# The Conformational State of Actin Filaments Regulates Branching by Actin-related Protein 2/3 (Arp2/3) Complex\*\*

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**Background:** The Arp2/3 complex preferentially forms branches from newly polymerized actin filaments, but the mechanism is unknown.

**Results:** Caldesmon bound to polymerizing actin preserves this preferred binding and branching activity of Arp2/3. **Conclusion:** By stabilizing filaments in their nascent state, caldesmon potentiates actin nucleation and branching by the Arp2/3 complex.

Significance: This work describes a novel mechanism regulating actin dynamics.

Actin is a highly ubiquitous protein in eukaryotic cells that plays a crucial role in cell mechanics and motility. Cell motility is driven by assembling actin as polymerizing actin drives cell protrusions in a process closely involving a host of other actin-binding proteins, notably the actin-related protein 2/3 (Arp2/3) complex, which nucleates actin and forms branched filamentous structures. The Arp2/3 complex preferentially binds specific actin networks at the cell leading edge and forms branched filamentous structures, which drive cell protrusions, but the exact regulatory mechanism behind this process is not well understood. Here we show using in vitro imaging and binding assays that a fragment of the actin-binding protein caldesmon added to polymerizing actin increases the Arp2/3-mediated branching activity, whereas it has no effect on branch formation when binding to aged actin filaments. Because this caldesmon effect is shown to be independent of nucleotide hydrolysis and phosphate release from actin, our results suggest a mechanism by which caldesmon maintains newly polymerized actin in a distinct state that has a higher affinity for the Arp2/3 complex. Our data show that this new state does not affect the level of cooperativity of binding by Arp2/3 complex or its distribution on actin. This presents a novel regulatory mechanism by which caldesmon, and potentially other actin-binding proteins, regulates the interactions of actin with its binding partners.

The actin cytoskeleton is a dynamic structure that regulates numerous important cellular events (1). A plethora of actinbinding proteins participates in this dynamic process, among which several modulate the stability of actin filaments and their interaction with myosin motors by binding along the sides of actin filaments, thereby controlling actin cytoskeleton assembly and dynamics (2). Caldesmon  $(CaD)^3$  is one such actin "side binder" that exists in nearly all vertebrate cells. Although the muscle-specific isoform of CaD plays a regulatory role in smooth muscle contractility (3), nonmuscle CaD is more broadly implicated in cell motility (4), including processes such as cell migration (5), focal adhesion assembly (6), podosome dynamics (7), and neointimal formation (8). Recently, CaD was also suggested to control a key structural transition occurring during actin polymerization, known as "maturation" (9, 10). We found that CaD or its C-terminal actin-binding fragment, when present before, but not after, the structural transition, inhibits the maturation process and arrests the filament at the pretransitional state (9). Importantly, the same effect was also observed in solutions containing only ADP, indicating that this maturation differs from the conventional aging process of actin filaments that depends on nucleotide hydrolysis.

The concept of actin maturation has been proposed previously (11–14), but because the "immature" (or "nascent") state is transient and not well characterized, its significance remains controversial. CaD-stabilized nascent actin filaments had a rather long lifetime on the order of hours and could therefore be subjected to further characterization. These filaments appeared irregular and rough when imaged with negative staining electron microscopy (10). The unusual morphology raised the possibility that nascent actin filaments might bind certain accessory proteins more easily and thus be more amenable to remodeling. In this work, we tested this possibility and found that the CaD-bound actin filaments had a higher affinity for the Arp2/3 complex and exhibited a higher branching activity if



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<sup>&</sup>lt;sup>3</sup> The abbreviations used are: CaD, caldesmon; Arp2/3, actin-related protein 2/3; D3D4, a mutant that mimics Erk-phosphorylated H32K; H32K, a C-terminal fragment of CaD; pH32K, Erk-phosphorylated H32K.

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CaD was added during actin polymerization. If, however, CaD was added to prepolymerized ("mature") filaments, no effect on Arp2/3-induced branching was observed, suggesting that only the CaD-stabilized nascent actin filament had an increased affinity for the Arp2/3 complex. The Arp2/3 complex exhibited little or no cooperativity when binding to either nascent or mature filaments. There also appeared to be no particular regions on the mother filaments that the Arp2/3 complex preferred to bind, suggesting that these filaments do not have internal structural heterogeneity. These results are discussed as a novel nucleotide-independent regulatory mechanism in which the state of the actin regulates the affinity for Arp2/3 complex and in which CaD acts as an age marker able to extend the lifetime of the nascent actin state by binding to newly polymerized actin filaments.

#### **EXPERIMENTAL PROCEDURES**

Actin and the Arp2/3 Complex—Actin, except rhodamine G-actin, which was purchased from Cytoskeleton (Denver, CO), was extracted and purified from acetone-dried rabbit skeletal muscle (15). Pyrene-labeled actin was prepared as described previously (9). Alexa Fluor 488 actin was prepared by labeling surface lysine residues according to published procedures (16). The labeling ratio was kept at 10-14% so that the labeled actin was able to polymerize without interference from the dye (16). The Arp2/3 complex was isolated from bovine brain (17).

*CaD Fragments*—Recombinant His<sub>6</sub>-tagged C-terminal fragment (residues from Leu<sup>604</sup> to Val<sup>793</sup>) of human CaD (H32K) was expressed in *Escherichia coli* and purified as described previously (18, 19). H32K was phosphorylated by Erk2 (New England Biolabs) and validated by mass spectrometry as described previously (18). A phospho-mimicking mutant of H32K, D3D4, was prepared by replacing the two Erk phosphorylation sites (Ser<sup>759</sup> and Ser<sup>789</sup>) with Asp residues. His<sub>6</sub>-tagged D3D4 was purified using a similar procedure as for H32K. Purified D3D4, like Erk-phosphorylated H32K, did not inhibit actomyosin ATPase activity and caused the same attenuation of pyrene fluorescence enhancement when added to polymerizing actin.<sup>4</sup>

Fluorescence Imaging-Real-time two-color fluorescence imaging of the Arp2/3 complex-mediated actin filament branching was performed similarly to previous studies (20, 21), except that CaD was added to interfere with the maturation process. Imaging experiments were conducted in two steps. In the first step, Alexa Fluor 488 actin was polymerized at 1.5  $\mu$ M in 25 mм KCl, 25 mм imidazole, 1 mм EGTA, 4 mм MgCl<sub>2</sub>, 1 mM ATP, and 10 mM dithiothreitol either with or without phosphorylated H32K (pH32K) at a 1:3 molar ratio to actin for 3 h to ensure attainment of steady-state conditions. Filaments prepared in the absence of the CaD fragment were used for control experiments. In the second step, a sample was prepared immediately before imaging by mixing rhodamine G-actin (final concentration 500 nm), Arp2/3 complex (final concentration 300 nM), Alexa Fluor 488 actin drawn from the prepolymerized stock (final concentration 15 nm), and additional pH32K to maintain a constant CaD:actin molar ratio of 1:3. The constit-

uents were mixed in an actin polymerization buffer, identical to the buffer previously used in total internal reflectance fluorescence microscopy (10). The sample was then immediately loaded into a flow chamber, which was assembled and incubated with bovine serum albumin, as was done previously for total internal reflectance fluorescence microscopy (10). Controls were performed in which Arp2/3 complex or pH32K was absent or in which pH32K was added before imaging but not during the prepolymerization of the Alexa Fluor 488 actin. Fluorescence images were obtained with a Leica SP5 confocal microscope, a  $100 \times / 1.4$  numerical aperture oil immersion objective, a 488 nm argon ion laser, and a 543 nm helium neon laser, captured at random locations on the flow cell surface within 11 min of sample mixing. Analysis was performed using ImageJ software (rsbweb.nih.gov/ij). Contrast was enhanced by 0.4%, and a Gaussian filter with  $\sigma = 0.025 \,\mu\text{m}$  was applied to the original images to reduce noise. Rhodamine-actin growing on Alexa Fluor 488 actin was counted as branching under the criteria that: (*a*) it formed a 70  $\pm$  10° angle with the Alexa Fluor 488 actin (22), (b) this angle was aimed at the mother filament barbed end evident from the growth of new rhodamine-actin (23), and (*c*) it was not apparently elongating off other Alexa Fluor 488 actin or branching off rhodamine-actin filaments. In addition to the density of branching, the distribution of branches (their relative separations, distances to mother filament ends, and the number of branches on each mother filament) was also noted. Bundled actin was omitted from all analyses.

*Monte Carlo Simulations*—The observed numbers of branches and mother filament length distributions from fluorescence microscopy imaging were fed into a custom-written MATLAB Monte Carlo simulation (MathWorks, Natick MA). To establish the distributions of branches for random Arp2/3 binding, the program randomly placed branches on the filaments and recorded the resulting distributions corresponding to each particular distribution of mother filament lengths. A total of 280 branches and 613 separate mother filaments were measured and input into the simulation for pH32K-stabilized nascent actin, whereas 276 branches and 985 filaments were measured and input for plain actin. The simulation was repeated until the distributions converged, which occurred after 100,000 iterations.

*Co-sedimentation and Immunoblot Assays*—To determine the binding of the Arp2/3 complex to actin filaments by co-sedimentation assays, varying amounts of Arp2/3 complex were incubated with rabbit skeletal actin filaments (1.5  $\mu$ M) polymerized with pH32K (0.5  $\mu$ M) or the phospho-mimicking mutant, D3D4 (0.5  $\mu$ M), added at either the beginning (t = 0min) or the end (t = 240 min) of polymerization. The mixtures were centrifuged at 80,000 × g for 30 min. The pellet fractions were reacted with monoclonal anti-Arp2 antibody (Sigma) followed by reaction with IRDye800-conjugated anti-mouse IgG from donkey (Rockland Immunochemicals).

#### **RESULTS AND DISCUSSION**

To investigate the dynamic properties of actin filaments before and after maturation as well as their interaction with Arp2/3 complex, we have visualized actin filament assembly



<sup>&</sup>lt;sup>4</sup> R. Huang and C.-L. A. Wang, unpublished observations.

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using fluorescently labeled actin of two different colors similarly to previous studies (20, 21), except that CaD was added to interfere with the maturation process. An Erk-phosphorylated CaD fragment pH32K was used for this purpose. The use of phosphorylated H32K minimized the problem of actin bundling, which is promoted by unphosphorylated CaD (24).

For direct imaging of actin assembly in the presence of Arp2/3 complex, rabbit skeletal actin, labeled with the green fluorescent dye Alexa Fluor 488, was first polymerized either with or without pH32K at a 1:3 molar ratio to actin for 3 h to ensure attainment of steady-state conditions. Immediately before imaging, a fraction of this prepolymerized actin was mixed with rhodamine G-actin, Arp2/3 complex, and additional pH32K (to maintain a constant CaD:actin molar ratio of 1:3). Samples without pH32K added at this stage were used as a control. The mixture was then loaded into a flow chamber for imaging.

Two-color fluorescence confocal microscopy imaging of actin assembly was carried out while rhodamine-actin was assembling (Fig. 1) until the field of view became too crowded with polymerized actin, which generally occurred about 10 min after sample mixing. To quantify the effects of pH32K on Arp2/3 complex-mediated branching, rhodamine-actin (red) branches originating from prepolymerized Alexa Fluor 488 actin mother filaments (green) were counted. Although considerable branching off of newly polymerized red mother filaments was also observed (20), we only considered branching on prepolymerized green F-actin, as the lengths of these were constant during the experiment and as we could control their age and interaction with pH32K during the prepolymerization step. The level of branching was quantified as the number of red branches per unit length of green filaments polymerized either in the presence or in the absence of pH32K. As compared with the branching activity in the absence of pH32K, we observed a 2-fold increase in dendritic branching density on Alexa Fluor 488 actin filaments prepolymerized in the presence of pH32K (Fig. 2). However, when pH32K was instead added right before imaging at a ratio of 1:3 to Alexa Fluor 488 actin filaments prepolymerized in the absence of pH32K, the level of branching was indistinguishable from the CaD-free control.

Several actin-binding proteins have been shown to cooperatively interact with actin, including tropomyosin (25), myosin (26), ADF (actin-depolymerizing factor)/cofilin (27, 28), and gelsolin (29). Although we have shown that actin polymerized with pH32K more readily forms Arp2/3-induced branches (Fig. 2), the branching density alone is insufficient to determine whether the increased branching arose from a change in affinity only or from a change in cooperativity of the Arp2/3 complex binding to actin. If the degree of cooperativity was altered, we would expect to see a difference in the distribution of branches on the Alexa Fluor 488 actin, evident either as clusters of branches on the Alexa Fluor 488 actin or as a few Alexa Fluor 488 actin filaments with a high density of branches, thus increasing the overall average branching density. To determine whether the additional branch formation resulted from changes in cooperative binding, we compared the cooperativity of branching activity between pH32K-stabilized nascent actin filaments and actin alone in two ways. First, we counted the



FIGURE 1. **Representative images from the actin assembly two-color imaging assay.** Alexa Fluor 488 actin (*green*) was first polymerized without (*A*) or with (*B*) pH32K for 3 h to ensure attainment of steady-state conditions. A combination of rhodamine G-actin (*red*) and Arp2/3 complex, prepolymerized Alexa Fluor 488 F-actin, and additional pH32K (maintaining a 1:3 molar ratio to actin) was then added and loaded into the flow chamber. *Arrows* indicate possible branches of rhodamine-actin on the Alexa Fluor 488 actin. *White arrows* indicate branches that were counted; *gray arrows* indicate branches that were discarded (see "Experimental Procedures" for criteria details). Note also the barbed ends of several Alexa Fluor 488 actin filaments being discernible by the cap of rhodamine-actin growing at these ends. *Scale bar*. 5  $\mu$ m.

number of branches per filament to see whether a few filaments with many branches were responsible for the increased branching density observed when actin was polymerized with pH32K. Most mother filaments with branches had only one branch, whereas fewer filaments had multiple branches per filament





FIGURE 2. Actin polymerized with pH32K showed increased branching induced by Arp2/3 complex under fluorescence microscopy. Branching was quantified as the number of branches on Alexa Fluor 488 actin per unit length of Alexa Fluor 488 actin. Bars represent mean values for all imaged fields (between 23 and 61 for each condition) weighted by the amount of Alexa Fluor 488 actin present  $\pm$  S.E. *Buffer* and *H32K* are control experiments and indicate apparent branching in the absence of Arp2/3 complex, either with or without pH32K. The last three bars indicate, from *left* to *right*: branching Mith Arp2/3 complex alone, with Arp2/3 complex and pH32K present using Alexa Fluor 488 actin prepolymerized without pH32K, and with Arp2/3 complex and pH32K present using Alexa Fluor 488 actin prepolymerized without pH32K, and with Arp2/3 complex (*pol. w. H32K*).

(Fig. 3). Furthermore, polymerizing actin with pH32K had no discernible effect on this distribution, suggesting that the increased branching density did not originate from a few filaments with a disproportionally high number of branches. Because the exact distributions depended on the length distributions of mother filaments in each experiment, we ran a Monte Carlo simulation to generate the distribution of Arp2/3-induced branches representing random, noncooperative binding of the Arp2/3 complex. As noted in Fig. 3, the observed distributions hardly differed from this random distribution, suggesting that Arp2/3 complex exhibits little cooperativity in binding to actin both with and without pH32K present.

Secondly, we quantified the distance between adjacent Arp2/3 branches to look for clusters of branches (Fig. 4). As above, the observed distributions were compared with a simulation-generated random distribution, each of which was specific to the particular filament lengths observed. Because a polymer in dynamic equilibrium, such as actin, tends to have an exponential length distribution with more short filaments and fewer long ones, the simulated random distribution is not even across all mother filament lengths. This disproportionate abundance of shorter filaments in turn makes randomly placed branch points more likely to be more closely spaced, explaining the asymmetric shape of the simulated data in Fig. 4. Importantly, when comparing the simulated and measured distributions, no marked differences were evident, suggesting that neither the actin polymerized with pH32K nor the plain pH32K-free actin produces more regions with closely spaced branches than would be expected from a random distribution. Taken together, Figs. 3 and 4 indicate that although pH32K-



FIGURE 3. Actin polymerized with pH32K did not have a different distribution of Arp2/3-induced branches per filament as compared with actin alone. Each bar represents the measured (*black bars*) or simulated based on random distribution (*gray bars*) relative number of filaments with 0–6 branches. Shown are two conditions: Arp2/3-induced branching on actin without any pH32K present (*top panel*) and Arp2/3-induced branching on actin polymerized with pH32K in a ratio to actin of 1:3 (*bottom panel*). Although the total number of branches per  $\mu$ m of Alexa Fluor 488 actin was doubled in the latter case (Fig. 2), the data for both conditions differ little from the calculated distributions for randomly distributed branches, suggesting that the Arp2/3 complex exhibits little cooperativity in binding both with and without pH32K present.

stabilized nascent actin has a higher density of branches (Fig. 2), it induces no marked differences in the distribution of branches. Thus, the increased branching was not the result of a CaD-induced alteration in binding cooperativity, but rather a uniform increase in the affinity of Arp2/3 complex for actin.

We also compared the relative locations of branches to their mother filament barbed end. As noted for Fig. 4, the simulated random distribution is skewed toward shorter distances due to the uneven length of actin mother filaments. We observed little difference in the distribution of branches along the mother filament as compared with a simulated random distribution of branches for both pH32K-stabilized nascent actin and mature actin filaments (Fig. 5). This was expected as all mother filaments were either fully nascent or mature and thus had no internal heterogeneity. Although the barbed end of growing actin consists of an ATP-actin or ADP-P<sub>i</sub>-actin cap, which has been noted to exhibit a higher affinity for the Arp2/3 complex



FIGURE 4. Actin polymerized with pH32K did not have a different relative distribution of Arp2/3-induced branches as compared with actin alone. The distribution of branches was measured as the distance between adjacent branching points on every actin filament without pH32K (*top panel*) or on every actin filament with pH32K in a ratio to actin of 1.3 (*bottom panel*). Only filaments with two or more branches contributed to this statistic. Shown are both the measured data (*black bars*) and the simulated distributions of frandomly generated branches (*gray bars*). The measured distributions differ little from those calculated for randomly localized branches, suggesting that the Arp2/3 complex does not preferentially bind in clusters on either pH32K-stabilized nascent actin or actin alone.

(20), our mother filaments had no such structural asymmetry and thus did not preferentially form branches along certain regions of the mother filament.

It has been reported previously that the Arp2/3 complex preferentially nucleates dendritic branching on newly polymerized actin filaments (20). Our observations here demonstrated that CaD added to polymerizing actin preserves the nascent properties of the aged actin filament and maintains its higher affinity for the Arp2/3 complex. Because the state of the actinbound nucleotide often serves as an aging marker of the filament as actin hydrolyzes ATP to ADP during polymerization with subsequent release of inorganic phosphate, it was thought that the nucleotide state affects the affinity of Arp2/3 complex for actin and results in preferred branching on nascent filaments. However, in our experiments, the increased branching occurs on actin filaments aged on a time scale significantly longer than that of phosphate release from actin (30). As has previously been shown, the effect induced by CaD does not involve

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FIGURE 5. Arp2/3-induced branching was not sensitive to the mother filament polarity. The distance between all branches and their mother filament barbed ends was measured both for actin alone (*top panel*) and for actin polymerized with pH32K in a ratio to actin of 1:3 (*bottom panel*). Only mother filaments whose barbed ends were discernible were counted. Shown are both the measured data (*black bars*) and the simulated distributions of randomly generated branches (*gray bars*). The measured distances differ little from those calculated for branches localized randomly on the mother filaments, suggesting that the Arp2/3 complex does not preferentially bind to certain locations on either pH32K-stabilized nascent mother filaments or plain actin mother filaments.

ATP hydrolysis or phosphate release (9), meaning that virtually all Alexa Fluor 488 actin during the imaging assay was already in the ADP state. Interestingly, a transient post-phosphate release intermediate conformation of actin prior to mature ADP-Factin formation has been detected before (12); such a conformation may be stabilized by CaD. These results suggest that the observed increase of the Arp2/3 complex-mediated branching by pH32K is associated with a mechanism independent of the hydrolysis of actin-bound ATP.

To validate the differential affinities of the Arp2/3 complex for the nascent and mature actin filaments, we performed binding studies by co-sedimentation assays. Because the amounts of Arp2/3 used in these experiments were relatively small, quantification was done by immunoblotting. Actin filaments polymerized in the presence or absence of H32K were first incubated with the Arp2/3 complex and were ultracentrifuged; Arp2/3 in the pellet was then allowed to react with anti-Arp2





FIGURE 6. Actin filaments polymerized with H32K or D3D4 showed increased Arp2/3 complex binding. Binding was monitored using co-sedimentation assays. Varying amounts of Arp2/3 complex were incubated with actin filaments (1.5  $\mu$ M) polymerized with H32K (0.5  $\mu$ M; *circles*) or D3D4 (0.5  $\mu$ M; *squares*) added at the beginning (t = 0 min; *closed symbols*) or the end (t = 240 min; *open symbols*) of polymerization. The mixtures were centrifuged at 80,000  $\times$  g for 30 min. The pellet fractions were reacted with anti-Arp2 antibody followed by secondary antibody reaction. *Inset*: Arp2/3 (150 nM) mixed with actin filaments (1.5  $\mu$ M) polymerized in the presence (*closed circles*) or absence (*open circles*) of H32K (0.5  $\mu$ M) followed by ultracentrifugation and immunoreaction with anti-Arp2 as described above.

after serial dilutions. We found that the level of Arp2/3 cosedimented with actin filaments polymerized in the presence of H32K was higher than that bound to CaD-free F-actin (Fig. 6, *inset*), indicating stronger binding of Arp2/3 to the H32K-stabilized nascent actin filaments than to the mature CaD-free filaments. These results also established the appropriate concentration ranges for both the Arp2/3 complex and anti-Arp2, in which a linear relationship between the immunoreactivity and the Arp2/3 concentration could be obtained. We then used these concentration ranges in the binding study of Arp2/3 to actin filaments. The immunoreactivity of filament-bound Arp2/3 with anti-Arp2 was further calibrated against a known amount of the Arp2/3 complex.

Because Arp2/3-mediated actin branching requires an excess of actin from which to form new branches, it was not feasible to saturate actin with Arp2/3 complex; thus, we did not attempt to determine the actual binding constant. Nevertheless, the amount of Arp2/3 co-sedimented with the H32K-stabilized nascent filaments was consistently higher than that with the H32K-bound mature filaments, indicating that the Arp2/3 complex indeed has a higher affinity for nascent actin filaments than for mature filaments (Fig. 6), in agreement with our direct imaging experiments (Fig. 2). We have also tested a phosphomimicking mutant of H32K, D3D4, in which the two Ser residues for Erk-mediated phosphorylation are replaced by Asp. Binding of the Arp2/3 complex to the D3D4-stabilized nascent filaments was also stronger than the D3D4-bound mature filaments. In fact, the amount of the Arp2/3 complex co-sedimented with the nascent filament stabilized by D3D4 was 10-fold higher than that with the H32K-bound filaments. This is consistent with the notion that CaD, but not phosphorylated

CaD, competes with Arp2/3 for actin binding (31). It also agrees with the fact that in cells, it is the phosphorylated form of CaD that is present at the leading edge and encounters dynamic actin filaments and the Arp2/3 complex.

Besides competing with Arp2/3 for actin binding (31), unphosphorylated CaD is also known to bundle actin filaments (24). To check the effect of actin bundling, we have performed low speed centrifugation prior to ultracentrifugation; we found that both pellet fractions contained H32K with similar ratios between H32K and actin, and the differences in the Arp2/3 complex also persisted in both bundled and unbundled actin filaments (data not shown). We therefore ruled out the possibility that CaD-induced bundling of mature F-actin might exclude Arp2/3 binding.

Several structural transitions besides that related to the nucleotide state are known for polymerizing actin, which in turn could modulate the affinity of actin-binding proteins for actin. For example, FRET measurements have revealed that individual actin monomers within a filament alternate on a time scale of seconds between structural states that are discernible by myosin (32); electron microscopy has also shown distinct structural states existing within F-actin (33). Notably, CaD has been found to attenuate pyrene fluorescence enhancement of polymerizing actin without altering the rates of elongation and to prolong the early stage rough appearance of actin filaments under negative staining electron microscopy (9, 10). Because these effects were not seen when CaD was added to aged filaments, it was argued that they correspond to a conformational transition of F-actin occurring naturally during polymerization (9). It was further suggested that CaD blocks this transition, thus keeping the actin filament in the nascent state. The time scale of this maturation process agrees with the time scale over which we observed the increased branching activity when actin is polymerized with pH32K, supporting the idea that the nascent state of actin filaments stabilized by CaD causes increased dendritic branching by the Arp2/3 complex. Moreover, the maturation process appears to be irreversible (9). This is also consistent with our observation that pH32K failed to increase dendritic branching by the Arp2/3 complex when added to aged, prepolymerized filaments. Although we do not know of any Arp2/3 interaction sites on CaD, the increased branching could reflect either a direct increase in affinity of the Arp2/3 complex for nascent F-actin or, conversely, an indirect increase in affinity through a nascent actin-induced stabilization of CaD in an alternative Arp2/3 complex binding conformation. Because the in vitro dissociation rate of actin branches by the Arp2/3 complex is too long for any appreciable dissociation to occur during our experiments (34), our data suggest that the increased affinity of Arp2/3 complex for pH32K-decorated nascent actin filaments reflects an increased association rate rather than a decreased dissociation rate.

That CaD promotes Arp2/3-mediated branching when present during actin polymerization presents a novel mechanism by which actin assembly and actin interactions with actin-binding proteins can be regulated. The stronger tendency of the Arp2/3 complex to initiate branches from nascent actin filaments than from mature filaments also provides an explanation for the lower than expected binding constant determined by *in vitro* 



studies that used actin filaments in the mature state (35). When cells are stimulated to migrate, CaD is phosphorylated and translocated from the cytosol to the cell periphery (36), including regions of membrane ruffling at the leading edge (37), where the Arp2/3 complex is abundant. The proximity of phosphorylated CaD to high amounts of newly polymerized actin may allow CaD to maintain actin filaments in a state that binds the Arp2/3 complex more readily. Our findings also have a more general implication for actin-binding protein regulation. The state of the actin filament itself could be a determining factor of the affinity of actin-binding proteins for actin. A number of actin-binding proteins (e.g. cofilin) are thought to target actin depending on its nucleotide state (1). Our work demonstrates that at least one additional mechanism besides the state of the nucleotide on actin should be considered as a filament-aging marker for actin targeting by its partner proteins.

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