Arp2/3 (actin-related protein 2/3) complex is a seven-subunit complex that nucleates branched actin filaments in response to cellular signals. Nucleation-promoting factors such as WASp/Scar family proteins activate the complex by facilitating the activating conformational change and recruiting the first actin monomer for the daughter branch. Here we address the role of the Arp2 subunit in the function of Arp2/3 complex by isolating a version of the complex lacking Arp2 (Arp2Δ Arp2/3 complex) from fission yeast. An x-ray crystal structure of the ΔArp2 Arp2/3 complex showed that the rest of the complex is unperturbed by the loss of Arp2. However, the Arp2Δ Arp2/3 complex was inactive in actin nucleation assays, indicating that Arp2 is essential to form a branch. A fluorescence anisotropy assay showed that Arp2 does not contribute to the affinity of the complex for Wsp1-VCA, a Schizosaccharomyces pombe nucleation-promoting factor protein. Fluorescence resonance energy transfer experiments showed that the loss of Arp2 does not prevent VCA from recruiting an actin monomer to the complex. Truncation of the N terminus of ARPC5, the smallest subunit in the complex, increased the yield of Arp2Δ Arp2/3 complex during purification but did not compromise nucleation activity of the full Arp2/3 complex.

Dynamic rearrangements of the actin cytoskeleton are essential for cellular responses to the environment, and cells employ a host of proteins to control nucleation, polymerization, capping, severing, bundling, cross-linking, and depolymerization of actin (1). Fission yeast have two well characterized nucleators (Cdc12 and For3) and Arp2/3 complex (consisting of seven subunits, Arp3, Arp2, and ARPC1–5) (2). Formins assemble unbranched filaments present both in cables that span the length of interphase cells and in the contractile ring, which constricts during cytokinesis (3, 4). Arp2/3 complex nucleates branched filaments in cortical structures called actin patches, which are sites of endocytosis located at cell poles during interphase and the cleavage furrow during cytokinesis (5–7).

Arp2/3 complex nucleates actin filament branches on the sides of pre-existing (mother) filaments (8). The new (daughter) filament grows at an angle of 78° on the side of the mother filament (9). Arp2/3 complex from most species is intrinsically inactive but is stimulated to form an actin filament branch through interactions with proteins called nucleation-promoting factors (NPFs), ATP, an actin monomer, and the side of a mother filament (2). WASp/Scar family proteins, the prototypical NPFs, contain a C-terminal region termed VCA (verprolin homology, central, acidic), which is the minimal fragment required to activate nucleation by Arp2/3 complex. Crystallographic and electron microscopic data suggest that a conformational change reorients the two actin-related subunits, Arp2 and Arp3, like two successive actin subunits along the short pitch helix of an actin filament to create the nucleus for polymerization of the daughter filament (9, 10). A model built by fitting crystal structures into reconstructions of electron tomograms of branch junctions shows that all seven subunits of Arp2/3 complex contact the mother filament and that the barbed ends of Arp2 and Arp3 interact with the pointed end of the daughter filament (9).

Numerous questions remain about the mechanism of branching nucleation despite many structural and kinetic studies. Nucleotide binding favors a conformation of Arp3 that may contribute to activation (11, 12). The V region of NPFs binds an actin monomer (13, 14), recruiting it to the branch point, whereas the C and A regions bind to Arp2/3 complex and are thought to facilitate conformational changes required for nucleation (14, 15). Cross-linking, radiation footprinting, and NMR experiments have implicated all but two subunits (ARPC2 and ARPC4) in interactions with VCA (16–19), but no high resolution structural information on VCA binding is available. A model based on small angle x-ray scattering of Arp2/3 complex bound to actin and VCA has the actin monomer located at the barbed end of Arp2 (20). Expression of recombiant human Arp2/3 complex subunits in insect cells demonstrated that ARPC2 and ARPC4 are essential for the integrity of the complex and that both of these subunits and Arp3 are necessary to assemble a complex that can nucleate actin filaments.
(22). This study did not address the role of Arp2 in the stability and nucleation activity of Arp2/3 complex. 

Here, we show that a complex lacking the Arp2 subunit (ΔArp2 Arp2/3 complex) can be isolated from fission yeast. Except for the absence of Arp2, the loss of Arp2 did not perturb the crystal structure of Arp2/3 complex. ΔArp2 Arp2/3 complex bound Wsp1-VCA but did not nucleate actin filaments, so Arp2 is essential to initiate a branch. Mutational analysis showed that branching nucleation does not require the N-terminus of ARPC5 to anchor Arp2 as proposed in one model of activation (22).

EXPERIMENTAL PROCEDURES

Purification of *S. pombe* Arp2/3 Complex—We purified native Arp2/3 complex from *Schizosaccharomyces pombe* strain TM011 typically starting with 500 g of wet cells. Ten milliliters of a turbid culture of cells was inoculated per liter of media made from 35 g/liter YES (Q-biogene) and grown with vigorous shaking overnight at 30 °C. In the morning, an additional 70 g/liter YES was added, and the cultures were grown to an optical density of ~6.0 at 600 nm. Cells were then pelleted and washed in lysis buffer (20 mM Tris, pH 8.0, 50 mM NaCl, and 1 mM EDTA). Pellets of cells were resuspended in 1 ml of lysis buffer per gram of wet cells and stored at −80 °C until lysis. After thawing, an additional 0.4 ml of lysis buffer plus DTT (to 1 mM) and Complete protease inhibitor tablets (Roche Applied Science, 1 tablet per 50 ml) were added per gram of cells. All subsequent steps were at 0–4 °C. Cells were lysed using a Microfluidizer (Microfluidics, model 110EH). Phenylmethylsulfonyl fluoride was added to 1 mM, and the lysate was centrifuged at 30,000 × g for 30 min. Supernatant was centrifuged a second time at 125,000 × g for 1 h, filtered through cheesecloth, and loaded onto a 150-ml Q-Sepharose column equilibrated with lysis buffer and loaded with a linear 300-ml gradient of 25–700 mM NaCl. Fractions containing recombinant protein were pooled and diluted with lysis buffer lacking NaCl to reduce the salt to 140 mM. The sample was then loaded onto a 10-ml column of glutathione-Sepharose 4B equilibrated with 20 mM Tris, pH 8.0, 140 mM NaCl, 2 mM EDTA, and 2 mM DTT (binding buffer) and washed with 5 column volumes of the same buffer. The wash buffer was allowed to drain to the top of the column bed, and 30 µl of 75 mM tobacco etch virus protease (from *E. coli* by elution from the pRK1043 tobacco etch virus vector kindly provided by D. Waugh (25)) was mixed into the glutathione-Sepharose slurry. Protein was released from the column during incubation with gentle rocking overnight at 4 °C. Eluted protein was pooled and loaded onto a 0.5-× 5-cm Mono Q column equilibrated with 20 mM Tris, pH 8.0, 100 mM NaCl, and 2 mM DTT. Protein was eluted with a 20-ml linear gradient of 100–800 mM NaCl, concentrated, and purified by size-exclusion chromatography onto a Superdex 75 HR16/60 column (GE Healthcare) in 20 mM Tris, pH 8.0, 100 mM NaCl, and 1 mM DTT before re-concentrating and flash freezing in liquid nitrogen.

Preparation of Rhodamine-labeled Sp-Wsp1/VCA Constructs—Sp-Wsp1/VCA was purified as the SpWsp1-VCA construct, except that DTT was excluded from the final gel filtration buffer. A fresh 20 mM stock of tetramethylrhodamine-6-maleimide (Invitrogen) in dimethylformamide was added dropwise to the pooled peak fractions of Sp-Wsp1/Cys-VCA while stirring on ice. Reaction was stopped after 1 h by the addition of 1 mM DTT. Labeled protein was separated from free dye by ion-exchange chromatography on a 0.5-× 5-cm Mono Q column and through repeated concentration and dilution in an Amicon Ultra protein concentrator. Concentration of SpWsp1-Rho-VCA (Rho-VCA) was determined by measuring absorbance at 552 nm with an extinction coefficient of 44,660 M–1 cm–1 (14).
Preparation of Labeled and Unlabeled and Actin Monomers—We purified actin by established procedures: chicken skeletal muscle actin (26), pyrene-labeled actin (27), and Oregon Green-actin (OG-actin) (28).

Construction of Mutant Strains of S. pombe—We constructed mutant strains by PCR-based gene targeting (29). For ARPC5 mutants, cassettes containing a KanMX6 module and the P3ntm1 thymine-repressible promoter were amplified with long primers to generate a 5′-flanking sequence complimentary to a region 330 nucleotides upstream from the ARPC5 start codon. The 3′-flanking sequence was complimentary to either the first 60 nucleotides of the ARPC5 coding region (with codon 3 mutated to change arginine to glutamic acid) or to a region 42 nucleotides downstream from the start codon (for ARPC5ΔN). Cassettes were transformed into the TM011 strain of S. pombe using the lithium acetate method (29, 30). Transformed cells were plated on YES plates and incubated for ∼18 h at 30 °C before re-plating onto YES plates containing 100 mg/ml G418/Geneticin (Invitrogen). Replica plates were incubated for 2–3 days, and large colonies were re-streaked on fresh YES plates and incubated for another 2–3 days, and large colonies were re-streaked on fresh YES plates and incubated for another 2–3 days. Genomic DNA was isolated from mutant strains (29), were plated on YES plates and incubated for 3 days, and large colonies were re-streaked on fresh YES plates. Genomic DNA was isolated from mutant strains (29), were plated on YES plates and incubated for 3 days, and large colonies were re-streaked on fresh YES plates. Genomic DNA was isolated from mutant strains (29), were plated on YES plates and incubated for 3 days, and large colonies were re-streaked on fresh YES plates. Genomic DNA was isolated from mutant strains (29), were plated on YES plates and incubated for 3 days, and large colonies were re-streaked on fresh YES plates.

Determination of Equilibrium Binding Constants—Binding constants were determined by fitting the anisotropy curves to Equation 1.

\[
r_f = r_b + (r_b - r_f) \cdot \frac{(K_d + [R] + [L])^2 - 4(K_d + [R] + [L])^2 - 4K_d[4r_f]}{2L}
\]

where \( r_f \) is the signal of the free receptor (\( R \)), \( r_b \) is the signal of the bound receptor, and \( [L] \) is the total concentration of the lipid (species titrated). \( r_b \) and \( K_d \) were fit using KaleidaGraph (Synergy Software). Determination of binding constants for unlabeled SpWsp1-VCA measured using competition assay is described in the supplemental materials.

Crystal Growth and Structure Determination—Crystals of ΔArp2 Arp2/3 complex grew in 750 mM ammonium sulfate, 30 mM sodium citrate, and 7% glycerol at 4 °C to an average size of 150 × 80 × 80 μm. ATP and calcium chloride were present in the crystallization drop at 0.5 mM each. The crystals diffracted weakly and were indexed to the P4,22 space group with a large unit cell (a = 219.1 Å, b = 219.1 Å, c = 315.2 Å) and two molecules in the asymmetric unit (76% solvent). Attempts to dehydrate crystals by increasing the precipitant concentration did not improve diffraction. We used a homology model of the S. pombe Arp2/3 complex based on the crystal structure of bovine Arp2/3 complex (31) with the Arp2 subunit removed as a molecular replacement search model. We also removed inserts and regions with high B-factors in the bovine complex structure from the search model. We found one solution using the program Phaser (32) with a translational Z-score of 25.6. The initial model was improved through three successive rounds of rebuilding and restrained refinement carried out using REFMAC with a weighting parameter of 0.005. B-factors were refined by TLS refinement using one TLS group for each subunit (33). The two molecules in the asymmetric unit were symmetrically constrained throughout the refinement. To improve density for model building, B-factors were sharpened using a value of 70 Å. The final model had an \( R_{sym} \) of 34.4% and an \( R_{work} \) of 32.4%. Coordinates were deposited in the Protein Data Bank with accession code 3DWL.

Fluorescence Microscopy—Cells were grown to \( A_{595} = 0.2–0.5 \) in YE5S at 25 °C and washed in Edinburgh minimal medium (potassium phthalate (3 g/liter), Na2SO4 (0.04 g/liter), ZnSO4 (0.4 mg/liter), Na2HPO4 (2.2 g/liter), pantothenic acid (1 mg/liter), FeCl2 (0.2 mg/liter), NH4Cl (5 g/liter), nicotinic acid (10 mg/liter), molybdic acid (40 μg/liter), dextrose (20 g/liter), myo-inositol (10 mg/liter), potassium iodide (0.1 mg/liter), MgCl2 (1.05 g/liter), biotin (1 mg/liter), CuSO4 (40 μg/liter), CaCl2 (14.7 mg/liter), boric acid (0.5 mg/liter), citric acid (1 mg/liter), KCl (1 g/liter), MnSO4 (0.4 mg/liter)) before mounting on 25% gelatin pads (34). Images were acquired on a UltraView RS (PerkinElmer Life Sciences) spinning disk confocal system installed on an Olympus IX-71.
Isolation of S. pombe Arp2/3 Complex Lacking Arp2—Arp2/3 complex from S. pombe purified as a single component during ammonium sulfate precipitation, ion-exchange chromatography (Q-Sepharose) and affinity chromatography on GST-N-WASp-VCA on a glutathione-Sepharose 4B column but split into two peaks during anion exchange chromatography on Mono Q. The first Mono Q peak eluted at a conductivity of 22 mS/cm, and the second peak eluted at 29 mS/cm (Fig. 1A). We completed the purification of the two fractions of Arp2/3 complex by gel filtration on a column of Superdex 200. Both peaks contained Arp2/3 complex subunits, but the band on SDS-PAGE previously shown to contain both Arp2 and ARPC1 (7) was less intense in pool A (Fig. 1B). Two-dimensional PAGE (Fig. 1C) showed that pool A lacked a cluster of four spots corresponding to Arp2 (theoretical pI = 5.8). Immunoblots with an S. pombe Arp2 antibody verified that Arp2 was missing (data not shown). The fraction of complex lacking Arp2 (ΔArp2/Arp2/3 complex) ranged from 5 to 35% (average = 13%) of total Arp2/3 complex in five preparations from wild-type cells.

Arp2 Is Required for Arp2/3 Complex Nucleation Activity—Polymerization assays showed that 200 nM ΔArp2 Arp2/3 complex did not nucleate actin filaments, whereas 200 nM of complete Arp2/3 complex (pool B) increased the concentration of barbed ends to a maximum of 3.5 nM in the presence of 1.6 μM SpWsp1-VCA and 4 μM chicken skeletal muscle actin (Fig. 2A). Even 1 μM ΔArp2Arp2/3 complex had no detectable nucleation activity (data not shown).
The ΔArp2 Arp2/3 Complex Binds SpWsp1-VCA and Actin Monomer—To determine the basis for the inactivity of ΔArp2 Arp2/3 complex, we used fluorescence anisotropy to measure the affinity of rhodamine-labeled SpWsp1-VCA (Rho-VCA) for complete and ΔArp2 Arp2/3 complex. Rho-VCA bound complete Arp2/3 complex with a dissociation equilibrium constant ($K_d$) of 49 ± 5 nM and the ΔArp2 complex with an affinity of 120 ± 13 nM (Fig. 2B, inset). Because the rhodamine label can affect the affinity of VCA for Arp2/3 complex (14), we carried out competition assays using unlabeled SpWsp1-VCA (VCA) (Fig. 2B). VCA had a slightly higher affinity ($K_d = 0.4 ± 0.1 \mu M$) for ΔArp2 Arp2/3 complex than native Arp2/3 complex ($K_d = 0.9 ± 0.1 \mu M$). Therefore, the Arp2-less complex is not inactive due to a loss of ability to bind this nucleation-promoting factor.

We next sought to determine if VCA bound to ΔArp2 Arp2/3 complex could recruit an actin monomer to form the ternary complex of Arp2/3 complex, NPF, and an actin monomer. This ternary complex is thought to assemble before binding to an actin filament to initiate a branch (14). To detect interaction of the VCA NPF with actin, we used fluorescence energy resonance transfer (FRET) from an Oregon Green 488 label on Cys-374 of actin (OG-actin) to Rho-VCA (Fig. 2C (35)). This assay gave a $K_d$ of 16 ± 2 nM for Rho-VCA binding OG-actin in the absence of Arp2/3 complex (Fig. 2D). FRET was then measured in the presence of 3 μM Arp2/3 complex, so that >98% of the Rho-VCA was bound to Arp2/3 complex. The presence of 3 μM complete Arp2/3 complex slightly increased the $K_d$ of Rho-VCA and OG-actin to 24 ± 1 nM, whereas the presence of 3 μM ΔArp2Arp2/3 complex had no effect on the binding ($K_d = 12 ± 2 \text{ nM}$). These results indicate that both complete and ΔArp2 Arp2/3 complexes can form a ternary complex with a VCA nucleation-promoting factor and an actin monomer. Because excess Arp2/3 complex does not increase the affinity of actin for Rho-VCA, we conclude that actin does not make productive contacts with Arp2/3 complex in the ternary complex.

Structure of ΔArp2 Arp2/3 Complex—To determine whether loss of the Arp2 subunit perturbs the structure of Arp2/3 complex, we solved the crystal structure of ΔArp2Arp2/3 complex using molecular replacement with a homology model based on the crystal structure of bovine Arp2/3 complex (31) to estimate initial phases. We used this model to generate a solvent-flattened electron density map averaged using the two molecules in the asymmetric unit. The map showed density for a number of backbone segments that adopt dramatically different conformations of the Arp2 complex with an affinity of 1.85 Å for 1166 aligned Cα atoms. Therefore, dissociation of Arp2 does not cause major changes in the rest of the complex (see supplemental Fig. S2 for detailed analysis).

The overall structure of the ΔArp2 Arp2/3 complex from S. pombe closely resembles bovine Arp2/3 complex, except for the absence of Arp2 (Fig. 3A). Arp2/3 complexes from these two species can be overlaid with an overall root mean square deviation of 1.85 Å for 1166 aligned Cα atoms. Therefore, dissociation of Arp2 does not cause major changes in the rest of the complex (see supplemental Fig. S2 for detailed analysis).

The complete S. pombe Arp2/3 complex did not crystallize under the same conditions used to grow crystals of ΔArp2 Arp2/3 complex. Modeling Arp2 into the ΔArp2 Arp2/3 complex crystal revealed that Arp2 sterically clashes with the Arp3 subunit from a symmetry-related complex, explaining why the packing arrangement is not possible if Arp2 is present.

The presence of the ARPC1 insert was the most striking feature of the electron density map of S. pombe ΔArp2 Arp2/3 complex (Fig. 3, A and B, and 4). Electron density for this region was present in the first $F_o - F_c$ map and subsequent rounds of rebuilding/refinement allowed us to build 19 of 43 residues of the insert. This region forms a random coil that inserts into the groove between subdomains 2 and 4 of Arp3 from the other molecule in the asymmetric unit (supplemental Fig. S3A). In some crystals of bovine Arp2/3 complex, the most conserved portion of the ARPC1 insert forms an α-helix, which packs against the barbed end of Arp3 between subdomains 1 and 3 in a symmetry-related molecule (supplemental Fig. S3B) (10). The conformations of the ARPC1 inserts differ markedly in crystals of S. pombe and bovine Arp2/3 complexes (Fig. 3A). The ordered region of the insert in the structure of ΔArp2 Arp2/3 complex consists of residues unique to S. pombe (Fig. 3C), whereas the conserved region of the insert that forms an α-helix in some bovine Arp2/3 complex structures is disordered in the

### Table 1: Data collection and refinement statistics

| Data collection statistics |  |
|---------------------------|--|
| Resolution limits (Å)     | 29.0-3.80 |
| Space group               | P4,22    |
| Cell constants            | a = b = 218.98 |
|                          | c = 315.05 |
| Mosaicity (°)             | 0.46     |
| Measured reflections      | 1,179,649 |
| Unique reflections        | 72,502   |
| Mean I/σ                  | 9.5 (2.6) |
| $R_{	ext{free}}$ (%)     | 19.8 (49.5) |
| Completeness (%)          | 99.4 (95.2) |

| Refinement statistics     |  |
|--------------------------|--|
| Modeled atoms            | 18,717 |
| $R_{	ext{free}}$, reflections | 3.84% (5%) |
| Average $B$-factor (Å²)  | 74.7   |
| Root mean square from ideal Bond lengths (Å) | 0.007 |
| Bond angles (°)          | 1.042  |
| Ramachandran statistics  |  |
| Most favored             | 1,751 (76.2%) |
| Additionally allowed     | 492 (21.4%) |
| Generously allowed       | 54 (2.3%) |
| Disallowed               | 0       |
| $R_{	ext{free}}$ (%)    | 34.4   |
| $R_{	ext{work}}$ (%)    | 32.4   |
Arp2/3 complex (\textit{S. pombe}) conformation (Fig. 5, from Arp3, ARPC1, and ARPC4 and migrates into the active terminus of ARPC5 serves as a tether for Arp2 as it dissociates.

The "migration model" for activation (22) proposed that the N terminus of ARPC5 is not essential for activity. Therefore, both mutants were defective in expelling Phloxin-B at 25 °C and 36 °C, but not at 30 °C (data not shown).

We tested this hypothesis by mutating the conserved arginine from the N terminus of ARPC5 to a glutamic acid (ARPC5R3E) or deleting the N terminus entirely (ARPC5ΔN). Both mutant strains grew normally at 30 °C on YES plates but slower than wild type at 25 °C and 36 °C on YES + 1 M NaCl (data not shown). Additionally, both mutants were defective in expelling Phloxin-B at 25 °C and 36 °C, but not at 30 °C (data not shown). The ARPC5ΔN strain yielded ~50% more purified complex lacking Arp2 than wild-type cells (data not shown). Purified ARPC5ΔN Arp2/3 complex (Fig. 5C) nucleated filaments as efficiently as native SpArp2/3 complex (Fig. 5D).

Arp2 Co-localizes with Other Arp2/3 Complex Subunits throughout the Cell Cycle—To determine if Arp2/3 complex in some parts of cells lacks Arp2, we imaged haploid strains of \textit{S. pombe} expressing both Arp2 C-terminally labeled with cyan fluorescent protein (CFP) and ARPC3 C-terminally labeled with yellow fluorescent protein (YFP). Both tagged genes were expressed from native promoters and provided the sole copy of the gene. Cells depending on both tagged proteins were viable but often misshapen. Their actin patches turned over slower than patches in cells with only one tagged Arp.3

Arp2 and ARPC3 co-localized in actin patches during all stages of the cell cycle (Fig. 6A). The relative fluorescence intensities of Arp2-CFP and ARPC3-YFP in patches were well correlated (Fig. 6B), showing that subpopulations of patches depleted of Arp2 were not present. The ratios of Arp2-CFP and ARPC3-YFP fluorescence were unequal in some patches moving away from the plasma membrane, owing to the time lapse between acquisition of CFP and YFP images. These results suggest that, if Arp2 dissociates from the rest of Arp2/3 complex in \textit{S. pombe}, it does not leave the actin patches.

DISCUSSION

Isolation and Activity of the ΔArp2 Arp2/3 Complex—We considered multiple hypotheses to explain why some of purified fission yeast Arp2/3 complex lacks Arp2. Measurements of fluorescently tagged Arp2/3 complex subunits in fission yeast indicated that all subunits were present in the cytoplasm at near equal concentrations, with Arp2 the second most abundantly expressed subunit (36). Therefore, low levels of Arp2 expression cannot explain the existence of the ΔArp2 Arp2/3 complex.

3 V. Sirotnik, personal communication.
Dissociation of Arp2 during purification is the most likely explanation for the absence of ΔArp2 Arp2/3 complex. No information is available on the affinity of individual subunits for Arp2/3 complex in S. pombe, but no Arp2 dissociated from the complete fission yeast complex during gel-filtration chromatography, indicating that the complete complex is stable under these conditions. A similar experiment with Acanthamoeba Arp2/3 complex showed that the highest dissociation equilibrium constant for any subunit is 70 nM (37). However, 7% of Arp2 dissociated when the complete complex was subjected to a second round of Mono Q purification. We conclude that Arp2 is the least tightly associated subunit in the S. pombe Arp2/3 complex (see supplemental Fig. S4). The mutated ARPC5 subunit has a higher mobility than the native subunit (arrow). D, comparison of the effects of native and ARPC5ΔN Arp2/3 complex on the time course of the polymerization of pyrene-labeled actin. Conditions: same as those in Fig. 2A with 80 nM wild-type (red) or ARPC5ΔN (green) Arp2/3 complex, 1.0 μM SpWsp1-VCA, 4 μM 15% pyrene-labeled Mg-ATP actin. The black line shows actin polymerization in the absence of Arp2/3 complex and activator.

from the rest of the complex for reconstitution experiments but could not isolate adequate amounts of purified Arp2 under either native or denaturing conditions (data not shown).

Previous work suggested that Arp2 may not be essential in some organisms, but no one had studied the biochemical properties of Arp2/3 complex lacking Arp2. Winter et al. (38) found that deletion of ARP2 from Saccharomyces cerevisiae is not lethal, suggesting that actin might substitute for Arp2 during filament branching. However, the E316K mutation of S. pombe Arp2 causes dissociation of Arp2 from the complex and septation defects at 36 °C indicating the importance of Arp2 (39). Gournier et al. (21) expressed human Arp2/3 complex without the Arp2 subunit in insect cells, but could not isolate the Arp2Δ Arp2/3 complex.

Our preparation of fission yeast Arp2/3 complex lacking Arp2 did not nucleate actin filaments in an assay with SpWsp1-VCA and chicken skeletal muscle actin (Fig. 2A), so actin cannot substitute for Arp2 in the branching reaction. S. pombe Arp2 and chicken skeletal muscle actin are 45% identical, but many residues of Arp2 that contact ARPC4, ARPC1, or ARPC5 are different in actin. For example, the αG helix in Arp2 (residues 226–236) and the loop immediately following it (residues 237–242) make extensive contacts to ARPC4, and only four residues in this region are identical in actin and Arp2. In addition, both Arp2 and Arp3 contain an insert in the αK/β15 (Arp2 residues 320–334) loop not present in actin. In Arp2, this insert forms a major part of the interaction surface with the N terminus of ARPC5. In the model based on the reconstruction of branch junctions (9), the DNase-binding loop of Arp2 (residues 39–51) interacts with an actin subunit in the mother filament. The DNase-binding loop of Arp2 differs in sequence and is two residues longer than in actin. These differences provide a convincing structural basis for the failure of actin to substitute for Arp2 in the nucleation reaction.

The presence of Arp2/3 complex lacking Arp2 in extracts of S. pombe led us to wonder if dissociation of Arp2 plays a role in regulating Arp2/3 complex activity in vivo. The inactivity of ΔArp2 Arp2/3 complex is consistent with the observation that the E316K mutation in Arp2 causes defects in cell septation, a process thought to require Arp2/3 complex (39, 40). Fluorescence microscopy of wild-type fission yeast strains expressing Arp2-CFP and ARPC3-YFP showed that the ratio of Arp2 to ARPC3 was uniform in actin patches throughout the cell cycle.
FIGURE 6. Arp2-CFP and ARPC3-YFP co-localize to actin patches. A, spinning disk confocal fluorescence micrographs of a haploid strain of S. pombe with Arp2 tagged on its C terminus with CFP and ARPC3 tagged on its C terminus with YFP, both expressed from their native promoters. Maximum projection images created from fourteen 0.6 μm Z-sections. Left panel, Arp2-CFP intensity; center panel, ARPC3-YFP intensity; and right panel, merged images with Arp2-CFP intensity shown in red and ARPC3-YFP intensity shown in green. B, correlation of the intensity of CFP and YFP fluorescence in individual actin patches. Plot of mean CFP and YFP intensity for 163 patches in 3 cells. The linear correlation coefficient = 0.81.

Interaction of VCA and Actin Monomers with Arp2/3 Complex Lacking Arp2—We found that the loss of Arp2 does not decrease the affinity of SpWsp1-Rho-VCA for SpArp2/3 complex. Similarly, GST-Bee1-VCA (a budding yeast WASp homolog construct) pulled down similar amounts of Arp3 from extracts of wild-type and Δarp2 strains of budding yeast (41). This is consistent with experiments that showed that most of the binding energy of GST-Bee1-VCA for ScArp2/3 complex comes from its interaction with the ARPC1 (Arc40) subunit (41). On the other hand, VCA constructs protect Arp2 of bovine Arp2/3 complex from oxidation in synchrotron radiation footprinting experiments (16) and have been chemically cross-linked to the Arp2 subunit of Arp2/3 complex from multiple species (17–19). All of these observations may be reconciled, if VCA contacts Arp2 without contributing strongly to the binding affinity.

Our FRET assays confirmed that the affinity of OG-actin for rhodamine-VCA is slightly weaker in the presence of the full Arp2/3 complex (Kd = 16 ± 2 nm without Arp2/3 complex, 24 ± 1 nm with Arp2/3 complex) (35). This indicates that VCA, actin, and Arp2/3 complex can interact simultaneously, but that the actin monomer in this ternary complex does not make productive contacts with Arp2/3 complex. We suggest that actin in the ternary complex must reorient relative to Arp2/3 complex subunits during the activation step (35) to establish a nucleus for the polymerization of the daughter filament. The lower affinity of actin for VCA in the presence of Arp2/3 complex also suggests that actin and Arp2/3 complex compete for a common binding site on VCA. The C region is the best candidate for this common site (14), because it binds weakly to both actin (Kd = 12 μm) and Arp2/3 complex (Kd > 200 μm) in a mutually exclusive manner (42).

The FRET assay showed that the affinity of VCA for OG-actin is 2-fold stronger (Kd = 12 ± 2 nm) with ΔArp2 Arp2/3 than full Arp2/3 complex (Kd = 24 ± 1 nm). We speculate that dissociation of Arp2 either relieves steric inhibition that prevents the C region from interacting with actin and/or that Arp2 subunit completes with actin directly by weakly binding the C region.

We note that our results differ from the interpretation of a small angle x-ray scattering model of Arp2/3 with bound activator and monomeric actin (20), in which the C-region of the activator makes extensive interactions with Arp2. Although we do not rule out weak interactions between Arp2 and the activator, our data show that the loss of Arp2 increases the affinity of VCA for Arp2/3 complex and does not affect recruitment of actin to VCA bound to Arp2/3 complex.

Insights into the Mechanism of Arp2/3 Complex Activation—Our structure of the ΔArp2 Arp2/3 complex shows that dissociation of the Arp2 subunit did not perturb the remaining subunits in the complex. This establishes the feasibility that Arp2 partially dissociates during the conformational change that activates the complex (9). However, the N terminus of ARPC5, which has been proposed to tether Arp2 to the complex during the proposed conformational rearrangement (22), is not necessary for Arp2/3 complex activity in vitro, suggesting that Arp2 maintains contacts with other subunits during activation. This observation supports a model where a twisting motion rotates a rigid body composed of Arp2, ARPC1, ARPC4, and ARPC5 into the active conformation (10). The interpretation of small angle x-ray scattering from Arp2/3 complex with bound activator and actin monomer also supports the rotation model (20).

Although the N-terminal tether of ARPC5 does not play a fundamental role in the activation of Arp2/3 complex, it may be important in stabilizing interactions of Arp2 with the rest of the complex. Consistent with this hypothesis, we recovered more ΔArp2 Arp2/3 complex from the ARPC5ΔN strain than wild-type cells. The residues involved in the interface between ARPC5 and Arp2 are conserved in most species (supplemental Fig. S4), but in most plant species the N terminus of ARPC5 is
too short to reach Arp2 in models based on structures of inactive bovine Arp2/3 complex (10–12) or the model of the branch junction based on electron tomography (9). Therefore, the stabilizing function of the N terminus of ARPC5 is unlikely to occur in plants.

We were surprised that Arp2 does not contribute to the affinity of VCA for Arp2/3 complex and that, in fact, the affinity is slightly higher without Arp2. This observation suggests that VCA facilitates the movement of Arp2 next to Arp3 without strongly interacting with Arp2. Perhaps Arp2 is a passive participant in activation, with interactions of other subunits of Arp2/3 complex with NPFs and a mother filament providing most of the free energy for the conformational change that creates a favorable binding site for the first actin subunit of the daughter filament with Arp2 and Arp3. Alternatively, VCA may interact strongly with Arp2 only after the complex is bound to mother filament. Consistent with this hypothesis, kinetic and thermodynamic data demonstrated multiple modes of NPF binding to Arp2/3 complex (42), and sequence similarities between C and V regions suggest that the C region may interact with Arp2 just as V interacts with actin during activation (43). Elucidation of this complex activation mechanism will require much more structural, biophysical, and biochemical information than is currently available.

Acknowledgments—We thank Kathleen Gould for the S. pombe Arp2 antibody, Chris Beltzner for yeast strains with Arp2 and Arp3 tagged with CFP and YFP, Vladimir Sirotkin for help with microscopy, Aaron Downs for help with 2D gels, Yong Xiong for advice on analyzing low resolution data, Hongli Chen for assistance with protein preparations, Aditya Paul for comments on the manuscript, and Shih-Chieh Ti and Julien Berro for deriving the formula for fitting the competition binding curve.

REFERENCES

1. Pollard, T. D., Blanchoin, L., and Mullins, R. D. (2000) Annu. Rev. Biophys. Biomol. Struct. 29, 545–576
2. Pollard, T. D. (2007) Annu. Rev. Biophys. Biomol. Struct. 36, 451–477
3. Chang, F., Drubin, D., and Nurse, P. (1997) J. Cell Biol. 137, 169–182
4. Feierbach, B., and Chang, F. (2001) Curr. Biol. 11, 1656–1665
5. McCollum, D., Fekotkstova, A., Morphew, M., Balasubramanian, M., and Gould, K. L. (1996) EMBO J. 15, 6438–6446
6. Toshima, J. Y., Toshima, J., Kaksonen, M., Martin, A. C., King, D. S., and Drubin, D. G. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 5793–5798
7. Sirotkin, V., Beltzner, C. C., Marchand, J. B., and Pollard, T. D. (2005) J. Cell Biol. 170, 637–648
8. Amann, K. J., and Pollard, T. D. (2001) Nat. Cell Biol. 3, 306–310
9. Rouiller, I., Xu, X. P., Amann, K. J., Egile, C., Nickell, S., Nicolato, D., Li, R., Pollard, T. D., Volkmann, N., and Hanein, D. (2008) J. Cell Biol. 180, 887–895
10. Robinson, R. C., Turbedsky, K., Kaiser, D. A., Marchand, J. B., Higgs, H. N., Choe, S., and Pollard, T. D. (2001) Science 294, 1679–1684
11. Nolen, B. J., Littlefield, R. S., and Pollard, T. D. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 15627–15632
12. Nolen, B. J., and Pollard, T. D. (2007) Mol. Cell 26, 449–457
13. Chereau, D., Kerff, F., Graceffa, P., Grabarek, Z., Langsetmo, K., and Dominguez, R. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 16644–16649
14. Marchand, J. B., Kaiser, D. A., Pollard, T. D., and Higgs, H. N. (2001) Nat. Cell Biol. 3, 76–82
15. Panchal, S. C., Kaiser, D. A., Torres, E., Pollard, T. D., and Rosen, M. K. (2003) Nat. Struct. Biol. 10, 591–598
16. Kieselar, J. G., Mahaffy, R., Pollard, T. D., Almo, S. C., and Chance, M. R. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 1552–1557
17. Kreisman-Deitrick, M., Goley, E. D., Burdine, L., Denison, C., Egile, C., Li, R., Murrali, N., Kodadek, T. J., Welch, M. D., and Rosen, M. K. (2005) Biochemistry 44, 15247–15256
18. Zalevsky, J., Grigorova, L., and Mullins, R. D. (2001) J. Biol. Chem. 276, 3468–3475
19. Zalevsky, J., Lempert, L., Krantz, H., and Mullins, R. D. (2001) Curr. Biol. 11, 1903–1913
20. Boczowska, M., Rebowsi, G., Petoukhov, M. V., Hayes, D. B., Svergun, D. I., and Dominguez, R. (2008) Structure 16, 695–704
21. Gournier, H., Goley, E. D., Niederstrasser, H., Trinh, T., and Welch, M. D. (2001) Mol. Cell 8, 1041–1052
22. Irobi, E., Aguda, A. H., Larsson, M., Guerin, C., Yin, H. L., Burtnick, L. D., Blanchoin, L., and Robinson, R. C. (2004) EMBO J. 23, 3599–3608
23. Higgs, H. N., Blanchoin, L., and Pollard, T. D. (1999) Biochemistry 38, 15122–15222
24. Gill, S. C., and von Hippel, P. H. (1989) Anal. Biochem. 182, 319–326
25. Kapust, R. B., Tozer, J., Fox, J. D., Anderson, D. E., Cherry, S., Copeland, T. D., and Waugh, D. S. (2001) Protein Eng. 14, 993–1000
26. MacLean-Fletcher, S., and Pollard, T. D. (1980) Biochem. Biophys. Res. Commun. 96, 18–27
27. Pollard, T. D. (1984) J. Cell Biol. 99, 769–777
28. Kuhn, J. R., and Pollard, T. D. (2005) Biophys. J. 88, 1387–1402
29. Bahler, J., Wu, J. Q., Longtine, M. S., Shah, N. G., McKenzie, A., 3rd, Steever, A. B., Wach, A., Philippen, P., and Pringle, J. R. (1998) Yeast 14, 943–951
30. Keeney, J. B., and Boeke, J. D. (1994) Genetics 136, 849–856
31. Beltzner, C. C., and Pollard, T. D. (2004) J. Mol. Biol. 336, 551–565
32. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, L. C., Storoni, L. C., and Read, R. J. (2007) J. Appl. Crystallogr. 40, 658–674
33. Winn, M. D., Isupov, M. N., and Murshudov, G. N. (2001) Acta Crystallogr. D. Biol. Crystallogr. 57, 122–133
34. Wu, J. Q., Kuhn, J. R., Kovar, D. R., and Pollard, T. D. (2003) Dev. Cell 5, 723–734
35. Beltzner, C. C., and Pollard, T. D. (2007) J. Biol. Chem. 283, 7135–7144
36. Wu, J. Q., and Pollard, T. D. (2005) Science 310, 310–314
37. Mullins, R. D., Stafford, W. F., and Pollard, T. D. (1997) J. Cell Biol. 136, 331–343
38. Winter, D. C., Choe, E. Y., and Li, R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7288–7293
39. Morrell, J. L., Morphew, M., and Gould, K. L. (1999) Mol. Biol. Cell 10, 4201–4215
40. Welch, M. D., Holtzman, D. A., and Drubin, D. G. (1994) Curr. Opin. Cell Biol. 6, 110–119
41. Pan, F., Egile, C., Lipkin, T., and Li, R. (2004) J. Biol. Chem. 279, 54629–54636
42. Kelly, A. E., Krantz, H., Dotsch, V., and Mullins, R. D. (2006) J. Biol. Chem. 281, 10589–10597
43. Aguda, A. H., Burtnick, L. D., and Robinson, R. C. (2005) EMBO Rep. 6, 220–226
44. Boujemaa-Paterski, R., Gouin, E., Hansen, G., Samarin, S., Le Clainche, C., Didry, D., Deboux, P., Cossart, P., Kocks, C., Carlier, M. F., and Pantaloni, D. (2001) Biochemistry 40, 11390–11404