Actin turnover–dependent fast dissociation of capping protein in the dendritic nucleation actin network: evidence of frequent filament severing

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Abstract

Actin forms the dendritic nucleation network and undergoes rapid polymerization-depolymerization cycles in lamellipodia. To elucidate the mechanism of actin disassembly, we characterized molecular kinetics of the major filament end-binding proteins Arp2/3 complex and capping protein (CP) using single-molecule speckle microscopy. We have determined the dissociation rates of Arp2/3 and CP as 0.048 and 0.58 s$^{-1}$, respectively, in lamellipodia of live XTC fibroblasts. This CP dissociation rate is three orders of magnitude faster than in vitro. CP dissociates slower from actin stress fibers than from the lamellipodial actin network, suggesting that CP dissociation correlates with actin filament dynamics. We found that jasplakinolide, an actin depolymerization inhibitor, rapidly blocked the fast CP dissociation in cells. Consistently, the coexpression of LIM kinase prolonged CP speckle lifetime in lamellipodia. These results suggest that coflin-mediated actin disassembly triggers CP dissociation from actin filaments. We predict that filament severing and end-to-end annealing might take place fairly frequently in the dendritic nucleation actin arrays.

Introduction

Migrating cells form a dynamic, thin, veil-like structure called lamellipodia at the leading edge. The dendritic nucleation model describes the reorganization processes of actin arrays in lamellipodia (Carlier et al., 2003; Pollard and Borisy, 2003). Within the lamellipodia, actin filaments display a branched network (Svitkina and Borisy, 1999). The Arp2/3 complex nucleates actin filaments off the sides of preexisting filaments (Mullins et al., 1998; Welch et al., 1998; Amann and Pollard, 2001; Fujiwara et al., 2002). Nucleated filaments elongate toward the leading edge (Small et al., 1978), and their growth is terminated by capping protein (CP; Mejillano et al., 2004; Wear and Cooper, 2004). Actin then depolymerizes to replenish the monomeric actin pool for the next round of polymerization. Quantitative modeling of the dendritic nucleation actin array is a logical goal in cell migration research, but it will require precise knowledge on the mechanisms governing actin filament turnover in vivo.

Our previous study using single-molecule speckle microscopy investigated actin filament lifetime distribution in lamellipodia of spreading Xenopus laevis fibroblasts (Watanabe and Mitchison, 2002). The filament lifetime spanned a wide range of time with a maximum of 148 s. Notably, one third of the filaments had a short lifetime of $<10$ s. This raised the question of what mechanisms may account for the observed lifetime of actin filaments.

In this study, we further extend our single-molecule speckle analysis to major actin end-binding proteins to elucidate the filament turnover mechanism. Single-molecule observations allow us to precisely measure the dissociation kinetics of a molecule in cells given that the molecule binds cellular structures on the order of seconds. Surprisingly, the dissociation of CP from actin was found to occur three orders of magnitude faster than in vitro and even much faster than the actin disassembly rate. We demonstrate that the CP dissociation rate is prolonged under the several conditions in which actin filaments are stabilized. Our data indicate that...
cofilin-mediated actin disassembly is required for the fast CP dissociation in lamellipodia. Based on the marked difference in the dissociation rate between actin (0.03 s⁻¹) and CP (0.58 s⁻¹), we predict that fairly frequent filament severing and end-to-end annealing might take place in the dendritic nucleation actin arrays.

Results

Fast dissociation kinetics of CP in lamellipodia

CP has been attributed to most of the actin capping activity in cell lysates (DiNubile et al., 1995; Hug et al., 1995) and has been found as an essential protein in the regulation of lamellipodium morphology (Rogers et al., 2003). Therefore, we analyzed the dynamics of CP to elucidate the state of barbed ends in the dendritic nucleation network. We generated three expression constructs by tagging either the α or β subunit of Xenopus CP with an EGFP. Of the three, two probes, EGFP-CPβ1 and CPβ1-EGFP, distributed to lamellipodia, which is consistent with previous studies (Schafer et al., 1998; Mejillano et al., 2004). EGFP-CPβ1 and CPβ1-EGFP speckles moved along with the retrograde actin flow, which suggested their association with actin structures (Fig. 1 A and Videos 1 and 2, available at http://www.jcb.org/cgi/content/full/jcb.200604176/DC1).

We selected these two probes for single-molecule speckle analysis of CP.

We measured the lifetime distribution of single-molecule CP speckles in lamellipodia of XTC cells spreading on poly-l-lysine (PLL)-coated glass coverslips. EGFP-CPβ1 and CPβ1-EGFP showed a similar lifetime distribution (Fig. 1, B and C). Our measurement revealed that 53–60% of CP dissociated from actin within 1 s. When the CP lifetime distribution was fit with a single exponential curve, the half-life was 1.20 and 1.23 s for EGFP-CPβ1 and CPβ1-EGFP, respectively, which is in marked contrast to the slow dissociation of CP from barbed ends in vitro \( (t_{1/2} = \sim 28 \text{ min}; \text{Schafer et al., 1996}) \). CP displayed similar lifetime distributions throughout lamellipodia (Fig. 1 D).

We next examined the spatial location of CP recruitment.

We recorded positions of newly appearing CP speckles and measured the distance from the cell edge. Although CP speckles appeared most frequently in the tip region, appearance was widely distributed throughout the lamellipodia (Fig. 1 E). This bias toward the lamellipodium tip region was weaker than that observed for the Arp2/3 complex (see Fig. 3 E).

Given this unexpectedly fast dissociation, we needed to test whether our EGFP-tagged CP probes retained high affinity to barbed ends. We characterized biochemical properties of the recombinant Cpx2/EGFP-CPβ1 heterodimer expressed in Escherichia coli (Fig. 2 A). First, we determined the on-rate constant \( (k_{on}) \) of Cpx2/EGFP-CPβ1 using a pyrene-actin-polymerization assay (Fig. 2 B). Low concentrations of Cpx2/EGFP-CPβ1 efficiently inhibited barbed end elongation from filamentous actin (F-actin) seeds. The on-rate constant was calculated to be 3.9 μM⁻¹s⁻¹, which was comparable with that of native CP (3.5–5.7 μM⁻¹s⁻¹; Schafer et al., 1996).

Second, we measured the dissociation constant \( (K_d) \) using a pyrene-actin steady-state assay (Fig. 2 C). The \( K_d \) of Cpx2/EGFP-CPβ1 with barbed ends was 1.3 nM, which was comparable with the \( K_d \) of native CP (0.06–1 nM; Caldwell et al., 1989; Casella and Torres, 1994; Schafer et al., 1996). The \( K_d \) and on-rate constant predict an off-rate constant of \( 5.1 \times 10^{-3} \text{ s}^{-1} \), which is \( \sim 100\text{-fold smaller than the dissociation rate of our CP probes in lamellipodia (Fig. 1, B and C). We also noted that recombinant EGFP-CPβ1, which was expressed and purified in the absence of Cpx subunits, did not interfere with actin elongation (unpublished data). This is consistent with previous findings (Casella and Torres, 1994). Therefore, CP speckles observed in cells should correspond to EGFP-CPβ1 coupled with endogenous Cpx subunits but not deficient EGFP-CPβ1 monomers.}

To further evaluate the fidelity of our CP probes, we permeabilized cells expressing EGFP-CPβ1 using Triton X-100 and followed the decay of EGFP fluorescence. The decay rate of fluorescence intensity was \( 1.7 \times 10^{-4} \text{ s}^{-1} \) in lamellipodia.
Arp2/3 localization (Mullins et al., 1997; Welch et al., 1997; punctate actin structures, which is consistent with the reported p21-EGFP—were localized to lamellipodia and cytoplasmic them, four probes—EGFP-p40, p40-EGFP, EGFP-p21, and

testing N- and C-terminal fusions in several cases. Among

dissociation kinetics of CP speckles represent the dynamics
of endogenous CP.

To test whether other barbed end interactors might cap actin tightly, we visualized the molecular dynamics of Eps8, VASP, and gelsolin (Fig. S1 and Videos 3–7, available at http://www.jcb.org/cgi/content/full/jcb.200604176/DC1; Witke et al., 1995; Caldwell et al., 1989; Casella and Torres, 1994; Schafer et al., 1998; Svitkina and Borisy, 1999). Using an anti-p40 antibody, we confirmed that distributions of EGFP-CP and p21-EGFP were identical to that of the endogenous p40 subunit of Arp2/3 (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200604176/DC1). We also compared localization of the four probes with each other by generating probes tagged with monomeric red fluorescent protein 1 (mRFP1; Campbell et al., 2002). All four probes showed identical localization (Fig. S2) and moved inward at the same rate as the retrograde actin flow. Based on these observations, we selected these four probes for further analysis.

We analyzed the kinetics of Arp2/3 dissociation from the actin network in lamellipodia. We followed each newly emerged single-molecule Arp2/3 speckle and measured the duration between its appearance and disappearance (Fig. 3, A–D; and Video 8, available at http://www.jcb.org/cgi/content/full/jcb.200604176/DC1). Arp2/3 interacts with the side of actin filaments with micromolar affinity (Mullins et al., 1997; Gourrier et al., 2001). Thus, Arp2/3 is expected to dissociate from the side of the filament on a subsecond timescale after leaving the pointed end, and rapidly dissociating side binding would not be detected using our current experimental settings. Therefore, we interpret that the lifetime of Arp2/3 speckles represents the duration of Arp2/3 association with the pointed end, although we do not know whether Arp2/3 dissociation occurs simultaneously with branching. The lifetime distribution of Arp2/3 speckles could be approximated by a single exponential curve, which suggests that the dissociation of Arp2/3 is governed by a single rate-limiting step. Half-life determined for four Arp2/3 probes yielded similar values that spanned 12.5 to 16.9 s. The half-life of Arp2/3 speckles is about half of
that of actin filaments in lamellipodia of XTC cells (Watanabe and Mitchison, 2002).

Next, we examined the spatial location of Arp2/3 recruitment by recording positions of newly appearing speckles. Although Arp2/3 speckles appeared throughout lamellipodia, appearance was heavily biased to a zone within 0.5 μm from the cell edge (Fig. 3 E). This bias was greater than that previously observed for actin speckles (Watanabe and Mitchison, 2002) and suggests that most Arp2/3-driven nucleation occurs in the tip region of the leading edge, where known Arp2/3 activators are localized (Nakagawa et al., 2001). The mean lifetime of Arp2/3 did not change throughout lamellipodia, although Arp2/3 with prolonged lifetime was observed more frequently in the cell edge region than in the rest of lamellipodia (Fig. 3 F).

Less dynamic behavior of CP bound to actin stress fibers

Under our normal observation conditions, up to 2 h after cells were seeded on PLL-coated coverslips, our CP probes were predominantly colocalized with Arp2/3 in lamellipodia and cytoplasmic punctate actin structures (Fig. 4 A). When cells were allowed to grow for another several hours, CP probes were also associated with actin stress fibers (SFs; Fig. 4 B). The similar localization of CP to myofibrillar structures in cultured cardiomyocytes has been reported previously (Schafer et al., 1994). We noticed that a fraction of CP speckles stayed stably associated with SFs, whereas CP speckles that did not localize with Arp2/3 speckles appeared throughout lamellipodia, appearance was heavily biased to a zone within 0.5 μm from the cell edge (Fig. 3 E). This bias was greater than that previously observed for actin speckles (Watanabe and Mitchison, 2002) and suggests that most Arp2/3-driven nucleation occurs in the tip region of the leading edge, where known Arp2/3 activators are localized (Nakagawa et al., 2001). The mean lifetime of Arp2/3 did not change throughout lamellipodia, although Arp2/3 with prolonged lifetime was observed more frequently in the cell edge region than in the rest of lamellipodia (Fig. 3 F).
SFs in the lamella region emerged and disappeared as quickly as in lamellipodia. When subjected to speckle regression analysis (Watanabe and Mitchison, 2002), the decay rate of persistent CP speckles located on SFs was slower than that of CP in the other area of lamella (Fig. 4 B). These results suggest that CP dissociation is differentially regulated in distinct actin structures and that the dissociation rate of CP may be correlated with the actin filament turnover rate.

**Attenuation of fast CP dissociation by the inhibition of actin disassembly**

Next, we examined the effect of stabilization of actin filaments on the dissociation kinetics of CP. We tested jasplakinolide (Jas), an actin depolymerization inhibitor (Bubb et al., 2000) that induces a three- to fourfold increase in the half-life of actin filaments within 1 min in lamellipodia (Watanabe and Mitchison, 2002). We observed a rapid, marked prolongation of CP speckle lifetime upon treatment with 1 μM Jas (Fig. 5 and Video 9, available at http://www.jcb.org/cgi/content/full/jcb.200604176/DC1). This finding is striking, as the half-life of actin filament turnover (~30 s; Watanabe and Mitchison, 2002) greatly (~20-fold) exceeds that of CP, and it is a counterintuitive phenomenon that inhibiting the disassembly of a network system has a strong impact on the kinetics of its associated molecule, which displays much faster binding-dissociation kinetics.

To confirm this, we tested whether the inactivation of coflin, a major actin depolymerizing factor implicated in lamellipodium formation (Bamburg, 1999; Chan et al., 2000; Nagata-Ohashi et al., 2004), may also have similar effects. LIM kinase (LIMK) phosphorylates coflin and prevents the interaction between coflin and actin (Yang et al., 1998). We generated an expression construct for mRFP1-tagged human LIMK (mRFP1–hLIMK-1) to examine the effect of various levels of LIMK-1 expression on CP. It was confirmed that cells expressing mRFP1–hLIMK-1 displayed slower actin filament turnover (Fig. S3 and Video 10, available at http://www.jcb.org/cgi/content/full/jcb.200604176/DC1), which is consistent with previous findings (Hotulainen et al., 2005). The decay rate of persistent CP speckles in lamellipodia was also markedly prolonged in cells expressing mRFP1–hLIMK-1, and this effect was correlated with the expression level of mRFP1–hLIMK-1 (Fig. 6). These results indicate that coflin-mediated actin disassembly is responsible for the fast dissociation kinetics of CP in lamellipodia. Because three different conditions of actin filament stabilization led to the prolongation of CP speckle lifetime (Figs. 4–6), filament severing may frequently take place in lamellipodia, which could lead to the fast dissociation of CP from the actin network.

**Discussion**

This study reveals the astoundingly fast dissociation kinetics of CP in lamellipodia. In vitro, native CP binds barbed ends with subnanomolar affinities, and CP dissociates from barbed ends at a very slow rate (~0.0004 s⁻¹; Caldwell et al., 1989; Casella and Torres, 1994; Schafer et al., 1996). In contrast, our CP probes displayed three orders of magnitude faster dissociation kinetics in cells. Several studies have already suggested the weak interaction of CP with barbed ends in cell lysates. Nearly 1 μM of free barbed ends are present despite ~1–2 μM CP being present in lysates obtained from *Dictyostelium discoideum* cells (Hug et al., 1995) and neutrophils (DiNubile et al., 1995). The dissociation constant of CP in cell lysates was estimated to be ~0.1 μM (Hug et al., 1995). The weak affinity of CP to barbed ends has now been confirmed by our data obtained using intact cells. Moreover, our results conclude that the fast dissociation of CP, but not its slow association as postulated (Hug et al., 1995), is responsible for the weak capping activity. Attenuation of the fast dissociation of CP probes upon permeabilization can be explained by the removal of CP dissociation promoters such as phosphatidylinositol bisphosphate (Schafer et al., 1996) and CARMIL (Yang et al., 2005). An unidentified anti-CP factor that differs from VASP and CARMIL has also been reported previously (Huang et al., 2005).
Twinfilin, a barbed end–interacting protein with a G-actin sequestering activity (Helfer et al., 2006), interacts directly with CP without affecting the interaction of CP with barbed ends (Falck et al., 2004). These molecules may contribute to the fast dissociation of CP. However, based on our findings of the strong dependency of the CP dissociation rate on actin filament turnover (Figs. 4–6), we propose that coflin-mediated filament severing may trigger the dissociation of CP from the dendritic nucleation actin arrays.

Possible mechanisms for actin filament turnover–dependent CP dissociation are depicted in Fig. 7 (A–C). Jas might strengthen CP–actin interaction by affecting filament conformation (Fig. 7 A). However, because CP alone can bind F-actin with a very high affinity, further stabilization of CP–barbed end interaction by Jas seems questionable.

Alternatively, filament severing induced by coflin may possibly trigger the dissociation of CP attached to a small actin oligomer. Jas, which binds actin filaments competitively with phalloidin (Bubb et al., 1994), probably exerts antisevering effects in a similar manner to phalloidin. If filament severing by coflin triggers fast CP dissociation, there must be a mechanism that prevents the actin network from equally fast breakdown. Disassembly of actin filaments ($t_{1/2} = 30$ s; Watanabe and Mitchison, 2002) is $\sim 20$-fold slower than the dissociation of CP in lamellipodia of XTC cells. One possible explanation is that coflin may sever actin filaments preferentially near the barbed end (Fig. 7 B). Another possibility is that coflin-mediated severing occurs frequently throughout the actin arrays, and rapid end-to-end annealing may prevent severed actin oligomers from leaving the filament network (Fig. 7 C). In vitro, rapid reannealing of sheared actin filaments has long been recognized (Nakaoka and Kasai, 1969; Carlier et al., 1984; Andrianantoandro et al., 2001). Annealing appears to be a favorable reaction given the high density of actin filaments, which is $\sim 1,000$ μM in lamellipodia (Abraham et al., 1999).

To gain evidence for filament annealing, we examined the distribution of free filament ends using recombinant EGFP-tagged CP and tropomodulin (Weber et al., 1994) in permeabilized XTC cells. From the binding density of these fluorescent probes to lamellipodia, the concentrations of free barbed ends and pointed ends were estimated to be 0.99 and 4.6 μM, respectively (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200604176/DC1). The coexistence of high amounts of free barbed ends and pointed ends may support our hypothesis of frequent end-to-end filament annealing (Fig. 7 C).

The current dendritic nucleation models did not incorporate end-to-end filament annealing as a dominant reaction because CP has been believed to terminate barbed end growth within 1–2 s after the nucleation of filaments (Carlier et al., 2003; Pollard and Borisy, 2003). Now, our finding of fast CP dissociation suggests that CP is not sufficient to strictly block barbed ends in lamellipodia. The present study also tested whether other molecules exist that are capable of tightly capping a major fraction of filaments. Gelsolin−/− fibroblasts display a reduced membrane ruffling response to EGF (Witke et al., 1995; Azuma et al., 1998), Eps8−/− fibroblasts are defective in membrane ruffling induction mediated by PDGF (Scita et al., 1999), and Caenorhabditis elegans lacking Eps8 displays severe defects in the organization of intestinal brush border microvilli (Croce et al., 2004). However, our data show that none of these barbed end factors, including gelsolin, Eps8, and VASP, cap actin tightly in lamellipodia. Notably, the dissociation constant of Eps8 with barbed ends determined in vitro (Disanza et al., 2004) was also considerably lower than that observed in vivo. Collectively, these results suggest that free barbed ends are abundant in lamellipodia despite these cappers, whose affinities to barbed ends are within the nanomolar range in vitro. Our findings of the fast dissociation kinetics of these cappers from the actin network have opened up a possibility that end-to-end filament annealing may ubiquitously occur in lamellipodia.

Fig. 4 D summarizes kinetics in regulation of the dendritic nucleation actin arrays. Our single-molecule speckle analysis revealed 0.048 and 0.58 s⁻¹ for the dissociation rates of Arp2/3 and CP, respectively. Another important notion is the fast actin
elongation in living cells as revealed by the actin polymerization-driven movement of mDia1 (Higashida et al., 2004). Based on the speed of mDia1F2 (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200604176/DC1), we estimate that free barbed ends may elongate at ~66 subunits/s in lamellipodia. Together with the fast dissociation kinetics of CP, these results suggest that barbed end growth is not strictly restricted as previously postulated. Given the relatively free and fast barbed end growth, we also predict the requirement of filament severing in catalyzing the fast actin disassembly in lamellipodia based on kinetic modeling (unpublished data). Filament severing is also relevant, as it seems indispensable to facilitate fast actin turnover (Theriot and Mitchison, 1991; Watanabe and Mitchison, 2002) in the presence of actin filaments of several microns in length found in lamellipodia (Small et al., 1995).

Collectively, we predict that filament severing and its countering reaction, end-to-end annealing, may take place frequently in lamellipodia. Filament severing and end-to-end annealing could play a pivotal role in leading edge dynamics by rapidly changing the direction of growth, length distribution, disassembly, and polarity of the actin filament arrays. Currently, it is difficult to estimate the frequency of these two inverse reactions in cells. However, our finding of the fast CP dissociation kinetics may imply a severing frequency of up to one per second for an actin filament of several tens of subunits in length. Further studies will be required to test this high frequency severing-annealing hypothesis.

Materials and methods

Plasmids and reagents

EST clones encoding subunits of Xenopus Arp2/3, CP, and other actin-related proteins were obtained from the IMAGE consortium. GenBank/EMBL/DDBJ accession no. for sequence data are as follows: EF011871 (Arp3), EF011865 (Arp2), EF011872 (p40), EF011864 (p34), EF011866 (p21), EF011870 (p20), EF011868 (p16), BC060481 (CPα), BC072853 (CPβ2), EF011869 (CPβ1), BC072836 (VASP), BC048059 (gelsolin), and EF011867 (tropomodulin). Each cDNA was cloned into the pEGFP-C1 (CLONTech Laboratories, Inc.)-derived vector harboring the defective cytomegalovirus (CMV) promoter (Watanabe and Mitchison, 2002). DelCMV-EGFP-C1: mrfP1 fusion constructs were generated by replacing EGFP sequences with mrfP1 cDNA (gift from R.Y. Tien, University of California, San Diego, San Diego, CA; Campbell et al., 2002). Full-length pEGFP-Eps8 was described previously (Scita et al., 2001). pmRF1-hLMK-1 was constructed by introducing wild-type human LMK-1 cDNA (gift from K. Mizuno, Tohoku University, Sendai, Japan; Yang et al., 1998) into the mrfP1 expression vector. Jas was purchased from Calbiochem.

Antibody and immunocytochemistry

The guinea pig anti-Ar1 antibody was raised against the 6xHis-tagged p40 subunit using pET-30a (Novagen). For immunocytochemistry, cells were extracted with 0.1% Triton X-100 in Cytoskeleton buffer (CB; 10 mM Tris-Cl, pH 7.5, 300 mM NaCl, 1 mM MgCl2, 1 mM EDTA, and 1 mM DTT) for 10 min. Illumination was attenuated using neutral density filters so that fluorescence decay caused by photobleaching was negligible. EGFP fluorescence intensity was measured in a cell peripheral region after the subtraction of background values obtained in an area outside of the cell.

Purification of the recombinant CPα2/EGFP-CPβ1 heterodimer

pGEX-CPβ2 was generated by inserting CPα2 DNA into pGEX-2T (GE Healthcare). pET-30-EGFP-CPβ1 was generated by replacing 6xHis sequences in pET-30a (Novagen) with the EGFP-CPβ1 coding sequences. Using electroporation, B21 Star (DE3; Invitrogen) harboring the pG-KJE8 chaperonin plasmid (Takara) was transformed with pGEX-CPα2 and pET-30-EGFP-CPβ1 simultaneously. Subsequent cultures were performed under the selection pressure of ampicillin, kanamycin, and chloramphenicol. Cells were grown at 37°C in Luria-Bertani medium overnight. After subculturing into fresh media, chaperonin proteins were induced, and, at OD600 = 0.6, 1 mM IPTG was added. Cells were cultured at 30°C for an additional 6 h and collected by centrifugation. Cells were then sonicated in buffer C (10 mM Tris-Cl, pH 7.5, 300 mM NaCl, 1 mM MgCl2, 1 mM DTT, and 1 mM ATP) supplemented with 0.5% Triton X-100, 200 μM PMSF, 2 μg/ml leupeptin, and 1 μg/ml peptatin A. The sonicate was clarified by centrifugation. The supernatant was incubated with glutathione-Sepharose 4B (GE Healthcare), and the beads were washed repeatedly with centrifugation with buffer C. The beads were incubated with thrombin (Sigma-Aldrich) in buffer C, and CPα2/EGFP-CPβ1 cleaved from GST was collected in the supernatant. Thrombin activity was neutralized with hirudin (Sigma-Aldrich).

Pyrene-actin polymerization assay

Pyrene-labeled actin and rabbit skeletal muscle actin were purchased from Cytoskeleton, Inc. Before use, the protein concentrations were verified using densitometry on the SDS-PAGE gel stained with Coomassie Brilliant blue. Unlabeled actin was polymerized at 5 μM in buffer F (10 mM Tris-Cl, pH 7.5, 100 mM KCl, 2 mM MgCl2, 1 mM ATP, 0.2 mM EGTA, and 0.2 mM DTT) for 1 h. For assembly from Factin seeds, a 12.5-μl aliquot of unlabeled, preassembled actin filaments was inserted into each well. To start the reaction, 6.67 μl of a 15-μM pyrene-actin droplet in buffer G containing 0.2 mM ATP was washed into the Factin seeds with 71 μl of 1.2× buffer F and 10 μl of buffer C containing a range of concentrations of CPα2/EGFP-CPβ1. Pyrene fluorescence was monitored at room temperature using Fluoroskan Ascent FL (Labsystem).

Determination of rate constants of the CPα2/EGFP-CPβ1 heterodimer

The rate constants for capping barbed ends were determined from kinetic parameter optimization using data collected in actin polymerization assays. The concentration of Factin seeds, [S], was first determined by fitting the fluorescence data without CPα2/EGFP-CPβ1 with the equation

\[ A_{\text{Pyr}} = A_{\text{Free}} \cdot P_{\text{on}} / (1 - e^{-k_{\text{on}} [S]}) \]

where \( A_{\text{Pyr}} \) is the concentration of polymerized actin, \( A_{\text{Free}} \) is the initial concentration of free actin monomers, \( P_{\text{on}} \) and \( P_{\text{off}} \) are on and off rates of barbed ends, and \( P_{\text{on}} \) and \( P_{\text{off}} \) are on and off rates of pointed ends.

Polymization in the presence of CPα2/EGFP-CPβ1 is described as \( B + A_{\text{Free}} \rightarrow B + A_{\text{Free}} \), \( B + C = BC \), and \( P + A_{\text{Free}} = P \), where \( A_{\text{Free}} \) is free actin monomers, \( B \) is free barbed ends, \( P \) is free pointed ends, \( C \) is free CP, and \( BC \) is capped barbed ends. Using \( [S]_0 \), actin polymerization curves...
were calculated numerically by Euler’s method. The on and off rates of CPα2/EGFP-CPβ1 were varied to fit calculated polymerization curves with pyrene-actin fluorescence data.

Determination of the dissociation constant (Kd) of CPα2/EGFP-CPβ1 heterodimer

2 μM pyrene-labeled actin was polymerized for 2 h in buffer E. Various amounts of CPα2/EGFP-CPβ3 in 10 μL of buffer C was added to 90 μL of the polymerized pyrene-actin solutions. Samples were incubated for 24 h at 22°C, and pyrene fluorescence was measured. At steady state, 

\[ B_{on}[B] + P_{off}[P] \rightarrow B_{off}[B] + P_{on}[P] \quad \text{and} \quad K_d = \frac{[B][C]}{[BC]}, \]

where \([A]_{on}\) is the concentration of free actin monomers, \([B]\) is the concentration of free barbed ends, \([P]\) is the concentration of free pointed ends, \([C]\) is the concentration of free CP, and \([BC]\) is the concentration of capped barbed ends.

At CP concentrations of \( \leq 1 \text{nM} \), the concentration of free CP \([C]\), is almost equal to its total concentration, \([C]_0\). The concentration of polymerized actin is calculated by the following equation:

\[ [A]_{on} = [A]_{free} + B_{on}[B] \quad \text{where} \quad X = \frac{K_d}{K_d + C_0}, \]

The Kd for CPα2/EGFP-CPβ1 was determined by optimizing this equation to fluorescence data.

Online supplemental material

Fig. S1 shows the fast dissociation of barbed end–interacting proteins Eps8 and VASP from the actin network in lamellipodia. Fig. S2 shows a comparison of the localization of our Arp2/3 constructs and endogenous Eps8 and VASP from the actin network in lamellipodia. Fig. S3 shows a comparison of the localization of our Arp2/3 constructs and endogenous Eps8 and VASP from the actin network in lamellipodia. Fig. S4 shows the distribution of free barbed ends.

Submitted: 26 April 2006
Accepted: 20 November 2006

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