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Three-dimensional reconstructions of actin filaments capped by Arp2/3 complex

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Abstract

The primary function of Arp2/3 complex is the generation of free barbed ends by nucleating new filaments from the sides of pre-existing filaments. The pathway of branch formation is complex and involves nucleation promoting factors, actin monomers and nucleotides. A less prominent function of Arp2/3 complex is capping of actin filament pointed ends. Here we show, using electron microscopy, electron tomography, and image reconstruction of negatively-stained samples at ~2–3 nm resolution, that Arp2/3 complex bound to the pointed ends of actin filaments has a conformation similar to that in the branch junction with the Arps arranged in an actin-filament like configuration. This is direct evidence for the existence of two distinct activation pathways for Arp2/3 complex, one in the context of branch formation, one in the context of pointed-end capping, with essentially the same conformational end point.

Keywords

Cell motility; actin cytoskeleton; electron microscopy; image analysis; fitting

Introduction

Rearrangement of the actin cytoskeleton is an integral feature of a vast number of eukaryotic cellular processes including movement, adhesion, endocytosis and establishment of polarity. Because the rate-limiting step for spontaneous actin polymerization is the formation of small oligomers, controlling the number of free barbed ends that can rapidly elongate is a key

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mechanism to control actin polymerization. De novo formation of barbed ends has now become an attractive model for rapid assembly of different types of actin structures with the discovery of the actin nucleation activity of the Arp2/3 complex and formin-family proteins.

A de novo actin nucleator must have the ability to increase the rate of the formation of barbed ends from actin monomers. The Arp2/3 complex, an evolutionary conserved 220-kDa complex, consists of seven stoichiometric subunits that include two actin-related proteins (Arps), Arp2 and Arp3, and five proteins called ARPC1-5. The Arp2/3 complex purified from a variety of cell types, including yeast, *Acanthamoeba*, platelets, and bovine brain, show little or no activity in actin nucleation assays. Biochemical experiments showed that the main pathway to actin nucleation involves binding of nucleation promoting factors (NPFs), an actin monomer, pre-existing (mother) filaments, and ATP. In the presence of all the above factors, Arp2/3 complex initiates a new filament that grows as a branch at an angle of about 78° from the mother filament (Pollard and Borisy, 2003; Pollard, 2007; Bugyi and Carlier, 2010; Volkmann et al., 2001).

Recent structural studies provided direct evidence for the existence of various conformational states of the Arp2/3 complex along this activation pathway suggesting a multiple-step regulation of branch formation. In the branch junction, Arp2/3 complex is bound to the side of the mother filament, and Arp2 and Arp3 form the first two subunits of the new (daughter) filament (Rouiller et al., 2008). In this active conformation, all of the subunits of the Arp2/3 complex, including the pointed ends of both Arps, contact the mother filament. Crystal structures of Arp2/3 complex (Robinson et al., 2001; Nolen et al., 2004; Nolen and Pollard, 2007; Ti et al., 2011), however, showed conformations where the arrangement of Arp2 and Arp3 does not support the growth of a daughter filament. The overall conformation of NPF-bound Arp2/3 complex also does not appear to adopt a conformation that is fully capable of promoting new filament nucleation (Xu et al., 2011). In particular, in the NPF-bound state, Arp3 is stabilized in an intermediate conformational state where the nucleotide binding cleft is half way in between a filament-like closed cleft and the open conformation observed in the crystal structures. In addition, the NPF density in the NPF-bound complex is located at the pointed ends of Arp2 and Arp3 partially occluding the mother filament-binding site. These findings suggest that stepwise conformational changes occur within the Arp2/3 complex, triggered by its interaction with various molecules including nucleotide, NPFs, the mother filament, and actin monomers, eventually leading to the formation of the daughter filament.

An apparent contradiction to this-multistep activation model was the observation that the Arp2/3 complex can cap the pointed-ends of pre-formed actin filaments independent of the presence of NPFs or actin monomers (Mullins et al., 1998). While barbed-end polymerization provides the force of protrusion, disassembly of actin filaments is critical to the turnover of filament networks in lamellipodia and appears to be a rate-limiting step in the cycle of actin filament assembly and disassembly (Carlier, 1998). Modifications of pointed-end capping by tropomodulin-3 in lamellipodia has pronounced effects on cell migration (Fischer et al., 2003), indicating that regulation of pointed end dynamics by capping may be an important factor in actin network assembly and disassembly. Cell-based assays for investigating the effect of pointed-end capping by Arp2/3 complex are difficult to

design because these effects will generally be masked by the more dominant branching function of the complex. However, the unexpected behavior of tropomodulin 3 mutants in the context of lamellipodia dynamics, suggests that pointed end capping by Arp2/3 complex may play a role in regulation of actin network dynamics in lamellipodia (Fischer et al., 2003).

To determine if there exists an alternative conformational state of the Arp2/3 complex that caps pointed ends, we used electron microscopy and electron tomography to generate reconstructions of *Acanthamoeba* Arp2/3 complex bound to the pointed ends of actin filaments. The reconstructions show that the conformation of Arp2/3 complex bound to the pointed end is similar to the conformation of the Arp2/3 complex in the branch junction, with the two actin-related proteins in an actin-filament-like dimer formation. Thus, neither NPFs nor binding to the side of a mother filament is required for Arp2/3 complex to achieve this active conformation. Our studies provide direct evidence for two distinct pathways to activate Arp2/3 complex, one for branching and one for pointed-end capping.

Materials and Methods

Sample Preparation for Transmission Electron Microscopy Imaging

Acanthamoeba castellanii Arp2/3 complex and phalloidin-stabilized *Acanthamoeba* actin filaments were kindly provided by Dr. Mark Dayel (Mullins lab). These preparations were described previously (Dayel et al., 2001; Dayel and Mullins, 2004; MacLean-Fletcher and Pollard, 1980). We prepared actin filaments capped at the pointed end by Arp2/3 complex following, with some modifications, the procedures described in (Dayel 2004). In brief, 2 μ M phalloidin-stabilized *Acanthamoeba* actin filaments in KMEI buffer plus 100 μ M ATP (50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA and 10 mM imidazole (pH 7.0)) were mixed with 20 nM Arp2/3 complex, sonicated twice (30 s) in a water bath (FS20, Fisher Scientific) to shear the filaments and incubated at RT for one minute. Samples were then applied to glow discharged carbon-coated 400 mesh copper electron microscopy grids (SPI Inc). For negative staining, 2% uranyl acetate was applied to the grids, which was subsequently blotted and air-dried.

Electron Microscopy and 2D Image Analysis

Images of all samples were acquired using a Tecnai 12 G2 transmission electron microscope (FEI company) equipped with a Lab6 filament at 120 kV and a nominal magnification of 52,000X. Micrographs were recorded on Kodak ISO-163 plates (Eastman Kodak Co) with a total dose of 20–50 e⁻/Å² and under defocus ranging between 1.5 μ m and 2.0 μ m. The micrographs were developed in full-strength Kodak D19 developer, digitized using a SCAI scanner (Integrageph) at 7- μ m raster and binned to a final pixel size of 0.6 nm. Totals of 232 assemblies were interactively selected from 40 micrographs. Reference-free class averages were determined with EMAN (Ludtke et al., 1999). The data segregated into seven separate classes with the largest showing 52 contributing to the average. The Fourier Ring Correlation between two random halves of the class indicated a resolution of 2.4 nm (0.5 cut off).

Electron Tomography and Image Analysis

Tomographic tilt series (approximately -70 to $+70^\circ$, every 2°) were acquired on a Tecnai G2 F20 (FEI Company) equipped with an energy filter (zero-loss, Gatan Inc.) using a 2020 advanced tomography holder (Fishione Instruments). The data was collected using SerialEM (Mastronarde, 2005). To minimize beam damage, all data, were collected under strict low-dose conditions with a cumulative dose per tomogram not exceeding $300 \text{ e}^-/\text{\AA}^2$. Three-dimensional reconstructions were generated using alignment and backprojection algorithms implemented in the IMOD package (Kremer et al., 1996). Noise-reduction (van der Heide et al., 2007) segmentation (Volkman, 2002) and sub-volume averaging were performed using the CoAn package (Volkman and Hanein, 1999). The Fourier Shell Correlation between two random halves of the sub-volumes indicated a resolution of 3.5 nm (0.5 cutoff).

Model building

Three models for the pointed-end capped actin filaments were derived. The first one was constructed by deleting the mother filament from the branch junction model (Rouiller et al., 2008) and extending the daughter filament by several subunits using the actin symmetry. The second model was derived by aligning Arp3 of NPF-bound Arp2/3 (Xu et al., 2011) with Arp3 of the first model and then replacing the original Arp2/3 complex with the aligned NPF-bound Arp2/3 complex. The third model was derived in a similar fashion only using the inactive conformation with completed Arp2 (Xu et al., 2011) to replace the original Arp2/3 complex.

Fitting

All fitting calculations were performed using the CoAn suite (Volkman and Hanein, 1999; Volkman, 2009). For the tomographic average, the entire models were fitted into the observed three-dimensional density as described (Volkman and Hanein, 1999). To find the best fit of the models to match the observed class average projection density, the models were turned around the filament axis in successions of two degrees and projection densities were calculated using electronic form factors for the atoms and imposing a resolution limit of 2.5 nm using convolution with a Gaussian function. Correlations between the observed and the calculated projection densities were derived only in the filament region of the model (above the line in Fig. 2A). This allows an evaluation of the quality of fit in the Arp2/3 complex region without bias from the fitting in that region. The quality of the fits was estimated in the following way. A contour level was defined that fit well in the filament region of the reconstructions. Then, the number of Arp2/3 complex atoms inside that contour level was counted and compared to the total number of atoms. The higher the percentage of atoms that are accounted for by the density, the higher is the quality of the fit (Rossmann, 2000).

Results and Discussion

The Arp2/3 complex is known to cap the pointed ends of pre-formed actin filaments *in vitro*, inhibiting both polymerization and depolymerization from the pointed ends of actin filaments (Mullins et al., 1998; Blanchoin et al., 2000; Dayel and Mullins, 2004). To define the conformational state of Arp2/3 complex in this context, we derived structures of actin

filaments with pointed ends bound to Arp2/3 complex purified from *Acanthamoeba*. We used mechanical shearing (sonication) of long, phalloidin-stabilized actin filaments to generate many short filaments. The newly generated filament ends can in principle undergo rapid re-annealing (Murphy et al., 1988). However, after shearing in the presence of 20 nM Arp2/3 complex, pointed-end capping by the Arp2/3 complex inhibits re-annealing and results in significantly shorter filaments (Fig 1A).

The length distribution of these filaments is between 30 nm and 800 nm and resembles a negative binomial distribution ($n=3$, $P_{failure}=0.3132$) with a mode of 128 nm and a standard deviation of 134 nm. The majority of the filaments are clustered narrowly around the mode with 66% of the filament being shorter than 200 nm (Fig. 1B). This distribution is strikingly different from the length distribution in a control group of unsheared, phalloidin-stabilized actin filaments where the shortest filaments are several 100 nm long and most filaments are longer than 1 μ m (Fig 1C). Pointed-end capping by Arp2/3 complex can be clearly seen in micrographs and in reference-free class averages of negatively stained samples (Fig. 2), as well as in tomographic reconstructions (Fig. 3). Comparison of the class averages with matched projections calculated from a model of Arp2/3 complex bound to the new daughter filament taken from our model of the branch junction (Rouiller et al., 2008) show an excellent fit (Fig. 2). The same model fits well into a sub-volume average of the pointed-end capped actin-filament densities derived by electron tomography (Fig. 3).

Mechanisms for forming new filaments in cells include growing a branch on the side of an existing filament, severing filaments to create new ends, or starting up a filament from monomers. Several factors are now known to nucleate new actin filaments, including Arp2/3 complex, formin-family proteins and spire-like proteins. Arp2/3 complex contains a cryptic filament barbed end, constructed from the actin-related proteins, Arp2 and Arp3. Arp2/3 complex binds preexisting filaments and creates new filaments that grow as branches off the older ones. NPFs control the activity of the complex. Rather than functioning as a simple transition between the active and inactive states, a succession of conformations is induced during the activation pathway by various co-factors eventually leading to branch formation. The complex seems to require binding of two nucleation-promoting factors (Padrick et al., 2008), and a handoff of actin monomers from the activators to the complex (Dayel and Mullins, 2004; Padrick et al., 2011), as well as the presence of pre-existing filaments to induce the formation of a new filament from the side of an existing filament. The two NPF-binding sites on the complex have significantly different affinities and each appears to execute a distinct step in the activation pathway (Xu et al., 2011; Ti et al., 2011).

The reconstructions of the pointed-end-bound conformation presented here suggest that these activation steps can be bypassed upon interaction of the Arp2/3 complex with the pointed of an actin filament. It is possible that in the absence of inhibitors such as coronin (Humphries et al., 2002) the Arp2/3 complex naturally fluctuates between the active and inactive conformations, and that the encounter and capture by an actin filament pointed end stabilizes the complex in the same active conformation. This notion is consistent with the previous finding that Arp2/3 complex caps the pointed end filaments with no apparent need of NPF or pre-existing filaments (Mullins et al., 1998; Blanchoin et al., 2000). The affinity of the pointed-end binding of Arp2/3 complex is comparable to that of tropomodulin-3, a

pointed end capper that has been shown to regulate motility of endothelial cells through pointed end capping in the lamellipodia (Fischer et al., 2003). Interestingly, increased pointed-end capping induced by transient overexpression of tropomodulin-3 decreases both free barbed ends and actin filaments in the lamellipodia. This behavior is counterintuitive, because an increase in pointed end capping should allow actin filaments to persist longer and thus lead to a larger number of actin filaments. A potential mechanism to account for the decrease would be that tropomodulin-3 competes directly with Arp2/3 complex for the capture of free pointed ends of actin filaments and oligomers suggesting a functional role for Arp2/3 complex in the regulation of pointed-end dynamics.

At the current resolution of the reconstructions it is clearly evident that Arp2 and Arp3 form an actin-filament like template that binds to the pointed end of an actin filament. The configuration of Arp2 and Arp3 in the inactive conformations seen in the crystal structures (Robinson et al., 2001; Nolen et al., 2004; Nolen and Pollard, 2007; Ti et al., 2011) and in solution (Xu et al., 2011) is incompatible with the density of Arp2/3 complex bound to the pointed end: The observed density only accounts for 79% ($\pm 2\%$) of the structure when either Arp2 or Arp3 of the inactive complex are oriented as an additional subunit at the pointed end. In contrast, the observed density accounts for 92% of the branch junction conformation (Rouiller et al., 2008) when Arp2 and Arp3 are oriented as additional subunits at the pointed end. If a model derived from the NPF-bound state (Xu et al., 2011) is used instead of the branch conformation, the observed density accounts for 88% of the structure, thus favoring the branch junction conformation over the NPF-bound conformation of the complex as well. Additional relatively minor conformational differences from the branch junction model cannot be ruled out but – given the already high quality fit of the unmodified branch junction conformation (92%) – cannot be modeled with confidence at the resolution of the study.

It was suggested (Dayel and Mullins, 2004) that burying the large surface area of the filament pointed end (approximately $6800 \text{ \AA}^2$) by interaction with an Arp2–Arp3 dimer in the correct conformation would be sufficient to promote formation of the pointed-end assembly. However, the terminal actin subunit at the filament pointed end does not follow standard actin symmetry and has additional contacts with the neighboring filament subunit (Narita et al., 2011) that would need to be broken to allow incorporation of Arp2 and Arp3 as filament subunits at the pointed end. Our data provide direct evidence that this combined energy barrier can be overcome upon binding of the two Arp subunits of Arp2/3 complex to the pointed end of filaments, supporting the notion for the existence of two activation pathways for the complex.

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Figure 1.

Actin filaments capped at the pointed end by Arp2/3 complex. (A) Representative field of view showing several short filaments with Arp2/3 complex capping the pointed ends. Arrowheads point at some of the Arp2/3 complexes capping filaments.. (B) Length distribution of capped actin filaments (in nm). (C) Representative image of control actin filaments in the absence of Arp2/3 complex. Note that images in A and C were taken at different magnifications (3X difference). Scale bars represent 100 nm.

Figure 2.

Reference-free class average with matched projections of pointed end of F-actin capped by Arp2/3 complex. (A) The reference-free class average clearly shows actin-filament symmetry at the top and the Arp2/3 complex attached at the bottom. The line indicates the division between the regions used for fitting (above the line) and for evaluation of the fit (below the line). (B) If a model based on the Arp2/3 complex and the daughter filament obtained from the branch junction model (Rouiller et al., 2008) is rotated to optimize the overlap of the actin symmetry at the top the projection of the attached Arp2/3 complex model in the branch orientation matches well the observed projection density. The figure shows the observed projection density shown in (A) overlaid with the projection density calculated from the optimally oriented model (colored subunits) (C). Surface representation of the model in the optimal orientation (front and back). The observed projection density is shown in the background. (D) Ribbon diagram of the Arp2/3 complex based on crystal structures and modified to fit into the reconstruction of the branch junctions (Rouiller et al., 2008). This color code for the subunits is also used in Figures 2 and 3.

Figure 3.

Sub-volume average (transparent surface) of the pointed-end capped actin filaments density fitted with our atomic model of Arp2/3 complex and the daughter filament extracted from the branch junction model (Rouiller et al., 2008). Three different views are presented. The second (B) is turned 90 degrees anticlockwise from the first (A) around the vertical axis. The third (C) is turned by 90 degrees clockwise around the horizontal axis. The contour level shown is optimized to emphasize the quality of fit in the Arp2/3 complex region. To show optimal coverage in the filament region, the contour level would need to be lowered, indicating that the filament subunit densities are slightly less well-defined, especially when further away from the Arp2/3 complex, a consequence of minor variations in the attachment angles.