. 5A) and unprenylated GTPgS-Cdc42 (not shown) overcame the inhibition of WASp-WA by WASp152-309.
of WASp-WA by WASp152-309 in the pyrene-actin nucleation assay, whereas PIP$_2$ did not reverse this inhibition. We also tested the effect of WASp152-309 on Arp2/3 complex activation by full-length WASp. WASp152-309 did not block the effect of PIP$_2$ on WASp, but did block the effect of prenylated GTP$\gamma$S-Cdc42 (Fig. 5B). These results suggest that WASp152-309 might not contain the region responsive to PIP$_2$. In support of this conclusion, WASp152-309 did not bind to vesicles containing PIP$_2$ or other anionic lipids (not shown).

**Discussion**

This is the first study to characterize native WASp from leukocytes in terms of its cellular fractionation, cellular concentrations, and ability to activate Arp2/3 complex. The concentration of WASp in human neutrophils is surprisingly high (9 $\mu$M), almost equal to that of Arp2/3 complex (Higgs et al., 1999). This high concentration of WASp suggests that its ability to activate Arp2/3 complex must be tightly regulated since, at this concentration, even a low constitutive activity would cause significant Arp2/3 complex activation.

Evidence from cell extract studies also suggests that Arp2/3 complex activation is tightly regulated, being strongly activated by GTP-Cdc42 and PIP$_2$ (Zigmond et al., 1997; Ma et al., 1998a; Mullins and Pollard, 1999). WASp and N-WASP are leading candidates for mediators of this activation. However, previous studies showed that low concentrations of WASp isolated from insect cells potently activated Arp2/3 complex in the absence of any activators (Yarar et al., 1999). Other studies using the N-WASP isolated from insect cells showed considerable constitutive Arp2/3 complex activation in the absence of activators (Egile et al., 1999; Rohatgi et al., 1999). In the latter two cases, the candidate WASp and N-WASP stimulator, Cdc42, did increase Arp2/3 complex activation, but surprisingly did so with both GDP- and GTP-bound in one study (Rohatgi et al., 1999). PIP$_2$ enhanced GTP-Cdc42 activation of N-WASP in one study (Rohatgi et al., 1999), but was not tested by Egile et al. The combination of these results suggests that the WASp and N-WASP expressed in insect cells is not as tightly regulated as expected for the activator of Arp2/3 complex.

Our results with native WASp activating Arp2/3 complex differ in two ways from previous work on WASp and N-WASP in insect cells (Egile et al., 1999; Rohatgi et al., 1999; Yarar et al., 1999). First, the difference in Arp2/3 complex activation between nonactivated and fully activated WASp is much greater in our study. Second, PIP$_2$ micelles alone stimulate native WASp, whereas prenylated GTP$\gamma$S-Cdc42 alone does not at 0.5 $\mu$M. However, we found that prenylated GTP$\gamma$S-Cdc42 increased WASp stimulation by PIP$_2$ twofold. Cdc42 had to be GTP$\gamma$S-bound and prenylated for this effect.

In our hands, the WASp expressed in insect cells behaves in a heterogeneous fashion, but the WASp from bovine thymus and human neutrophils behaves much more homogeneously. Over 90% of WASp from extracts of either leukocyte source partitions into the 100,000 g supernatant, indicating that the majority is not associated with either the actin cytoskeleton or membranes under these conditions. This also suggests that most WASp molecules are not bound to GTP-Cdc42 or to PIP$_2$, because both of these ligands are associated with the membranes (Regazzi et al., 1992; Nomanbhoy et al., 1999). Purified WASp has a Stokes’ radius of 5.0 nm by gel filtration, and a mass of 122 kD by sedimentation equilibrium analytical ultracentrifugation. Because no other bands were visible in the clearest WASp preparation, the size suggests that WASp is a multimer, either a dimer or a trimer. The WASp multimer appears to be very stable, since only 40 nM of WASp was used for these analytical techniques. Others have proposed N-WASP to be a multimer, with a sedimentation coefficient of 5.45 S at 1 $\mu$M, by sedimentation velocity analytical ultracentrifugation (Carlier et al., 2000). These authors also proposed that N-WASP is in equilibrium between monomer and multimer, and that the monomer is the active state. Our results at 40 nM WASp suggest that the equilibrium strongly favors the multimer. Further work is necessary to determine the stoichiometry of the multimer, the interfaces involved in multimerization, and the effects of WASp activators on the multimeric state.

When stimulated by both PIP$_2$ and prenylated GTP$\gamma$S-Cdc42, native WASp activated Arp2/3 complex much more strongly than the constitutively active WA fragment. WASp at 4 nM activated Arp2/3 complex almost stoichiometrically, whereas 4 nM WA caused no detectable activation. Dimeric GST-WA had an activation efficiency similar to native WASp, and could fully activate Arp2/3 complex at a 50-fold lower concentration than WA. Dimeric GST-WA binds actin and Arp2/3 complex with an affinity similar to monomeric WA (Marchand, J.B., H.N. Higgs, and T.D. Pollard, unpublished data). The multimeric state of native WASp and GST-WA may contribute to a higher activation efficiency, but the mechanism is not yet clear.

Our observations suggest that the synergy between PIP$_2$ and Cdc42 is dependent on localization of both activators on a membrane surface. PIP$_2$ micelles or vesicles containing PIP$_2$ or PS were foci of intense actin filament nucleation in the presence of prenylated (but not unprenylated) GTP$\gamma$S-Cdc42 and native WASp. When prenylated, Cdc42 stably associates with membranes (Nomanbhoy et al., 1999). This is similar to other particulate activators of Arp2/3 complex including bacteria (Welch et al., 1997b), viruses (Frischknecht et al., 1999), intracellular vesicles (Taunton et al., 2000), beads coated with bacterial or eukaryotic activators of Arp2/3 complex (Cameron et al., 1999; Yarar et al., 1999), or beads coated with Cdc42 (Ma et al., 1998b). Others have also observed Cdc42- and PIP$_2$-dependent vesicle movement in whole cell extracts (Ma et al., 1998a; Moreau and Way, 1998), although the minimum requirements for this effect could not be determined in these studies. Vesicles with PIP$_2$ were more effective in producing these actin halos than micelles of PIP$_2$ or vesicles containing PS, PIP$_2$-containing vesicles alone promote dendritic nucleation by native WASp and Arp2/3 complex, but the filaments do not concentrate around vesicles. We do not know at present whether actin filaments nucleated by WASp and Arp2/3 complex in the presence of prenylated GTP$\gamma$S-Cdc42- and PIP$_2$-containing vesicles are associated noncovalently with the vesicle surface. If WASp binds actin filaments like N-WASP (Egile et al., 1999), some filaments may attach by this mechanism. However,
the actin filament halo is so thick around the vesicle that the majority of filaments are probably not bound to the membrane but held in place by the dendritic network formed during nucleation by Arp2/3 complex (Blanchoin et al., 2000).

Kim et al. (2000) proposed a mechanism for WASp regulation by GTP-Cdc42 based on earlier ideas on autoinhibition (Miki et al., 1998). A nuclear magnetic resonance structure and direct binding assays showed that WASp GBD binds to residues 461–492 of WASp-WA. This interaction was proposed to block binding of WASp-WA to Arp2/3 complex. Binding of GTP-Cdc42 to WASp GBD was proposed to release WASp-WA, allowing it to bind and activate Arp2/3 complex. Our experiments are consistent with the main features of this model. A construct (WASP152-309) containing the WASp GBD inhibited WASp WA activation of Arp2/3 complex by competing with Arp2/3 complex for WA binding. GTPγS-Cdc42 relieved inhibition by WASP152-309. Conversely, WASP152-309 blocked enhancement of native WASp stimulation by GTPγS-Cdc42. On the other hand, 0.5 μM prenylated GTPγS-Cdc42 alone does not overcome autoinhibition of native WASp since WA binds so tightly to WASp GBD that it reduces the affinity of the GBD for GTP-Cdc42 by 80–300-fold (Kim et al., 2000). To be an effective activator of WASp at a submicromolar concentration, prenylated GTPγS-Cdc42 requires a membrane containing an acidic lipid such as PIP2. However, our results do not rule out other mechanisms acting in concert with or separately from the autoinhibition model as proposed.

The PIP2 binding site on WASp is unknown. N-WASP and WASp were originally predicted to have an NH2-terminal PH domain (Miki et al., 1996), but atomic structures revealed a polyproline binding EVH1 domain (Fedorov et al., 1999; Prehoda et al., 1999) that overlaps, but is offset revealed a polyproline binding EVH1 domain (Fedorov et al., 1999; Prehoda et al., 1999) that overlaps, but is offset

Many aspects of this model remain to be tested including the following: the PIP2 binding site on WASp; WASp affinity for PIP2; and other lipids; the mechanism of partial WASp activation by PIP2; and the equilibrium and rate constants for WASp association with membranes containing PIP2 and/or Cdc42. Also, a full understanding of WASp activation will require accurate values for cellular concentrations of PIP2 and GTP-Cdc42 upon cell stimulation in addition to the knowledge of distributions of these molecules in cells. The proposed synergism between GTP-Cdc42 and PIP2, mediated by membrane binding, might provide a mechanism for the membrane-associated actin polymerization at the leading edge and on phagosomes/endosomes (Merrifield et al., 1999; Taunton et al., 2000). In addition, other WASp-binding proteins (Grb2, Nck, Src kinases, Btk) are membrane-associated upon cell stimulation, which might similarly affect WASp activation (Carrier et al., 2000; for review see Higgs and Pollard, 1999).

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References
Abdul-Manan, N., R. Aghazadeh, G.A. Liu, A. Majumdar, O. Ouwerkerk, K.A. Siminovich, and M.K. Rosen. 1999. Structure of Cdc42 in complex with the GTPase-binding domain of the ‘Wiskott-Aldrich Syndrome’ protein. Nature. 399:379–383.
Bailly, M., F. Macaluso, M. Cammer, A. Chan, J.E. Segall, and J.S. Condeelis. 1999. Relationship between Arp2/3 complex and the barbed ends of actin filaments at the leading edge of carcinoma cells after epithelial growth factor stimulation. J. Cell Biol. 145:331–345.
Balasubramanian, M.K., A. Feoktistova, D. McCollum, and K.L. Gould. 1996. Fission yeast Sop2p: a novel and evolutionarily conserved protein that interacts with Arp3p and modulates profilin function. EMBO (Eur. Mol. Biol. Organ.) J. 15:6426–6437.
Blanchion, L., and T.D. Pollard. 1998. Interaction of actin monomers with Acanthamoeba actophorin (ADF/cofilin) and profilin. J. Biol. Chem. 273: 25106–25111.
Blanchion, L., K.J. Amann, H.N. Higgs, J.B. Marchand, D.A. Kaiser, and T.D. Pollard. 2000. Direct observation of dendritic actin filament networks nucleated by Arp2/3 complex and WASp/Scar proteins. Nature. 404:1007–1011.
Cameron, L.A., M.J. Footer, A. van Oudenaarden, and J.A. Theriot. 1999. Mobility of ActA protein-coated microspheres driven by actin polymerization. Proc. Natl. Acad. Sci. USA. 96:4908–4913.
Carlier, M., P. Noeche, I. Brouin-L’Hermite, R. Boujemaa, C. Leclainche, C. Egile, C. Garbay, A. Darcux, P.J. Sansonetti, and D. Pantaloni. 2000. Grb2 links signalling to actin assembly by enhancing interaction of neural Wiskott-Aldrich Syndrome protein (N-WASP) with actin-related-protein (Arp2/3) complex. J. Biol. Chem. 275:21946–21952.
Egile, C., T.P. Loisel, V. Laurent, R. Li, D. Pantaloni, P.J. Sansonetti, and M.F. Carlier. 1999. Activation of the CDC42 effector N-WASP by the Shigella flexneri IcsA protein promotes actin nucleation by Arp2/3 complex and bacterial actin-based motility. J. Cell Biol. 146:1319–1332.
Fedorov, A.A., E. Fedorov, F. Gertler, and S.C. Almo. 1999. Structure of EVH1, a novel proline-rich ligand-binding module involved in cytoskeletal polymerization. Nonlipidated Cdc42 is ineffective since it does not bind vesicles.

The signaling pathway to WASp and Arp2/3 complex may operate through GTP activation of Cdc42 alone in the context of an anionic membrane surface, possibly containing PIP3, as follows. Binding to anionic lipid on the membrane surface partially activates WASp, possibly by exposing the acidic COOH terminus of WASp that interacts with Arp2/3 complex. Interaction of WASp with anionic lipid may also allow GTP-Cdc42 to bind the WASp GBD, perhaps concentrating WASp together with prenylated Cdc42 on the membrane surface, reducing their interaction potential. Interaction with Cdc42 fully stimulates WASp to activate Arp2/3 complex by releasing WA. WASp binding to Cdc42 may also stabilize active WASp on the membrane, resulting in localized actin polymerization.
Mullins, R.D., and D.T. Pollard. 1999. Rho-family GTPases require the Arp2/3 complex to stimulate actin polymerization in Acanthamoeba extracts. Curr. Biol. 9:405–415.

Nobes, C.D., and A. Hall. 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multicomponent focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell. 81:53–62.

Nomanbhoy, T.K., J.W. Erickson, and R.A. Cerione. 1999. Kinetics of Cdc42 membrane extraction by Rho-GDI monitored by real-time fluorescence resonance energy transfer. Biochemistry. 38:1744–1750.

Ochs, H.D. 1998. The Wiskott-Aldrich Syndrome. Semin. Hematol. 35:332–345.

Pollard, T.D., and J.A. Cooper. 1984. Quantitative analysis of the effect of Acanthamoeba profilin on actin filament nucleation and elongation. Biochemistry. 23:6631–6641.

Prehoda, K.E., D.J. Lee, and W.A. Lim. 1999. Structure of the Enabled/VASP Homology 1 domain-actin complex: a key component in the spatial control of actin assembly. Cell. 97:471–480.

Regazzi, R., A. Kikuchi, Y. Takai, and C.B. Wolheim. 1992. The small GTP-binding proteins in the cytosol of insulin-secreting cells are complexed to GDP dissociation inhibitor proteins. J. Biol. Chem. 267:17512–17519.

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

Spudich, J.A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the trepomyosin-tropomyosin complex with actin and the proteolytic fragments of myosin. J. Biol. Chem. 246:4866–4871.

Suescuna, S., H. Miki, and T. Takenawa. 1999. Identification of two human WASP/SCAR homologues as general actin regulatory molecules which associate with the Arp2/3 complex. Biochem. Biophys. Res. Commun. 260:296–302.

Svitkina, T.M., and G.G. Borisy. 1999. Arp2/3 complex and actin depolymerizing factor/cofilin in dendritic organization and tumor formation of actin filament array in lamellipodia. J. Cell Biol. 145:1009–1026.

Taunton, J., B.A. Rowning, M.L. Coughlin, M. Wu, R.T. Moon, T.J. Mitchison, and C.A. Larabell. 2000. Actin-dependent propulsion of endosomes and lysosomes by recruitment of N-WASP. J. Cell Biol. 148:519–530.

Ting-Beall, H.P., Needleman, D., and R.M. Hochmuth. 1993. Volume and osmotic properties of human neutrophils. Blood. 81:2774–2780.

Vinson, V.K., E.M. De La Cruz, H.N. Higgs, and T.D. Pollard. 1998. Interactions of Acanthamoeba profilin with actin and nucleotides bound to actin. Biochemistry. 37:10871–10880.

Winter, D.C., E.Y. Choe, and R. Li. 1999b. Genetic dissection of the budding yeast actin cytoskeleton. J. Cell Biol. 140:1125–1136.