Pathway of Actin Filament Branch Formation by Arp2/3 Complex

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A spectroscopic assay using pyrene-labeled fission yeast Arp2/3 complex revealed that the complex binds to and dissociates from actin filaments extremely slowly with or without the nucleation-promoting factor fission yeast Wsp1-VCA. Wsp1-VCA binds both Arp2/3 complex and actin monomers with high affinity. These two ligands have only modest impacts on the interaction of the other ligand with VCA. Simulations of a mathematical model based on the kinetic parameters determined in this study and elsewhere account for the full time course of actin polymerization in the presence of Arp2/3 complex and Wsp1-VCA and show that an activation step, postulated to follow binding of a ternary complex of Arp2/3 complex, a bound nucleation-promoting factor, and an actin monomer to an actin filament, has a rate constant at least 0.15 s⁻¹. Kinetic parameters determined in this study constrain the process of actin filament branch formation during cellular motility to one main pathway.

Arp (actin-related protein) 2/3 complex nucleates actin filaments as branches on pre-existing actin filaments so that growth of these filaments can transmit force for cellular motility through the cytoskeleton to the substrate (1). Extensive structural and biochemical data established that Arp2/3 complex is intrinsically inactive, so nucleation depends on interactions with proteins called nucleation-promoting factors (2), actin monomers, and actin filaments. Four reactants create many possible pathways to the birth of a daughter filament on the side of a mother filament (see Fig. 1). The only way to identify the favored pathway is to measure the rates of all of the reactions and the concentrations of each of the reactants.

An initial study by Marchand et al. showed that the VCA (verprolin homology, central, and acidic) region of the nucleation-promoting factor WASp (Wiskott-Aldrich-syndrome protein) interacts with both actin monomers and Arp2/3 complex in rapid equilibria (3). The verprolin homology and central region of these nucleation-promoting factors binds actin monomers, and the CA region binds Arp2/3 complex. Thus the C region binds both actin and Arp2/3 complex but not simultaneously (4). The dissociation equilibrium constant for human WASp-VCA binding actin monomers is 0.6 μM, and the rate of exchange at equilibrium (k⁺) is 3 s⁻¹. WASp-VCA binds bovine Arp2/3 complex with a Kd of 0.9 μM and k⁻ of 0.2 s⁻¹. Because a mutation in human WASp-VCA from a patient with Wiskott-Aldrich syndrome did not alter its affinity for actin monomers or Arp2/3 complex but decreased the number of actin filament barbed ends formed in bulk samples, Marchand et al. proposed a rate-limiting activation step, after a ternary complex consisting of Arp2/3 complex, VCA, and an actin monomer binds a mother filament and before the branch starts to grow.

In the absence of a full set of rate constants, the possible pathways from reactants to branches were not well constrained. In particular nothing was known about the rates of which Arp2/3 complex binds and dissociates on the sides of mother filaments. To define the pathway more rigorously, we sought new spectroscopic assays to measure the rates of interactions between Arp2/3 complex, nucleation-promoting factors, actin monomers, and actin filaments. Ideally, the signal from an assay to measure the interaction between any two of the reactants will be insensitive to the presence of other binding partners, so that any perturbation of the reaction by other binding partners can be determined. Fig. 1 shows a detailed kinetic mechanism for the formation of actin filament branches to provide a guide to the reactions that we studied.

EXPERIMENTAL PROCEDURES

Purification of S. pombe Arp2/3 Complex—Native Arp2/3 complex was purified from protease-deficient TM011 strain of Schizosaccharomyces pombe. Cells were suspended in 1 ml of buffer U (50 mM HEPES, 100 mM KCl, 3 mM MgCl₂, 1 mM EGTA, and 1 mM DTT, pH 7.5) per gram of wet cell pellet and stored at −80 °C. All subsequent steps were carried out at 0–4 °C. Complete protease inhibitor tablets (Roche Applied Science, 1 tablet per 50 ml) were added to thawed cell pellets, and cells were lysed by mechanical disruption in a Microfluidizer (model M-110S, Microfluidics). Lysates were centrifuged at 20,000 × g for 20 min, and the supernatant was centrifuged again at 100,000 × g for 1 h. Proteins in the supernatant were precipitated with 40% ammonium sulfate, resuspended in buffer PKME (10 mM PIPES, 50 mM KCl, 3 mM MgCl₂, 1 mM
EGTA, 0.1 mM ATP, 1 mM DTT, pH 6.5), and then dialyzed against PKME overnight. The dialyzed sample was clarified by centrifugation at 150,000 × g for 1 h before being applied to a column of 15 mg of GST-N-WASp-VCA immobilized on 2 ml of glutathione-Sepharose. The column was washed with 30 ml of PKME, 20 ml of PKME-150 (10 mM PIPES, 150 mM KCl, 3 mM MgCl₂, 1 mM EGTA, 0.1 mM ATP, 1 mM DTT, pH 6.5), eluted with buffer Q (10 mM PIPES, pH 6.8, 25 mM NaCl, 0.25 mM MgCl₂, 0.25 mM EGTA, 1 mM DTT) containing 1 mM NaCl and then dialyzed against buffer Q for at least 2 h. Arp2/3 complex was further purified by ion-exchange chromatography on a 1-ml column of Mono Q (AKTA FPLC, GE Healthcare). DTT was omitted from FPLC buffers, if the protein was to be labeled with a fluorescent dye. A typical yield was 1.2 mg from 50 grams of cells.

Labeling Arp2/3 Complex—Arp2/3 complex for fluorescent labeling was immediately pooled from the Mono Q fractions and concentrated to at least 1 mg/ml using an Amicon ultra-4, 50,000 MWCO (Millipore). Concentrated fractions were reacted with a 20-fold molar excess of N-(1-pyrenyl)iodoacetamide or Oregon Green 488 iodoacetamide on ice in the dark for up to 2 h. The reaction was quenched by addition of 10 mM DTT, centrifuged at 16,000 × g for 15 min at 4 °C to remove insoluble dye and dialyzed against KMEI (10 mM imidazole, pH 7.0, 50 mM KCl, 1 mM MgCl₂, 1 mM EGTA) with 0.1 mM ATP and 1 mM DTT overnight before use.

Purification and Labeling of Other Proteins—Skeletal muscle actin (5), pyrene-labeled actin (6), GST fusion of Wsp1 VCA (7), rhodamine-VCA (3), and capping protein (8) were purified and labeled with fluorescent dyes.

Fission Yeast Mutagenesis—Mutations were created by introducing nucleotide changes into the forward integration primer for fission yeast transformations using the pFA6a vector (9). Isolating the non-parental dittype in tetrad analysis identified the double mutant strain.

Mass Spectrometry—Purified S. pombe Arp2/3 complex was incubated with a 20-fold molar excess of Oregon-Green 488 iodoacetamide for 2 h on ice. Labeled and unlabeled samples were analyzed by SDS-PAGE. The fluorescence band (Fig. 2B) and the corresponding band on the unlabeled control were cut from the gel and subjected to in-gel trypsin digest. Analysis of peptide masses by matrix-assisted laser desorption ionization time-of-flight mass spectroscopy by the Yates laboratory at Scripps Research Institute revealed a single peptide differing by 550 atomic mass units between the two samples. Theoretical trypsin digest maps of ARPC3 and ARPC4 identified the peptide as the C-terminal peptide of ARPC4, containing a single cysteine, residue 167.

Pyrene Arp2/3 Complex Binding Assays—For equilibrium binding assays involving actin filaments, actin was polymerized for 1 h at room temperature before adding 50 nm pyrene-labeled Arp2/3 complex and incubating for an additional 2 h. Pyrene fluorescence was measured with an Alphascan fluorometer (Photon Technology International) with excitation at 365 nm and emission at 390 nm. The average counts per second for a 10-s scan are reported. For kinetic experiments, actin filaments were polymerized for 10–20 min before mixing by hand with 50 nm pyrene Arp2/3 complex and starting time courses.

Fluorescence emission was measured for 1 s at 5-s intervals. Time courses were fit to single or double exponentials to yield observed rate constants.

Fluorescence Anisotropy—Anisotropy measurements were made with an Alphascan fluorometer in a T-format. Fixed concentrations of rhodamine-VCA or VCA-rhodamine were mixed with actin or Arp2/3 complex and incubated for at least 30 min in KMEI or buffer G. Samples were excited with polarized light at 552 nm, and polarized emitted light was measured at 574 nm with 0.1 point/s for 30 s. The average anisotropy was calculated using PTI Felix software.

FRET Measurements—FRET measurements were made with an Alphascan fluorometer. For steady-state measurements a fixed concentration of actin labeled on Cys-374 with Oregon Green 488 (Oregon Green actin) was mixed with a range of concentrations of rhodamine-VCA in the presence or absence of 1.5 μM Arp2/3 complex in KMEI and incubated for at least 30 min at room temperature. Emission scans from 510 to 610 nm were made using an excitation wavelength of 470 nm. Percent donor fluorescence quenched was calculated by comparing the average fluorescence from 519 to 524 nm with OG-actin alone. Kinetic experiments were performed using a MiniMixer stopped-flow apparatus (KinTek). The samples were excited at 470 nm, and emission was measured at 519 nm with a 3-nm slit width on the Alphascan monochromator. Time courses were fit to single exponentials to yield observed rate constants.

Determination of Equilibrium and Kinetic Constants—Equilibrium dissociation constants (K₀) were calculated by fitting Equation 1 to the ligand concentration dependence of the signal (S) for pyrene fluorescence, anisotropy, or fraction donor fluorescence quenched,

\[
S = S_0 + (S_0 - S_l)([L] + K_0) - ([L] + K_0)^2 - 4*([L])^2/2*([L]) \text{ (Eq. 1)}
\]

where \(S_l\) is the signal of the free receptor (R), \(S_0\) is the signal of the bound receptor, and \([L]\) is the concentration of the ligand (species on the x-axis). \(S_0\) and \(K_0\) were left as parameters to be fit. For Fig. 4D, \(S_0\) was also left as a parameter to be fit.

We calculated the association (kₐ) and dissociation (k₋) rate constants by fitting Equation 2 to the observed rate constants \(k_{obs}\).

\[
k_{obs} = k_a[L] + k_-
\]

where \([L]\) is the total concentration of ligand (species on the x-axis).

Actin Filament Assembly Assays—Assembly of actin was measured with an Alphascan fluorometer from the fluorescence of pyrene-labeled actin (10) with 4 μM actin monomers (5% pyrene-labeled), 1 mM rhodamine-VCA, and a range of concentrations of fission yeast Arp2/3 complex. Fluorescence readings were converted to actin filament concentrations by normalizing the starting signal to 0 μM actin filaments and the average plateau value to 3.84 μM.

Simulations of Actin Filament Assembly—Models were built, and simulations were run using Vcell 4.2 from National Resource for Cell Analysis and Modeling (NRCAM, www.vcell.org).
RESULTS

Creation of Fluorescent Derivatives of S. pombe Arp2/3 Complex—Cysteine 167 of ARPC4 is the only reactive cysteine in S. pombe Arp2/3 complex, but it is not a good site to attach a fluorescent dye for biochemical experiments, because it interferes with Arp2/3 complex function (see supplemental text). Reaction of purified S. pombe Arp2/3 complex with Oregon Green 488 iodoacetamide resulted in dye incorporation into only the ARPC4 subunit (Fig. 2, A and B). Analysis of tryptic peptides by mass spectroscopy identified a single peptide of ARPC4 containing cysteine 167 that differed by ~550 atomic mass units between labeled and unlabeled samples. The other 22 cysteines in the complex did not react with Oregon Green 488 iodoacetamide.

Mutation of ARPC4 cysteine 167 to serine resulted in the loss of Oregon Green 488 incorporation into Arp2/3 complex without altering its biochemical activity in actin filament nucleation assays (data not shown). Fission yeast expressing this mutation had no noticeable growth or morphology defects. Therefore the C167S mutation in ARPC4 provided a suitable background for creating new labeling sites.

To develop a spectroscopic assay for interactions between Arp2/3 complex and actin filaments, we used our phylogenetic analysis of Arp2/3 complex (12) and existing biochemical evidence to pick a labeling site that might be close to the mother filament in branches. We chose to mutate the genome to replace a solvent-exposed, unconserved alanine (317) at the C terminus of ARPC2 with cysteine. We crossed the ARPC2 A317C strain with the strain containing the ARPC4 C167S mutation. The double mutant had normal growth and morphology under the conditions tested.

Reaction of Oregon Green 488 iodoacetamide with purified Arp2/3 complex from the double mutant (ARPC4-C167S/ARPC2-A317C) consistently labeled the ARPC2 subunit (Fig. 2B). The reaction went to completion in 2 h (data not shown). Oregon Green 488 occasionally labeled the band containing Arp2 and ARP1 (Fig. 2B). Reaction of ARPC4 C167S/ARPC2 A317C Arp2/3 complex with N-(1-pyrenyl)iodoacetamide cre-
ated “pyrene-labeled Arp2/3 complex,” which behaved the same as wild-type fission yeast Arp2/3 complex by SDS-PAGE and in actin assembly assays monitored with the fluorescence of pyrene-labeled actin. However, pyrene-labeled Arp2/3 complex contributes to the overall pyrene fluorescence, so we did not attempt a quantitative analysis of actin assembly with this assay.

Interaction of Arp2/3 Complex with Actin Filaments—Titration of pyrene-labeled Arp2/3 complex with unlabeled chicken skeletal muscle actin filaments gave a concentration-dependent increase in fluorescence up to 5-fold (Fig. 2C). Fitting a binding isotherm to this data yielded a dissociation equilibrium constant of 4.6 ± 0.5 μM (Fig. 2D), similar to the affinity of Acanthamoeba Arp2/3 complex for actin filaments measured with cosedimentation assays (11). The extent of fluorescence enhancement was not affected by the presence of capping protein or by extensive shearing of the actin filaments (data not shown), treatments that increase the number of filament ends. The fluorescence enhancement being sensitive to the concentration of polymerized actin, but not the number of filament ends, suggests that the fluorescence enhancement is due to Arp2/3 complex binding to the sides of actin filaments rather than barbed or pointed ends.

The fluorescence of pyrene-labeled Arp2/3 complex increased very slowly following the addition of actin filaments (Fig. 2E). A single exponential with a rate constant on the order of 10⁻³ s⁻¹ fit the time course of the fluorescence change. We measured the time courses of the fluorescence change over a range of actin filament concentrations and plotted $k_{obs}$ versus actin filament concentration (Fig. 2F). The plot was linear with
a slope corresponding to the association rate constant ($k_a$) of $1.5 \times 10^{-3} \mu\text{M}^{-1} \text{s}^{-1}$ and a $y$-intercept corresponding to the dissociation rate constant ($k_d$) of $1.0 \times 10^{-3} \text{s}^{-1}$ (Fig. 2F). The ratio of these rate constants ($K_d = k_d / k_a = 6.7 \mu\text{M}$) confirmed the equilibrium measurements of the affinity. The time course of binding did not differ significantly for actin filaments polymerized for 6 min (a mixture of ATP-, ADP-Pi, and ADP-actin) and 45 min (mostly ADP-actin) (data not shown). The presence of a saturating concentration, 3 M, of the nucleation-promoting factor *S. pombe* Wsp1 VCA fused to GST did not alter the association rate constant, but increased the dissociation rate constant to $3.4 \times 10^{-3} \text{s}^{-1}$ (Fig. 2F).

**Fluorescence Anisotropy Assays for Binding of a Nucleation-promoting Factor to Arp2/3 Complex or Actin Monomers**—We used fluorescence anisotropy (3) to measure interactions between *S. pombe* Wsp1 VCA labeled on a N-terminal cysteine residue with tetramethylrhodamine iodoacetamide (rhodamine-VCA) and chicken skeletal muscle actin monomers or *S. pombe* Arp2/3 complex (Fig. 3). Titration of a low concentration of rhodamine-VCA with unlabeled actin monomers (Fig. 3A) or unlabeled Arp2/3 complex (Fig. 3B) both gave binding isotherms with $K_d$s of 16 nM.

The anisotropy of rhodamine-VCA does not have a favorable signal-to-noise ratio to measure interactions of the complex of VCA and an actin monomer with Arp2/3 complex, so we used *S. pombe* Wsp1 VCA labeled on the C terminus (VCA-rhodamine). The fluorescent dye on the C terminus is far from the actin binding site, allowing the flexible VCA-rhodamine peptide to retain considerable rotational diffusion even when bound to an actin monomer. The rotational diffusion decreases when the C-terminal A region of VCA binds Arp2/3 complex, giving a favorable anisotropy change. VCA-rhodamine bound Arp2/3 complex with a $K_d$ of 130 nM by itself and a $K_d$ of 310 nM in the presence of 3 M actin monomers in a low ionic strength buffer (G-buffer, Fig. 3C). In a physiological strength buffer (KMEI), VCA-rhodamine bound Arp2/3 complex with a $K_d$ of 180 nM by itself and a $K_d$ of 100 nM in the presence of 3 M Latrunculin-A (Lat-A) actin (Fig. 3D). Therefore actin bound to VCA has only a small effect on the affinity of VCA for Arp2/3 complex, a 2-fold negative effect at low ionic strength, and a 2-fold positive effect with Lat-A actin at physiological ionic strength.

**FRET Assay for Binding of a Nucleation-promoting Factor to Actin Monomers**—We developed an assay based on FRET to measure interactions between nucleation-promoting factors and actin monomers without interference by Arp2/3 complex. Actin monomers labeled with Oregon-Green 488 on Cys-374 served as the “donor” and VCA containing an N-terminal cysteine labeled with tetramethylrhodamine (rhodamine-VCA) served as the “acceptor.” Equilibrium binding experiments showed that a saturating concentration of acceptor quenches the donor fluorescence by ~80% (Fig. 4, A and B). From the concentration dependence of the FRET signal we measured the affinity of rhodamine-VCA for Oregon Green actin monomers. The dissociation equilibrium constant was 15 nM in the absence and 29 nM in the presence of 1.5 M Arp2/3 complex (Fig. 4B).

We used competition experiments with unlabeled actin monomers to determine the dissociation rate of Oregon Green actin from rhodamine-VCA in the presence and absence of *S. pombe* Arp2/3 complex. In both experiments the rate of fluorescence increase due to the loss of FRET was 0.44 s$^{-1}$ (Fig. 4C). From the $K_d$ and $k_a$ measured in these experiments we calculate the $k_d$ for rhodamine-VCA binding an actin monomer to be 29.3 M$^{-1}$ s$^{-1}$ when rhodamine-VCA is free in solution and 15.2 M$^{-1}$ s$^{-1}$ when rhodamine-VCA is bound to Arp2/3 complex.

**Estimation of the Activation Rate Constant by Mathematical Modeling**—With our measurement of the kinetics of Arp2/3 complex interactions with actin filaments, we felt we had enough information to develop a mathematical model of actin assembly under standard *in vitro* conditions. Primarily, we wanted to measure the rate of an activation step, which was postulated to occur after the ternary complex of actin monomer, VCA, and Arp2/3 complex binds to the side of a mother filament and before the daughter filament starts to grow (3). This activation step was proposed to explain how a mutation in WASp VCA might reduce the rate of branch formation with...
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FIGURE 4. Measurement of interactions between VCA and actin monomers with FRET. A, emission spectrum (480 nm excitation) of 100 nM actin monomers labeled on cysteine 374 with Oregon Green 488 in the absence (open circles, solid curve) and presence of 1 μM rhodamine-VCA (open square, dashed curve). B, equilibrium binding experiment with 10 nM Oregon Green actin and a range of concentrations of rhodamine-VCA in the absence (open circles) or presence of 1.5 μM Arp2/3 complex (open squares). The fractional reduction of Oregon Green actin emission from 519–524 nm was plotted versus rhodamine-VCA concentration and fit to Equation 1, resulting in a $K_D$ of 15 nM in the absence and 29 nM in the presence of 1.5 μM Arp2/3 complex. C, time course of the dissociation of Oregon Green actin and rhodamine-VCA. An equilibrium mixture of 20 nM Oregon Green actin and 0.2 μM rhodamine-VCA alone (open circles) or with 2 μM Arp2/3 complex (open square) was rapidly mixed 1:1 with 10 μM Lat-A actin to compete the Oregon Green actin from rhodamine-VCA. The time courses of fluorescence recovery were fit to a single exponentials giving dissociation rates of 0.44 s$^{-1}$ for both conditions.

little impact on the affinity of VCA for Arp2/3 complex or actin monomers.

To estimate the activation rate constant we collected the full time course of actin assembly over a range of Arp2/3 complex concentrations (0–100 nM) (Fig. 5A). The assay contained 4 μM actin (5% pyrene-labeled) and a saturating concentration of rhodamine-VCA (1 μM). We used Virtual Cell software to simulate the time course of polymerization, using the reaction parameters in Table 1. Fig. 5C shows a graphical depiction of the reaction pathway.

We varied the value of the activation rate constant until the simulations matched the experimental data for the full range of concentrations of Arp2/3 complex. With activation rate constants <0.1 s$^{-1}$ the simulations were slower than the experimental observations. An activation rate constant of 0.15 s$^{-1}$ gave simulated time courses that matched the observations. Activation rate constants >0.15 s$^{-1}$ accelerated the simulated time course of polymerization only modestly beyond the experimental observations. For example, with an activation rate constant of 0.15 s$^{-1}$ and 50 nM Arp2/3 complex the simulated time course reached 80% completion in 318 s (similar to the experimental time of 324 s), whereas simulations with activation rate constants of 1.5 s$^{-1}$ to 5 s$^{-1}$ reached this point in 300 s. This analysis puts a lower limit of 0.15 s$^{-1}$ on the activation rate constant.

The simulations deviated systematically to a small extent from the experimental data (Fig. 5B). The residuals shared a non-random sinusoidal appearance, even for actin alone, because we used a simplified model of actin filament nucleation to allow us to focus on the reactions involved in branch formation. This simplification contributed most of the error but is unlikely to have a significant impact on the overall analysis.

With kinetic data on more of the interactions among Arp2/3 complex, VCA, actin monomers, and actin filaments, we sought to determine the predominant pathway of actin filament branch formation during cellular motility. For this simulation we used established rate constants for interactions between human WASp VCA and bovine Arp2/3 complex and skeletal muscle actin (3) along with rate constants for interactions of Arp2/3 complex and actin filaments, and activation of Arp2/3 complex from this study (Table 2). Marchand et al. determined equilibrium binding constants and dissociation rates for rhodamine-VCA and equilibrium binding values for unlabeled VCA. We wanted to use the rate constants for unlabeled VCA, so we adjusted the $k_-$ to correct for the effect of rhodamine on binding affinity. We also decreased by 2-fold the $k_+$ for actin monomer binding to VCA in the presence of Arp2/3 complex (reactions 5, 6, 8, and 10) based on our FRET results (Fig. 4, B and C). Marchand et al. determined that the presence of 5 μM actin filaments increased the affinity of human WASp VCA for bovine Arp2/3 complex 5-fold but did not determine if this was due to a decrease in the $k_-$ or an increase in the $k_+$ (Table 2). We included this observation in our model by decreasing the $k_-$ of reactions 4, 5, and 9 by 5-fold. With these adjustments, the free energy change is equivalent along each pathway to form an actin filament branch. We used estimated cellular concentrations of reactants found at the leading edge of motile cell types: 10 μM actin monomers (13), 200 nM activated nucleation-promoting factor (VCA) (our estimate), 5 μM Arp2/3 complex (10), and 1000 μM polymerized actin (14). The concentrations were held constant to mimic steady-state motility where recycling mechanisms actively turn over cytoskeletal components. We did not take into account the presence of profilin, which competes with VCA for binding actin monomers (10, 15, 16), because these reactions have not yet been fully characterized.

Fig. 1B lists the steady-state rates of the reactions. The slowest reaction (4) is 0.085 μM/s, and the fastest (reaction 7) is 1.3 μM/s among reactions of the competing pathways (reactions 1–10). At steady state the process produced 2.1 μM actin filament branches per second. Each of these barbed ends would grow at 116 subunits per second. Reaction 3 reached its steady-state value in 3 s; reactions 1, 2, 7, 8, 9, and 10 reached their steady-state values in ~10 s; reactions 4, 5, 6, and 11 reached their steady-state values within 50 s.
DISCUSSION

Interaction between Arp2/3 Complex and Actin Filaments—Fission yeast genetic techniques allowed us to create useful reagents for measuring interactions involved in actin filament branch formation. Removal of the single solvent-exposed cysteine in *S. pombe* Arp2/3 complex created the substrate to add single reactive cysteines to this large complex for the rational design of spectroscopic assays. The availability of quantitative assays for Arp2/3 complex in live fission yeast (7) allowed us to verify that these mutations did not compromise function and will offer the opportunity to test the mechanisms studied here in live cells in the future.

The association rate constant for Arp2/3 complex-binding actin filaments is slower than many macromolecular interactions. For example, the association rate constant of the subfragment 1 domain of myosin binding actin filaments is $10^4$ times larger (17) than for Arp2/3 complex binding actin filaments despite interacting with at least three rather than one

![Figure 5](image)

**FIGURE 5.** Mathematical model of actin assembly based on experimentally determined kinetic parameters. Simulations were performed using the program Virtual Cell, version 4.2. A, comparison of the time course of actin assembly measured by the fluorescence of pyrene actin and simulations based on the model in part C. The concentration of polymerized actin was plotted versus time for pyrene actin assembly data (solid lines) and simulations (dashed lines). Reactions contained 4 μM actin (5% pyrene-labeled), 1 μM rhodamine-VCA and the following concentrations of Arp2/3 complex: black, 0 nM; red, 2 nM; orange, 5 nM; light green, 10 nM; dark green, 20 nM; blue, 50 nM; and purple, 100 nM. B, plot of residuals between actin assembly data and simulations of the mathematical model. Residuals are taken as the difference between the assembly data and the simulation. C, graphical description of the reactions included in the mathematical model. Chemical species are similar to Fig. 1, and reactions are represented as yellow circles. Forward reactions are shown as arrowheads and chemical species that are treated catalytically in a reaction are represented as a dashed gray line. Forward and reverse rate constants used are shown in Table 1. We modeled nucleation of actin filaments from monomers approximately as the irreversible formation of a trimer at a rate that depends on the nucleation rate constant and the cube of the actin monomer concentration. The rate of nucleation (reaction 1) was varied to obtain a good fit with the 0 nM Arp2/3 complex curve and the rate of activation of Arp2/3 complex (reaction 7) was varied to obtain the best fit to the 50 nM Arp2/3 complex curve.

**TABLE 1**

Reactions and parameters used in the mathematical model shown in Fig. 5C

| Reaction | Description | $k_+$ | $k_-$ | Reference |
|----------|-------------|-------|-------|-----------|
| 1        | Nucleation of an unbranched filament from monomers | $8 \times 10^{-9}$ | 0 | Empirical |
| 2        | Elongation of a filament by addition of monomers to barbed and pointed ends | $1.5 \times 10^{-4}$ | $1.4 \times 10^{-3}$ | (28) |
| 3        | VCA bound Arp2/3 complex binding to filament | $1.5 \times 10^{-4}$ | $3.4 \times 10^{-3}$ | This study |
| 4        | Actin monomer binding to VCA bound Arp2/3 complex bound to filament | 15.2 | 0.84 | This study |
| 5        | Actin monomer binding to VCA bound Arp2/3 complex | 15.2 | 0.84 | This study |
| 6        | Activation | 0.15$^a$ | 0 | This study |
| 7        | Elongation of an actin filament branch from the barbed end | 11.6 | 1.4 | (28) |

$^a$ Units are μM$^{-1}$ s$^{-1}$.

$^b$ Units are s$^{-1}$.
subunit in the mother filament (18). As in the cases of phalloidin (19) and cofilin (20, 21) binding to actin filaments, few segments of the filaments may be in a conformation favorable for binding Arp2/3 complex at any given time. The inactive conformation of Arp2/3 complex may also be unfavorable for interacting with an actin filament. Alternatively, the initial interaction between Arp2/3 complex and actin filaments may be a fast reversible reaction that depends on a slow first order conformational change to produce the signal from pyrene-labeled Arp2/3 complex. Despite the slow rate of association, the affinity of Arp2/3 complex for a filament is relatively high due to the slow rate of dissociation. Perhaps conformational changes in both Arp2/3 complex and the mother filament together with the large contact area are responsible for the slow dissociation.

The presence of a bound nucleation-promoting factor on Arp2/3 complex only had a modest effect on the rate that Arp2/3 complex binds filaments. With bound VCA, Arp2/3 complex did not bind actin filaments faster, but it dissociated from them three times faster. Thus it differs from the finding that the presence of 5 μM actin filaments increases the affinity of human WASp rhodamine-VCA for bovine Arp2/3 complex 5-fold. This may reflect mechanistic differences in the activity of Arp2/3 complex from fungi and mammals, either in their mechanism of binding actin filaments or may reflect differences in the equilibrium between different conformations induced by VCA and/or actin filament binding.

Even with the development of pyrene-labeled Arp2/3 complex, we were unable to measure the rate of the interaction between an actin filament and the ternary complex of Arp2/3 complex with a bound nucleation-promoting factor and actin monomer. We doped a mixture of pyrene-labeled Arp2/3 complex and VCA with 1 μM actin monomers to make the ternary complex before mixing with actin filaments. The time course of binding to actin filaments was the same as VCA-bound pyrene-labeled Arp2/3 complex. This result is inconclusive, because of a side reaction; actin monomers assemble on the ends of the actin filaments faster than Arp2/3 complex binds to filaments, so actin monomers are depleted rather than remaining bound to VCA and Arp2/3 complex. Depletion of actin monomers by polymerization on a faster time scale than Arp2/3 complex binding to filaments also complicated FRET and anisotropy assays to measure interactions between actin monomers, VCA, and Arp2/3 complex in the presence of actin filaments. Capping barbed ends would overcome some of these problems but would complicate the experiments by adding competing reactions. Chemical cross-linking may be required to form a stable ternary complex for filament binding assays.

In the absence of better biochemical data, for the purposes of kinetic simulations of actin filament branch formation by Arp2/3 complex, we assumed that an actin monomer bound to VCA does not change the interaction between VCA bound Arp2/3 complex and an actin filament and that the presence of actin filaments do not influence the rate of actin monomer association and dissociation with VCA bound to Arp2/3 complex. This assumption is reasonable, because it only affects the third, and last, reaction in the pathway (Fig. 1, reactions 5, 6, and 9) leading to activation, whereas earlier steps have a stronger influence on the predominant pathway. In fact, modest changes of 2- to 10-fold in the rates of these reactions do not affect the overall interpretation of pathway 4 as the dominant one in the simulations. Therefore, errors in the parameters for these reactions have only a small impact in the overall process of actin filament branch formation.

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#### Table 2

| Reaction | Description | $k_+$ | $k_-$ | Reference |
|----------|--------------|-------|-------|-----------|
| 1        | Arp2/3 complex binding to VCA | 0.8   | 0.74  | (3)       |
| 2        | VCA bound Arp2/3 complex binding to filament | $1.5 \times 10^{-4}$ | $0.2 \times 10^{-3}$ | This study, (3) |
| 3        | Arp2/3 complex binding to filament | $1.5 \times 10^{-4}$ | $0.2 \times 10^{-3}$ | This study, (3) |
| 4        | Filament bound Arp2/3 complex binding to VCA | 0.8   | 0.06  | (3)       |
| 5        | Filament bound Arp2/3 complex binding to monomer bound VCA | 0.4   | 0.015 | This study, (3) |
| 6        | Filament bound Arp2/3 complex bound to VCA binding to actin monomer | 21.5  | 25.7  | This study, (3) |
| 7        | VCA binding to monomer | 42.9  | 25.7  | (3)       |
| 8        | VCA bound monomer binding to Arp2/3 complex | 0.4   | 0.74  | This study, (3) |
| 9        | Arp2/3 complex bound VCA to monomer binding to filament | $1.5 \times 10^{-4}$ | $0.2 \times 10^{-3}$ | This study, (3) |
| 10       | Arp2/3 complex bound to VCA binding to monomer | 21.5  | 25.7  | This study, (3) |
| 11       | Activation of Arp2/3 complex | 0.15  | 0     | This study |

*Units are s$^{-1}$. 

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action of VCA with Arp2/3 complex in a low ionic strength buffer but not with Lat-A bound to actin in a physiological buffer (Fig. 3, C and D). We conclude that Arp2/3 complex and actin monomers compete with each other for binding VCA as first pointed out by Kelly et al. (4), who used anisotropy to show that binding of C region of VCA to actin monomers and Arp2/3 complex is mutually exclusive. The assay with Lat-A gave a contrary result, however, the effect of Lat-A is unknown in the context of this interaction. The similarity in the time course of Oregon Green-labeled actin dissociation from rhodaminelabeled VCA in the presence and absence of Arp2/3 complex suggests that the mutually exclusive portion of the C region interacts with actin in the ternary complex of VCA, actin, and Arp2/3 complex.

Activation—Several factors are thought to activate Arp2/3 complex to allow formation of an actin filament branch. In addition to assembling a stable ternary complex of Arp2/3 complex with a nucleation-promoting factor and an actin monomer bound to the side of an actin filament, Arp2/3 complex requires ATP and is proposed to require several conformational changes (12, 22). Formation of a short-pitch dimer between Arp2 and Arp3 is consistent with two-dimensional reconstructions of electron micrographs (18, 23, 24), but these reconstructions do not show how the proposed clashes between subdomain 2 of Arp2 and ARPC3 or the helical insert in Arp3 and the first actin monomer of the daughter filament are accommodated in the branch junction (12).

A rate-limiting reaction with a rate constant of 0.15 s$^{-1}$ ($t_{1/2} = 4.6$ s) between the binding of Arp2/3 complex-VCA to the mother filament and elongation of the daughter filament improves the simulation of the time course of actin polymerization in bulk samples. This “activation reaction” converts an inactive barbed end into a barbed end that can elongate by addition of actin monomers. This step might correspond to the conformational change that rearranges Arp2 relative to Arp3, making an Arp dimer similar to the barbed end of an actin filament. Without a rate-limiting activation reaction following binding of Arp2/3 complex to the side of a filament, simulations proceed somewhat faster than the time course of actin polymerization in bulk samples. Only a few more branches are formed with an activation rate constant of 1.5 s$^{-1}$ rather than 0.15 s$^{-1}$.

Differences in the overall branching nucleation reactions suggest that some of the reaction rates differ for the vertebrate and fission yeast systems. Under optimal conditions bovine Arp2/3 complex forms over 4-fold more branches than fission yeast Arp2/3 complex (10 nM bovine Arp2/3 complex produced 8 nm ends (3) and 10 nm fission yeast Arp2/3 complex produced 2 nm ends (7) but 10-fold less fission yeast Wsp1-VCA was required than human WASp VCA, because of its higher affinity for actin monomers and Arp2/3 complex). Assuming a lower limit of 0.15 s$^{-1}$ for the activation rate constant, another reaction must increase the efficiency of nucleation by bovine Arp2/3 complex. A good candidate is faster binding of bovine Arp2/3 complex to actin filaments. In our simulations binding of Arp2/3 complex to actin filaments must be 15-fold faster to achieve a 4-fold increase in the number of ends formed. These simulated reactions reached completion in under 300 s similar as seen by Marchand et al. Adjusting the rate constant for dissociation of Arp2/3 complex from actin filaments to agree with the $K_d$ of 2 μM measured for recombinant human Arp2/3 complex (25) had little effect on the simulations.

Overview of the Branching Pathway—The events producing the full time course of polymerization of mixtures of actin monomers, Arp2/3 complex, and VCA are more complicated than usually recognized. Simulations of the reactions in Fig. 5C show that the reaction passes through several stages. Initially formation of new barbed ends is limited by the spontaneous nucleation of filaments from actin monomers to create binding sites for Arp2/3 complex. The formation of new barbed ends then speeds up as Arp2/3 complex binds to these first filaments and generates more ends in an autocatalytic manner. The branching reaction abruptly stops as growth of the filaments depletes actin monomers, which are no longer available to bind to VCA and Arp2/3 complex. The slow rate of association of Arp2/3 complex with a limited number of spontaneously formed actin filaments creates the initial lag in the time course of assembly. The rate of dissociation of Arp2/3 complex from actin filaments has little effect on the time course of the polymerization reaction. For example, increasing the rate of dissociation 10-fold does not change the simulations. When actin monomers are available, a large fraction of Arp2/3 complexes that bind an actin filament eventually form a new barbed end, because the activation rate constant is at least 40 times faster than the rate of dissociation of Arp2/3 complex from actin filaments ($t_{1/2} ≈ 200$ s). Elongation of nascent branches (Fig. 5C, reaction 7) lags behind binding of the ternary complex to a mother filament (Fig. 5C, reactions 4 and 6) owing to the first order activation reaction.

With critical details added to the pathway of actin filament branch formation, we reassessed the question of the dominant pathway in vivo, finding that it is the same pathway (#4 in Fig. 1) proposed by Marchand et al. (3). Despite very slow association of Arp2/3 complex with actin filaments, this binding reaction is fast in the cell owing to the high local concentration of filaments. Pathways 2 and 3, which begin with Arp2/3 complex associating with an actin filament (4), are unlikely to be productive collisions in vivo, due to nucleation-promoting factors being anchored to the membrane. Therefore, we discount these pathways as significant contributors to actin filament branch formation during cell motility. Pathways 1 and 5 begin with the same first step, but the second step is four times faster in pathway 5 than in pathway 1.

Pathway 4 is favored under the conditions of our simulations, but the effects of profilin have not been considered. Profilin competes with VCA for binding actin monomers (10, 15, 16) and binds to Arp2 (26). Further characterization of these reactions and their inclusion in the mechanism may favor pathway 5 over 4.

Pathways 4 and 5 produce 1.2 μm/s of actin filament branches in our simulation. With a half time for the activation reaction of 4.6 s, every second ~14%, or 170 nm, of inactive nascent branches are converted to growing actin filaments capable of pushing on the membrane or an intracellular bacterium to drive motility. Assuming that active nucleation-promoting factors are tethered to the plasma membrane in rapidly moving cells, it is likely that activation is restricted to a short
interval of time (perhaps <100 ms) after the ternary complex binds to an actin filament. The remaining inactive branches would be recycled to the pool of reactants for continued motility. Slow intrinsic association of Arp2/3 complex with actin filaments coupled with recycling mechanisms would maintain a soluble pool of Arp2/3 complex available to bind activated nucleation-promoting factors.

Nucleation-promoting factors stay behind on the surface of Listeria (2) and on the plasma membrane of yeast (7, 27) after interacting with Arp2/3 complex, so the VCA region must dissociate rapidly from Arp2/3 complex at branch junctions whether Arp2/3 complex is activated or not. This should allow a single, active nucleation-promoting factor to participate in multiple rounds of branching. The relative effects of physical coupling of nucleation-promoting factors to the plasma membrane and kinetics of interactions between VCA and Arp2/3 complex are unknown. An attractive hypothesis for regulation of cell motility is that cells control the amount of force generated for motility directly through the amount of nucleation-promoting factors activated by signaling pathways. It will be critical to determine the concentration of active nucleation-promoting factors during cellular motility to understand this process better.

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