

Activation of the Arp2/3 Complex by the *Listeria* ActA Protein

ActA BINDS TWO ACTIN MONOMERS AND THREE SUBUNITS OF THE Arp2/3 COMPLEX*

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ActA is a bacterially encoded protein that enables *Listeria monocytogenes* to hijack the host cell actin cytoskeleton. It promotes Arp2/3-dependent actin nucleation, but its interactions with cellular components of the nucleation machinery are not well understood. Here we show that two domains of ActA (residues 85–104 and 121–138) with sequence similarity to WASP homology 2 domains bind two actin monomers with submicromolar affinity. ActA binds Arp2/3 with a K_d of 0.6 μM and competes for binding with the WASP family proteins N-WASP and Scar1. By chemical cross-linking, ActA, N-WASP, and Scar1 contact the same three subunits of the Arp2/3 complex, p40, Arp2, and Arp3. Interestingly, profilin competes with ActA for binding of Arp2/3, but actophorin (cofilin) does not. The minimal Arp2/3-binding site of ActA (residues 144–170) is C-terminal to both actin-binding sites and shares sequence homology with Arp2/3-binding regions of WASP family proteins. The maximal activity at saturating concentrations of ActA is identical to the most active domains of the WASP family proteins. We propose that ActA and endogenous WASP family proteins promote Arp2/3-dependent nucleation by similar mechanisms and require simultaneous binding of Arp2 and Arp3.

The actin cytoskeleton participates in many essential functions in eukaryotic cells including motility, endocytosis, and cytokinesis. These processes rely on the rapid and localized assembly and disassembly of actin filaments. Cellular signals, such as activated Rho family G-proteins, direct construction of new actin filaments *de novo* by localizing and activating the nucleation machinery. The actin nucleation machinery consists of the Arp2/3 complex, a multiprotein complex that nucleates and cross-links actin filaments into orthogonal arrays, and a nucleation promoting factor that activates or enhances the activity of the Arp2/3 complex. Both the mechanism of Arp2/3-mediated nucleation and its enhancement by nucleation promoting factors are poorly understood. Cellular nucleation promoting factors identified to date are all members of the WASP family of proteins, which includes isoforms of WASP, N-WASP, and Scar. Other proteins, including fungal myosin I (1, 2) and p150 Spir (a possible c-Jun N-terminal kinase substrate in

humans and *Drosophila* (3)), have been identified as potential nucleation promoting factors based on genetic and biochemical interactions with Arp2/3 and/or sequence similarity with regions of WASP family proteins.

The bacterium *Listeria monocytogenes* recruits the host cell cytoskeleton to power its own motility and migrate from cell to cell to avoid the humoral immune system. The only bacterial protein required for this activity is ActA (4, 5), a nucleation promoting factor that recruits and activates Arp2/3. Although ActA was the first protein found to stimulate Arp2/3-dependent actin nucleation (6), we know much less about how actin and Arp2/3 interact with ActA than with the WASP family proteins. WASP family proteins contain an acidic C-terminal domain that binds Arp2/3 (7) and one or two WASP homology 2 (WH2)¹ domains that bind actin (8, 9). Biochemical analysis of truncation mutants suggests that both the acidic and WH2 domains are required to promote Arp2/3-dependent nucleation (10, 11). The nucleation promoting activity of ActA has been shown to reside in the N-terminal 262 amino acids of the molecule (6), but the location, number, and activity of actin- and Arp2/3-binding sites have not been determined.

Understanding the structure and function of ActA provides insight into the mechanism of Arp2/3-mediated actin polymerization. In addition, comparison of ActA with endogenous nucleation promoting factors shows us how relevant conclusions based on studying *Listeria* motility are to normal cellular motility. In the present study we find that ActA binds two actin monomers with submicromolar affinity using domains with some sequence similarity to WH2 domains from endogenous nucleation promoting factors. The binding site for Arp2/3 is located C-terminal to the actin-binding sites, and although it does not contain the characteristic cluster of acidic residues, it shares limited sequence homology with the Arp2/3-binding domains found in WASP family proteins. By chemical cross-linking and polarization anisotropy, we show that ActA and the WASP family proteins hScar1 and N-WASP contact the same subunits on Arp2/3 and compete for the same binding site. This binding site is much more extensive than previously thought and includes both of the actin-related proteins, Arp2 and Arp3. *In vitro* the maximal activity of full-length ActA is nearly identical to the endogenous nucleation promoting factor N-WASP.

MATERIALS AND METHODS

Protein Purification—*L. monocytogenes* 4035 genomic DNA was a generous gift from A. K. Benson, University of Nebraska, Lincoln. Oligonucleotide primers were designed that correspond to the following: FACTA30N, GATCGAATTCTCCAGTCTGAACACAGATGAATGGG; FACTA120N, GATCGGATCCATGGCTTCAGGAGCCGACCGACC;

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¹ The abbreviations used are: WH2, WASP homology 2; DTT, dithiothreitol; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; NHS, *N*-hydroxysuccinimide.

FACTA152N, GATCGGATCCATGATAGCATCATCGGATAGTGAGC; FACT180N, GGATCCATGTCAGTTGCGGATGCTTCTGAA; RACTA-170N, GATCGAATTCTTAACATTTTGGTTGTTATCCGGATAAGTAGGC; and RACTA612, GAATTCTTAACACGTCGTATGGTTCCCTGTCTTCTCCCTCGC. Polymerase chain reactions were performed with proofreading polymerase (Roche Molecular Biochemicals) under standard conditions. DNA fragments were subcloned into the pET-5a expression vector (Novagen, Madison, WI). ActA-(30–120) and ActA-(121–170) were constructed either by dropout and/or *EcoRI* partial digestion of the ActA-(30–170) construct.

All ActA proteins were expressed and purified as follows. Fresh transformations of BL-21(DE3) cells were grown at 37 °C in TPM (20 g of tryptone, 15 g of yeast extract, 8 g of NaCl, 2 g of Na₂HPO₄, 0.2% glucose, 50 µg/ml ampicillin) to A₆₅₀ of ~0.6, then induced with 0.25 mM isopropyl-1-thio-β-D-galactopyranoside (Sigma), and grown an additional 2 h. Cells were harvested at 5000 rpm and pellets stored –80 °C. Cells were thawed and resuspended in lysis buffer (10 mM Tris, pH 8, 0.2 M sucrose, 1000 units of DNase, 1000 units of RNase, 5 mM DTT, 5 mM phenylmethylsulfonyl fluoride) and lysed by three passes in a microfluidizer (Microfluidics International Corp., Newton, MA). The extract was spun at 15,000 rpm, and the supernatant was fractionated by gel filtration on a 500-ml Sephacryl S-300 column equilibrated in high salt buffer (10 mM Tris, pH 8, 500 mM KCl, 1 mM EDTA, 1 mM DTT). ActA-containing fractions were pooled and dialyzed against DEAE buffer (10 mM Tris, pH 8, 1 mM EDTA, 1 mM DTT). Protein was then purified by anion exchange chromatography using DE52 resin. Proteins were concentrated either by dialysis against solid sucrose or with a Centrprep spin column. Purified proteins were flash-frozen and stored at –80 °C. The WA constructs of hSCAR1 and N-WASP were expressed with C-terminal His tags and purified on nickel-nitrilotriacetic acid-agarose (Qiagen, Valencia, CA). Cell growth conditions, buffers, and lysis conditions were identical to those used for ActA mutants.

Arp2/3 was purified as described previously (12) but with a few modifications. Poly-L-proline flow-through was passed over a C-200 cation exchange column. The flow-through was adsorbed onto a C-500 anion exchange column and eluted with a linear gradient from 0 to 0.2 M KCl in column buffer. Protein-containing fractions were concentrated by dialysis against solid sucrose and run on an S-300 column equilibrated in high salt complex buffer (10 mM imidazole, pH 7.5, 150 mM NaCl, 750 mM KCl, 0.1 mM EGTA, 0.2 mM MgCl₂, 1 mM DTT, 0.2 mM ATP). Purified Arp2/3 was stored in complex buffer supplemented with 35–50% sucrose.

Acanthamoeba actin and rabbit skeletal muscle actin were purified according to Refs. 13 and 14. Plasma gelsolin was purified as described previously (15, 16).

Fluorescent Labeling—Purified proteins were dialyzed against 50 mM KCl, 1 mM MgSO₄, 1 mM EGTA, 10 mM imidazole, pH 7, and incubated with a 4–7-fold molar excess of rhodamine maleimide (Molecular Probes, Eugene, OR) for 1 h at room temperature and overnight at 4 °C. Samples were spun at 65,000 rpm, and the supernatant was chromatographed on a Sephacryl S-300 column to remove unreacted dye. In Arp2/3-mediated polymerization assays, the activity of the rhodamine-labeled ActA was indistinguishable from that of unlabeled ActA.

Freshly purified Arp2/3 was dialyzed into labeling buffer (10 mM imidazole, pH 7, 50 mM KCl, 0.1 mM MgCl₂, 0.1 mM ATP, 0.5 mM TCEP) overnight and centrifuged at 13,000 rpm to remove precipitated protein. The supernatant was concentrated to 1–2 mg/ml with a microcon concentrator and mixed with an equimolar amount of rhodamine maleimide for 1.5 h at 24 °C under cover of darkness. The reaction was quenched with excess DTT, and the sample was spun at 70,000 rpm for 30 min at 4 °C to remove precipitated dye. The supernatant was chromatographed on a 10-ml Sephadex G-25 column to remove unreacted dye. This labeling protocol results in ~15% labeling of Arp2/3, almost exclusively on the Arp3 subunit. Higher concentrations of rhodamine maleimide produce elevated percentages of labeling, and the complex becomes labeled on both the Arp3 and p18 subunits. To determine the effect of labeling on Arp2/3 activity, we tested 60% rhodamine-labeled Arp2/3 and found no effect on either nucleation or pointed end capping.

Acanthamoeba and Rabbit skeletal muscle actin were labeled with pyrene iodoacetamide as described (17).

Actin Polymerization—We mixed unlabeled *Acanthamoeba* actin with pyrene-labeled amoeba or rabbit skeletal muscle actin to 10–15% of final actin concentration. Polymerization reactions contained 50 mM KCl, 1 mM MgSO₄, 1 mM EGTA, 10 mM imidazole, pH 7. Ca²⁺-actin was converted to Mg²⁺-actin prior to each polymerization reaction by a 2-min incubation with 50 µM MgCl₂, 200 µM EGTA. Gelsolin-actin dimers were prepared according to Ref. 15. Pyrene fluorescence was

measured by a K2 Multifrequency Fluorometer (ISS, Champagne, IL). Data were analyzed using KaleidaGraph (Synergy Software, Reading, PA).

Polarization Anisotropy—Rhodamine-labeled ActA-(30–170) (500 nM) or Arp2/3 (750 nM) was mixed with the appropriate unlabeled proteins to the indicated concentrations. Samples were degassed under vacuum at room temperature before data collection. Data were collected with a K2 Multifrequency Fluorometer and analyzed using KaleidaGraph. Under the conditions used in our study, anisotropy is a measure of the rotational mobility of the fluorescently labeled protein. We excited the fluorophore with plane polarized light at 543 nm, and measured emission at 575 nm at polarizations both parallel (I_{\parallel}) and perpendicular (I_{\perp}) to the excitation source. We then calculated anisotropy (r) as shown in Equation 1,

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad (\text{Eq. 1})$$

We determined dissociation equilibrium constants by fitting anisotropy data to the function shown in Equation 2,

$$r = r_f + (r_b - r_f) \frac{(K_d + [R] + [A]) - \sqrt{(K_d + [R] + [A])^2 - 4[R][A]}}{2[R]} \quad (\text{Eq. 2})$$

where r_f and r_b are the anisotropies of free and bound rhodamine ActA; [A] and [R] are the total concentrations of ActA and Arp2/3, and K_d is the dissociation equilibrium constant. Fitting with this function makes no assumptions about the relative concentrations of ActA and Arp2/3.

In experiments where nonfluorescent ligands compete with rhodamine-labeled ActA (Fig. 4), we determined dissociation constants by fitting anisotropy data to the function (18) shown in Equation 3,

$$r = r_f + \frac{(r_b - r_f)}{K_d \frac{[C] + K_{d2}}{K_{d,2}[R_o]} + 1} \quad (\text{Eq. 3})$$

where $K_{d,2}$ is the dissociation constant of the nonfluorescent competitor; [C] is the total concentration of the competitor, and $[R_o]$ is the concentration of free Arp2/3 when [C] = 0. For this analysis $[R_o]$ and K_d are determined from the anisotropy in the absence of competitor and $K_{d,2}$ is determined from fitting Equation 3 to experimental data. This function is an approximation and only valid when Arp2/3 and the competitor are in excess over labeled ActA. These conditions were met in all our competition binding experiments.

Chemical Cross-linking—The indicated concentrations of ActA-(121–170) and Arp2/3 were dialyzed into 50 mM KCl, 1 mM MgSO₄, 1 mM EGTA, 10 mM imidazole, pH 7, to facilitate cross-linking. Stock solutions of 100 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) (Pierce) were prepared in Me₂SO. Cross-linking reactions were carried out at room temperature at the indicated concentrations of proteins and cross-linkers for 45 min. The reaction was quenched with 10 mM Tris, 100 mM glycine. Reaction products were precipitated with methanol and chloroform and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting.

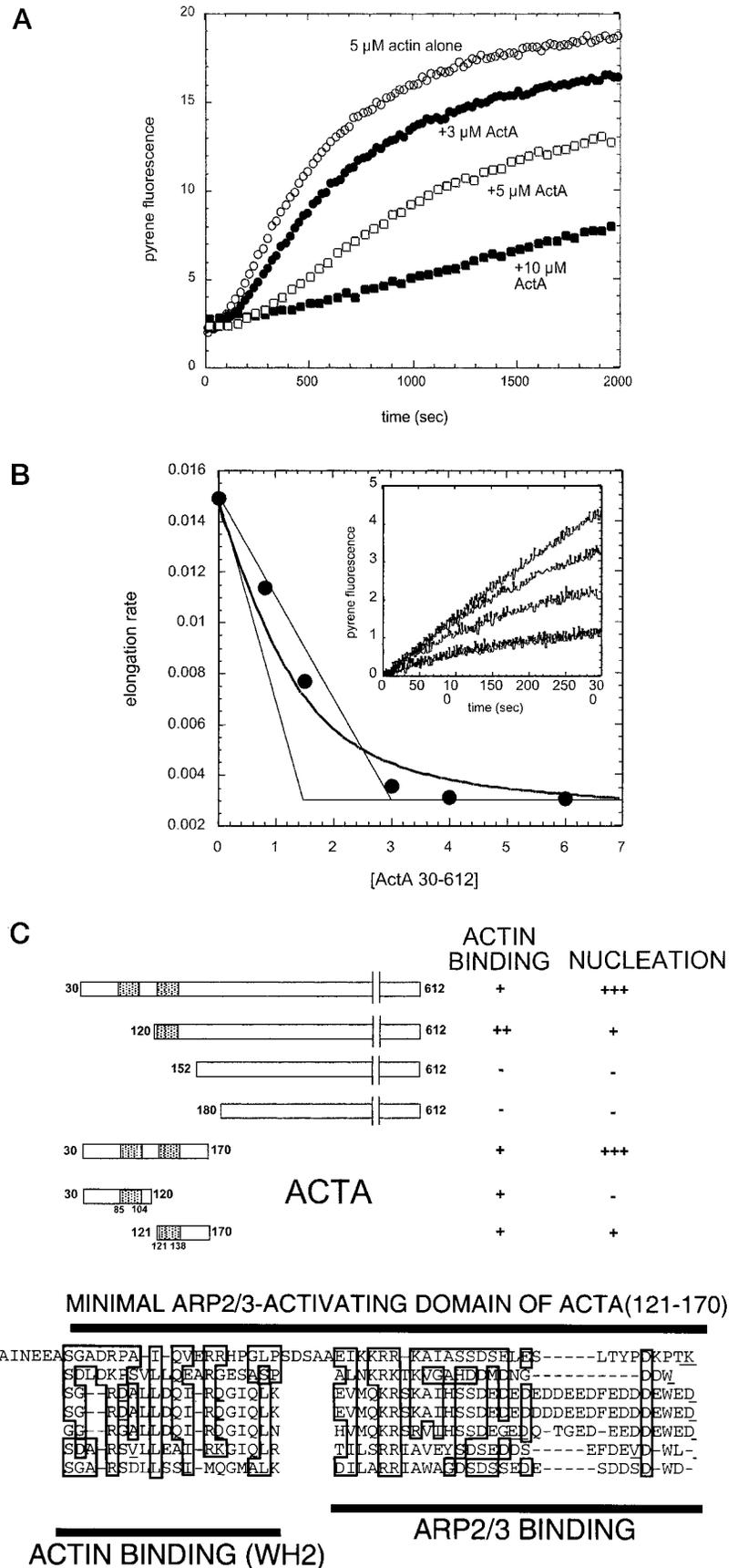
Atomic Models—The atomic model for actin was taken from Schutt *et al.* (19), and the model of Arp2 was constructed by Joe Kelleher based on homology with conventional actin combined with molecular dynamics simulations (20). Visualization and rendering were performed with Molscrip (21) and Raster3d (22).

RESULTS

ActA Binds Two Actin Monomers via WH2-like Motifs—Full-length ActA (amino acids 30–612) and several smaller truncation mutants inhibit spontaneous actin polymerization and elongation from the pointed end of existing actin filaments but have no effect on elongation from the barbed end (Fig. 1, *A* and *B*, *inset*). This activity is similar to that reported for fragments of N-WASP (23) and Scar1 (11). Unlike Scar and N-WASP, which almost completely inhibit pointed end elongation, saturating concentrations of ActA inhibit pointed end elongation by 80%.

We used the effect on pointed end elongation to quantitate actin monomer binding by ActA-(30–612) (Fig. 1*B*). The decrease in elongation rate as a function of ActA concentration is not well fit by a model in which an individual ActA molecule

FIG. 1. ActA binds two actin monomers. *A*, inhibition of spontaneous actin polymerization by ActA-(30–612). 5 μM actin monomers were polymerized by addition of KCl and MgCl_2 in the presence of the following concentrations of ActA-(30–612): \circ , 0.0 μM ; \bullet , 3 μM ; \square , 5 μM ; \blacksquare , 10 μM (conditions, actin was 15% pyrene-labeled; buffer, 50 mM KCl, 1 mM MgSO_4 , 1 mM EGTA, 10 mM imidazole, pH 7, temperature, 24 $^\circ\text{C}$). *B*, ActA-(30–612) blocks addition of actin monomers to the pointed ends of existing filaments. We polymerized 3 μM actin (10% pyrene-labeled) from the pointed ends of gelsolin-capped actin seeds in the presence of various concentrations of ActA-(30–612). *Inset*, time courses of polymerization in the presence of 0.0, 0.8, 1.6, 3.0, 4.0, and 6.0 μM ActA-(30–612) (conditions, same as *A*). *Straight lines* represent infinitely tight binding to either one (*right*) or two (*left*) binding sites. The *heavy curve* is the best fit to a two binding site model with equal dissociation equilibrium constants of $0.7 \pm 0.2 \mu\text{M}$. *C*, domain organization of ActA. ActA truncation mutants used in this study and their relative activities in inhibiting spontaneous actin polymerization and promoting Arp2/3-dependent nucleation activity. *D*, actin and Arp2/3-binding sites in the N-terminal portion of ActA. The *heavy dark lines* indicate the minimal fragment of ActA capable of promoting Arp2/3-dependent nucleation (*top*) and the putative actin and Arp2/3-binding sites identified by homology with domains from WASP family proteins (*bottom*). The domains from WASP family proteins begin at the following amino acids: *BeeIp*, 547, 570, and 613; *human N-WASP*, 345, 372, and 413; *Rat N-WASP*, 401, 428, and 469; *Mouse WASP*, 447 and 490; *Human Scar1*, 495 and 533; and *Dicty Scar*, 380 and 418.



binds a single actin monomer (*right line*). The data fit much better to a model in which one ActA binds two actin monomers, each with a K_d of $0.7 \pm 0.2 \mu\text{M}$ suggesting that ActA contains at least two actin monomer-binding sites. Previous studies iden-

tified a putative actin-binding site (24, 25) between amino acids 60 and 90. To extend this work we used truncation mutants to perform a systematic search for actin-binding sites throughout the entire molecule. We expressed truncation mutants span-

ning the entire molecule (Fig. 1C) and assayed the monomer binding activity of each by its effect in three assays as follows: spontaneous actin polymerization, elongation from the barbed end, and elongation from the pointed end of existing filaments.

The construct spanning amino acids 30–120 weakly inhibited spontaneous polymerization (data not shown), consistent with previous reports of an actin-binding site between amino acids 60 and 100 (24). In addition, the construct spanning amino acids 120–612 inhibited spontaneous polymerization and elongation from both the pointed and barbed ends of existing filaments. We further localized this second actin-binding site to a region of the protein between amino acids 121 and 152. We estimated a dissociation constant of $2.1 \pm 0.5 \mu\text{M}$ for the actin-binding site on ActA-(120–612) from barbed end elongation assays (data not shown). The lower affinity of this binding site compared with that predicted from ActA-(30–612) and the fact that it blocks barbed as well as pointed end elongations are probably artifacts caused by some degree of misfolding of the ActA-(120–612) construct. Cicchetti *et al.* (24) found that deletions in the N-terminal region of ActA perturb the secondary structure and decrease the stability of the protein.

We conclude that full-length ActA contains two actin monomer-binding sites. We further localized them based on sequence similarity to the actin-binding WH2 domains from WASP family proteins (Fig. 1D).

The Arp2/3-binding Site of ActA—Amino acids 143–169 of ActA constitute a minimal Arp2/3-binding site. Full-length ActA and truncations spanning residues 30–170, 120–612, and 121–170 all stimulated Arp2/3-dependent nucleation (Figs. 1C and 2A). Therefore, residues 121–170 contain the minimal requirements to promote nucleation - actin monomer binding activity and an Arp2/3-binding site. Within this region we identified residues 143–169 as the Arp2/3-binding site by sequence similarity to the C-terminal portions of WASP, Scar, and N-WASP (Fig. 1D). This domain contains very few acidic residues compared with the Arp2/3-binding sites of most WASP family proteins but is most similar to the yeast protein Las17p/Bee1p. At saturating concentrations the ActA-(120–612) truncation is less efficient at stimulating actin polymerization than proteins containing amino acids 30–120. Under our assay conditions ($2 \mu\text{M}$ actin, 50 nM Arp2/3), the time to half-maximal polymerization stimulated by saturating concentrations of ActA-(30–612) and ActA-(30–170) is $\sim 75 \text{ s}$ compared with $t_{1/2}$ of $\sim 500 \text{ s}$ for ActA-(120–612) (Fig. 2A).

We determined the affinity of the 30–612 and 30–170 ActA proteins for Arp2/3 by polarization anisotropy (Fig. 2B). To characterize the interaction with ActA-(30–612) we labeled Arp2/3 with rhodamine maleimide. Our labeling conditions produced 5–15% labeling, almost exclusively on the Arp3 subunit. From preparation to preparation, free Arp2/3 had an anisotropy that varied between 0.215 and 0.26. Addition of saturating concentrations of ActA-(30–612) consistently increased the steady-state anisotropy by 0.015. From this change we measured a K_d of $0.5 \pm 0.2 \mu\text{M}$.

To measure Arp2/3 binding to the smaller truncation mutants, we labeled ActA with rhodamine maleimide. We designed all our ActA proteins with a cysteine at the extreme C terminus for efficient labeling with sulfhydryl-reactive fluorophores. We labeled each construct with rhodamine maleimide and measured its anisotropy alone and in the presence of Arp2/3. ActA-(30–612) had a very low anisotropy (0.05) similar to that of free dye, which did not change upon addition of Arp2/3 suggesting that the C terminus of the protein is flexible and possibly disordered. ActA-(30–170) was much more well behaved, and in the absence of Arp2/3 it had an anisotropy of 0.095 and in the presence of saturating Arp2/3 concentrations

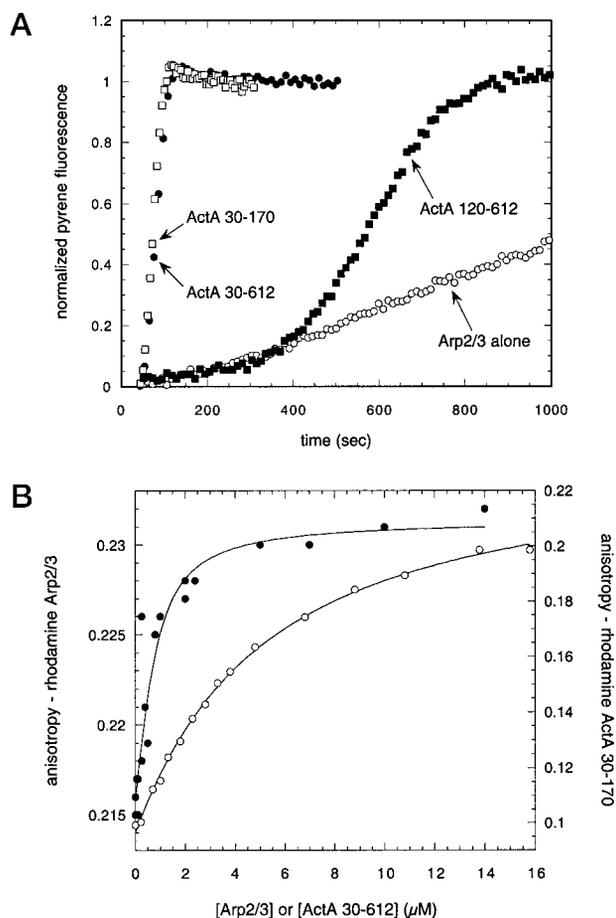


FIG. 2. Binding and activation of the Arp2/3 complex by ActA. A, comparison of the maximal activities of different ActA truncations. 50 nM Arp2/3 complex and $2 \mu\text{M}$ actin were polymerized in the presence of saturating concentrations of ActA proteins (conditions, amoeba actin was 15% pyrene-labeled; temperature, 24°C ; buffer conditions, 50 mM KCl, 1 mM MgSO_4 , 1 mM EGTA, 10 mM imidazole, pH 7). \circ , Arp2/3 alone; \bullet , 100 nM ActA-(30–612); \square , $5.7 \mu\text{M}$ ActA-(30–170); \blacksquare , 200 nM ActA-(120–612). B, polarization anisotropy measurements of Arp2/3 binding ActA-(30–612) and ActA-(30–170). We measured either the anisotropy of rhodamine-labeled Arp2/3 (750 nm) in the presence of unlabeled ActA-(30–612) (\bullet) or the anisotropy of rhodamine-labeled ActA-(30–170) (500 nm) in the presence of unlabeled Arp2/3 (\circ). Buffer conditions, 50 mM KCl, 1 mM MgCl_2 , 1 mM EGTA, 10 mM imidazole, pH 7.0.

of 0.21. From this anisotropy change, we measured a K_d of $4.8 \pm 0.3 \mu\text{M}$. The lower affinity of ActA-(30–170) suggests that residues C-terminal to 170 may also interact with Arp2/3.

ActA and Scar1 Bind Arp2/3 at the Junction between p40, Arp2, and Arp3—To localize the binding sites for nucleation promoting factors on Arp2/3, we carried out identical cross-linking experiments with the active fragment of hScar1 and the smallest fragment of ActA capable of stimulating Arp2/3-dependent nucleation, ActA-(121–170). We identified three Arp2/3 subunits that directly contact both ActA and hScar1 by cross-linking each protein to Arp2/3 with the zero-length cross-linker EDC together with NHS. After cross-linking, antibodies that recognize the T7 tag on ActA-(121–170) or the His₆ tag on hScar1 react with three prominent new bands (Fig. 3, A and B). The new bands are also recognized by monospecific antibodies against the p40, Arp2, and Arp3 subunits of Arp2/3.

We next investigated whether the binding site for ActA overlapped that of endogenous WASP family proteins by performing competition binding assays. Addition of the active C-terminal domain of N-WASP to a mixture of Arp2/3 and rhodamine ActA-(30–170) decreases the anisotropy of labeled ActA down

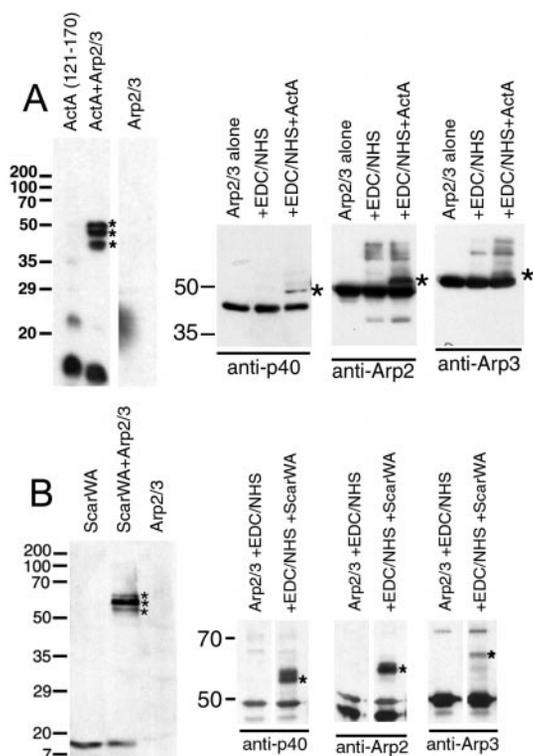


FIG. 3. ActA and Scar1 contact three subunits of the Arp2/3 complex. *A*, chemical cross-linking of ActA-(121–170) to the Arp2/3 complex reveals interactions with the p40, Arp2, and Arp3 subunits. We incubated $33.7 \mu\text{M}$ ActA-(121–170) with 1.5 mM EDC and NHS in the absence and presence of $1 \mu\text{M}$ Arp2/3 (conditions: buffer, 50 mM KCl, 1 mM MgSO_4 , 1 mM EGTA, 10 mM imidazole, pH 7; temperature, 24°C ; time, 45 min) and analyzed the results by immunoblot using an anti-T7 antibody that recognizes the recombinant ActA. We found three cross-links between ActA and Arp2/3 that ran at or just below the 50-kDa marker (*left panel*). We identified the Arp2/3 subunits involved by blotting with monospecific antibodies against Arp2/3 subunits (*right panel*, cross-linked products marked with an asterisk). *B*, hSCAR1WA also cross-links to the p40, Arp2, and Arp3 subunits of the Arp2/3 complex. $40 \mu\text{M}$ hSCAR1WA was incubated with $1 \mu\text{M}$ of Arp2/3 under the same conditions as described above, and the Western blot was probed with an anti-His₆ antibody that recognizes the recombinant hSCAR1WA protein. Three cross-links were detected between Arp2/3 and hSCAR1WA (*left panel*). These new bands were identified as described in *A*.

to the level of free ActA (Fig. 4), suggesting that the two proteins bind in a mutually exclusive manner. From these competition experiments we estimate a K_d of $1.9 \pm 0.3 \mu\text{M}$ for N-WASP binding to Arp2/3. Similar results were obtained with hScar1 ($K_d = 2.0 \pm 0.4 \mu\text{M}$, data not shown).

Our results suggest that Arp2 interacts directly with ActA and WASP family proteins. To further localize the binding site for nucleation promoting factors, we tested the effects of two proteins known to bind Arp2, profilin and actophorin, on the binding of ActA to Arp2/3. Interestingly, profilin decreases the fraction of ActA bound to Arp2/3 in a dose-dependent manner, suggesting that its binding site overlaps that of ActA. Fitting the data with a competitive binding model yields a K_d of 3.4 ± 0.3 for profilin binding, somewhat lower than our previous estimate of $7 \mu\text{M}$ from analytical ultracentrifugation (26). Profilin, however, is not as effective as N-WASP at displacing ActA from Arp2/3. In the presence of Arp2/3, saturating concentrations of profilin do not decrease the anisotropy of rhodamine ActA-(30–170) down to the levels of free protein suggesting that the binding sites do not completely overlap. The data are consistent with an increase in the K_d for ActA binding from 4.8 to $\sim 30 \mu\text{M}$. Although actophorin binds Arp2/3 with approximately the same affinity as profilin, actophorin does not dis-

place rhodamine ActA even at high concentrations (Fig. 4).

Kinetics of Arp2/3-dependent Nucleation—Although we do not understand the molecular details of Arp2/3-dependent nucleation, several characteristics of the process have been well described. Addition of WASP family proteins to Arp2/3 accelerates filament formation but, even at high concentrations, does not completely abolish the initial lag phase of polymerization (10, 27, 28). This suggests that nucleation promoting factors do not convert Arp2/3 into a *bona fide* actin nucleus but promote nucleation via a multistep mechanism. In addition, pre-existing filaments accelerate the nucleation reaction so that the majority of new actin filaments grow from the sides of pre-existing filaments in a characteristic branching pattern (7, 11, 15, 29). To compare the activity of ActA to that of endogenous nucleation promoting factors, we asked the following three questions. 1) Does addition of ActA abolish the lag phase of polymerization? 2) How does the maximal activity of ActA compare with that of an endogenous nucleation promoting factor? 3) Do pre-existing actin filaments enhance ActA-stimulated filament formation?

The maximal activity of either ActA-(30–612) or ActA-(30–170) is nearly identical to the most active C-terminal fragment of N-WASP (Fig. 5A). The initial lag phase of actin polymerization is reduced in the presence of both proteins but, even at saturating concentrations, is not abolished. We also find that prepolymerized actin seeds enhance the rate of ActA-stimulated polymerization (Fig. 5B). Under these conditions the lag phase is either abolished or so short that the dead time of our assay prevents us from detecting it.

DISCUSSION

Understanding the functional organization of the ActA protein is fundamental to the study of cellular actin dynamics. Motility of *Listeria* or beads coated with ActA is widely used as a model for *in vivo* actin assembly (30) and actin-based force generation (31). ActA, however, is a prokaryotic protein and a constitutive Arp2/3 activator and thus might operate by a different mechanism from that of the endogenous WASP family proteins. The present study, therefore, not only provides insight into the function of the Arp2/3 complex but also indicates the relevance of conclusions drawn from the study of *Listeria* motility to regulation of actin assembly by endogenous factors.

The structural and kinetic similarities identified by this study indicate that the constitutive Arp2/3 activator ActA and the tightly regulated WASP family proteins operate via a common mechanism and that ActA-dependent actin assembly closely mimics endogenous processes. In addition, for the first time we have identified the subunits of Arp2/3 that bind nucleation promoting factors, including ActA and the WASP family proteins.

Our results show that ActA binds two actin monomers and has actin-binding properties similar to WH2-containing WASP family proteins. The domain organization of ActA most closely resembles that of N-WASP (Fig. 2B) with two actin-binding sites N-terminal to an Arp2/3-binding site. The smallest ActA proteins that promote Arp2/3-dependent nucleation contain both an actin and an Arp2/3-binding site separated by a very short (five residues) and apparently flexible linker suggesting that nucleation promoting activity involves delivery of actin monomers to Arp2/3. The maximal activity of ActA is insensitive to a 10-fold change in the affinity for Arp2/3, but the maximal activity is very sensitive to the loss of residues 30–120. This may be caused by loss of one actin-binding site or possibly by a misfolding artifact. Cicchetti *et al.* (24) report that N-terminal truncations of ActA are unstable, and we find that, whereas ActA-(30–612) inhibits only pointed end elongation,

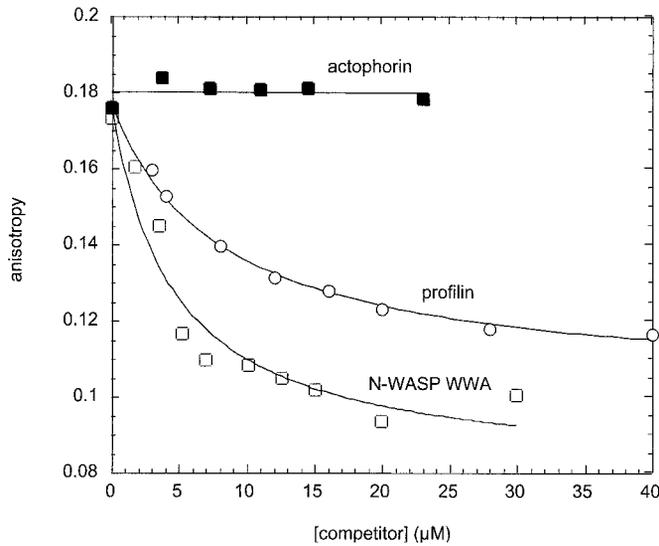


FIG. 4. N-WASP and profilin compete with ActA for binding to Arp2/3. Actophorin, which also binds Arp2, fails to compete. We mixed $4.8 \mu\text{M}$ Arp2/3 and $1 \mu\text{M}$ rhodamine ActA-(30–120) with the indicated concentrations of actophorin (■), profilin (○), or N-WASP (□), and we measured the polarization anisotropy of the labeled ActA. Displacement of ActA from Arp2/3 results in a concentration-dependent decrease in anisotropy. *Solid lines* are best fits to Equation 3 and predict dissociation equilibrium constants of $3.4 \pm 0.3 \mu\text{M}$ for profilin and $1.3 \pm 0.3 \mu\text{M}$ for N-WASP binding to Arp2/3 (conditions: buffer, 50 mM KCl, 1 mM MgSO_4 , 1 mM EGTA, 10 mM imidazole, pH 7; temperature, 24 °C).

ActA-(120–612) inhibits both pointed and barbed end elongation.

The most interesting result of our study is the identification of the binding site for nucleation promoting factors on the Arp2/3 complex. It is interesting because it tells us a lot about both the organization and activation of the Arp2/3 complex. We find that ActA and the endogenous WASP family proteins bind Arp2/3 in a mutually exclusive manner and by chemical cross-linking these proteins interact with the Arp2, Arp3, and p40 subunits of the Arp2/3 complex. Previous chemical cross-linking studies (32) identified interactions between Arp2 and p40 but found no evidence of a contact between Arp3 and either Arp2 or p40. The interaction of the small (49 residue) ActA-(121–170) protein with all three subunits suggests that they are in close physical proximity (Fig. 6A). Arp2 and Arp3 were previously predicted to form a heterodimer (20), but ours is the first experimental evidence that they are in close proximity within the Arp2/3 complex.

A yeast two-hybrid screen originally identified an interaction between the vertebrate p21arc subunit of the Arp2/3 complex and the Scar protein (7). We do not detect a direct interaction between p18 (the amoeba homolog of p21arc) and either ActA or hScar1, but our experiments do not rule it out. The p18-Scar-binding interface may not involve close proximity of amino and carboxyl groups required for chemical cross-linking by EDC. Regardless, p21arc does not appear to be required for Arp2/3 binding since Winter *et al.* (33, 34) found that a yeast Arp2/3 complex lacking p18 (yeast homolog of p21arc) and/or p40 can still bind Las17p/Bee1p (a yeast protein similar to N-WASP).

We propose that the essential interaction required for Arp2/3 activation is between the nucleation promoting factor and the actin-related protein Arp2 (Fig. 6). Three experiments argue for this. 1) We find that ActA, Scar, and N-WASP directly interact with Arp2. 2) Winter *et al.* (Winter, 1997) found that a yeast Arp2/3 complex lacking p40 coimmunoprecipitates with Las17p but that a complex lacking Arp2, p40, and p15 does not. 3) We previously showed that anti-Arp2 antibodies that do not affect the base-line nucleation or pointed end capping of puri-

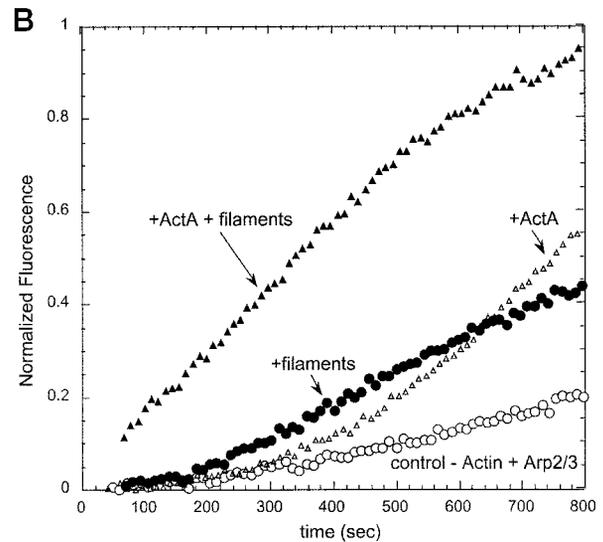
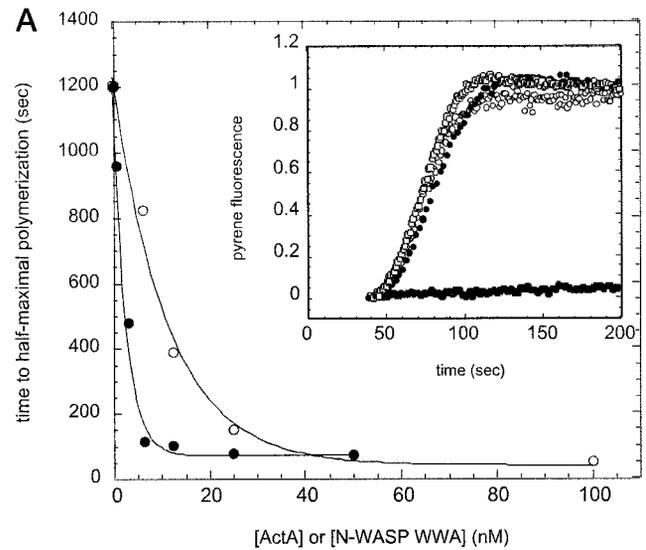


FIG. 5. Comparison of ActA activity with that of WASP family proteins. A, ActA exhibits similar actin polymerization kinetics to N-WASP VCA. Shown is a plot of the polymerization half-time with respect to either N-WASP VCA (○) or ActA-(30–612) (●) concentrations. *Inset* shows comparison of the maximal activities of ActA and N-WASP in the presence of 50 nM Arp2/3 and 2 μM actin. □, 100 nM ActA-(30–612); ○, 100 nM N-WASP VCA; ●, 5.7 μM ActA-(30–170); ■, Arp2/3 alone (conditions: amoeba actin was 15% pyrene-labeled; temperature, 24 °C; buffer conditions, 50 mM KCl, 1 mM MgSO_4 , 1 mM EGTA, 10 mM imidazole, pH 7). B, pre-polymerized actin filaments decrease the lag time. 15 nM Arp2/3 and 2.4 μM actin were mixed with Arp2/3 alone (○), 150 nM pre-polymerized actin (●), 200 nM ActA-(120–612) (□), 200 nM ActA-(120–612) (■), and 150 nM pre-polymerized actin (conditions: same as Fig. 4A).

fied Arp2/3 block its activation in cell extracts (44). We also detect direct interaction between ActA, Scar, N-WASP, and Arp3, but we can say less about the role of this interaction in nucleation. Our data are consistent with the two most likely models for Arp2/3 activation as follows: 1) nucleation promoting factor binding induces a reorganization of subunits and converts Arp2 and Arp3 into an actin-like nucleus (35), or 2) nucleation promoting factors deliver actin monomers to Arp2 or Arp3 (7).

Arp2 is an actin-related protein with 50% sequence identity to conventional actin. The surfaces of actin that interact with profilin and actophorin are conserved in Arp2 (20, 26) so the ability of profilin and the failure of actophorin to compete with ActA allow us to localize further the ActA-binding site on Arp2.

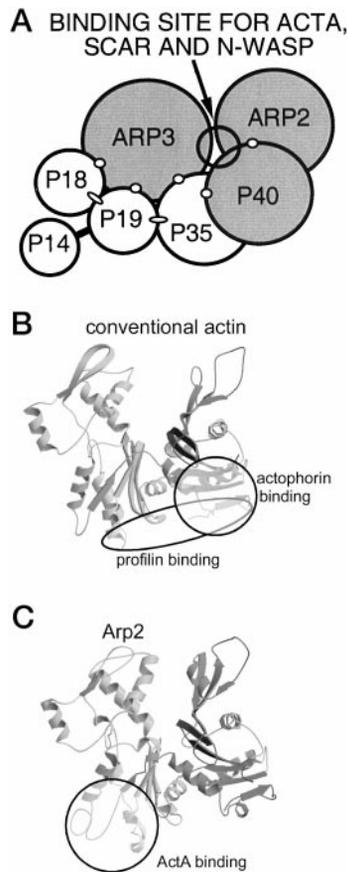


FIG. 6. Location of the ActA-binding site on the Arp2/3 complex and the Arp2 subunit. *A*, nearest-neighbor model of the Arp2/3 complex showing the location of the binding site for ActA and Scar. The model is based on chemical cross-linking (open circles and dark lines) (32) and two-hybrid analysis (ovals) (7). Subunits that cross-link to ActA, N-WASP, and Scar1 are shaded. *B*, atomic model of actin in the standard view (45) showing the regions involved in binding profilin and actophorin. *C*, homology model of Arp2 based on the crystal structure of conventional actin (20) showing the ActA-binding site deduced from competition binding experiments.

The profilin-binding site includes parts of subdomains 1 and 3 of the actin monomer (19), a surface that is exposed at the barbed end of a filament. The binding site for actophorin is less well characterized but is known to overlap the profilin-binding site (36) and to involve subdomain 1 (37). Based on our data we propose that ActA and the WASP family proteins interact with a site on Arp2 analogous to subdomain 3 in conventional actin (Fig. 6, *B* and *C*). This location is satisfying for two additional reasons. 1) It places the nucleation promoting factors in an ideal position to stabilize the interaction between the “barbed” end of Arp2 and a conventional actin monomer or filament. 2) This surface of Arp2 is not well conserved in conventional actin and contains an inserted loop that may allow ligands to discriminate between Arp2 and conventional actin (20, 38).

Profilin has been shown to decrease the efficiency of Arp2/3-mediated nucleation (10, 11). The effect was assumed to be caused by the binding of actin, and it was argued that profilin:actin is a poor substrate for the nucleation machinery. This creates a problem in understanding actin nucleation *in vivo* because in intact cells almost all polymerizable, monomeric actin is bound to profilin (39). We demonstrate that profilin competes directly with nucleation promoting factors for binding Arp2/3, and this competition may explain the previously observed inhibitory effects of profilin on nucleation. The ability of the nucleation machinery to create nuclei out of profilin:actin must be reinvestigated.

A similar study of ActA was published while this manuscript was under review. Based on analysis of deletion mutants, Skoble *et al.* (40) argue that actin binding is not essential for ActA activity *in vivo* and that perhaps the combination of VASP and profilin can deliver actin monomers to the Arp2/3 complex. We disfavor this model for two reasons. 1) The authors failed to remove the actin-binding site between amino acids 121 and 138 and so never directly tested an ActA molecule with no actin-binding sites in their motility assays. 2) Although VASP is known to enhance *Listeria* motility, this effect has been shown to be independent of its ability to bind profilin (41).

We do not understand the physiological significance of the interaction between profilin and Arp2/3. We estimated previously that *in vivo* a significant fraction of Arp2/3 is bound to profilin (26). The fact that profilin inhibits binding of nucleation promoting factors suggests that it may play a role in regulation of Arp2/3 activity.

During *Listeria* motility ActA remains attached to the surface of the bacterium (4), whereas Arp2/3 remains attached to the actin network (15). So for efficient motility ActA must dissociate rapidly from Arp2/3 after a new filament is formed. To avoid retarding bacterial motility, we estimate that the Arp2/3 must dissociate from ActA before the bacterium has moved 25 nm (or approximately ~ 10 monomers). We measure an affinity of $0.6 \mu\text{M}$ for Arp2/3-binding ActA. A diffusion-limited association rate constant of $10 \mu\text{M}^{-1} \text{s}^{-1}$ predicts an upper limit for the dissociation rate constant of 6s^{-1} and a lower limit of 0.13 s for the half-time of dissociation. The cytoplasmic concentration of polymerizable actin is $\sim 100 \mu\text{M}$ (42), and the rate constant for filament elongation at the bacterial surface is between 3 and $10 \mu\text{M}^{-1} \text{s}^{-1}$ (31, 43) so filaments associated with the bacterium should elongate at a rate of 300–1000 monomers/s. Thus the bacterium will move by more than 75 nm (30–100 actin monomers) by the time half the ActA has dissociated from Arp2/3. Thus we speculate that the *in vivo* rate of Arp2/3 dissociation is significantly faster than the rate *in vitro*. Perhaps some step in the nucleation reaction decreases the affinity of Arp2/3 for ActA.

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Activation of the Arp2/3 Complex by the *Listeria* ActA Protein: ActA BINDS TWO ACTIN MONOMERS AND THREE SUBUNITS OF THE Arp2/3 COMPLEX

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