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Actin Hydrophobic Loop (262-274) and Filament Nucleation and Elongation

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Summary

The importance of actin hydrophobic loop 262-274 dynamics to actin polymerization and filament stability has been shown recently using a yeast actin mutant, L180C/L269C/C374A, in which the hydrophobic loop could be locked in a “parked” conformation by a disulfide bond between C180 and C269. Such a cross-linked G-actin does not form filaments, suggesting nucleation and/or elongation inhibition. To determine the role of loop dynamics in filament nucleation and/or elongation, we studied the polymerization of the cross-linked actin in the presence of cofilin - to assist with actin nucleation - and with phalloidin, to stabilize the elongating filament segments. We demonstrate here that together, but not alone, phalloidin and cofilin co-rescue the polymerization of cross-linked actin. The polymerization was also rescued by filament seeds added together with phalloidin but not with cofilin. Thus, loop immobilization via cross-linking inhibits both filament nucleation and elongation. Nevertheless, the conformational changes needed to catalyze ATP hydrolysis by actin occur in the cross-linked actin. When actin filaments are fully decorated by cofilin the helical twist of F-actin changes by ~ 5° per subunit. Electron microscopic analysis of filaments rescued by cofilin and phalloidin revealed a dense contact between opposite strands in F-actin, and a change of twist by ~ 1° per subunit, indicating either partial or disordered attachment of cofilin to F-actin and/or a competition between cofilin and phalloidin to alter F-actin symmetry. Our findings show an importance of the hydrophobic loop conformational dynamics to both actin nucleation and elongation and reveal that the inhibition of these two steps in the cross-linked actin can be relieved by appropriate factors.

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Keywords

actin; hydrophobic loop; polymerization; cofilin; phalloidin

Introduction

Actin, which is an abundant protein and the main component of the cytoskeleton in eukaryotic cells, plays a key role in many cellular processes.¹⁻⁴ The ability of monomeric actin (G-actin) to polymerize reversibly into double stranded filaments (F-actin), which are further organized into higher order structures, is important for cell motility, cell morphology and the interaction with actin-binding proteins. The process of filament formation requires two steps: an energetically unfavorable nucleation step in which first two and then three monomers form a transient complex followed by addition of monomers which stabilizes the assembly, ultimately yielding the elongated filament. A large body of evidence indicates that the actin monomer must undergo a series of conformational changes before it becomes polymerization competent, and even within the filament, conformational changes in the protomer can lead to at least three different structures: the closed, open, and tilted forms of the filament.^{5, 6}

Although the crystal structure of the actin monomer has been solved on numerous occasions, no structure of the filament exists because of the inability to generate filament crystals. However, good working models of the filament have been generated by fitting the monomer crystal structure into a filament model to match X-ray diffraction of oriented actin gels.^{7,8} The first of these, constructed by Holmes, proposed that polymerization is accompanied by a major conformation change in a loop consisting of residues 262-274 between subdomains 3 and 4 of the actin monomer. In G-actin, this loop is parked along side of the major body of the monomer. Holmes proposed that during polymerization, this loop extended away from the monomer and inserted into a hydrophobic pocket formed by the interface of two monomers in the opposing strand of the actin double helix. This extension would then result in cross-strand helix stabilization. Interestingly, this loop is absent from the bacterial “actin-like” proteins MreB⁹ and ParM.¹⁰

An extensive body of work has produced no convincing evidence that such an extension of the loop to form a plug-pocket stabilizing interaction indeed occurs or is necessary for polymerization. However, these studies have clearly demonstrated that this loop must be able to detach from its parked position and undergo a conformational change if polymerization of the actin is to occur. Introduction of cysteine residues in the loop at position 269 and in the actin body at 180 followed by copper-catalyzed oxidation to form a disulfide bond between the two residues allowed the loop to be locked in its parked position against the body of the monomer preventing extension or conformational change. When the reaction was performed with G-actin, the cross-linked actin could no longer polymerize.¹¹

Subsequent works showed that the same cross-linking could be induced after the actin had polymerized. In this case, the filaments disassembled.^{6,12} Reversal of the cross-linking resulted in rescue of polymerization. The results of these studies showed that a conformational change involving this loop was important for actin polymerization but that

within the context of the polymer, the loop remained flexible, probably in equilibrium between one or more conformations. The equilibration of the loop among different conformational states was further documented via cross-linking and electron paramagnetic resonance studies with cysteine mutants of S265, V266, L267 and L269 yeast actin.¹³ Notably, these conformational states also include loop positions that do not maintain the filament structure, and when captured and locked by cross-linking - disrupt the filament. Although the importance of loop dynamics was established for overall polymerization, its contribution to the nucleation and elongation processes remains unknown.

The inability of the C180-C269 cross-linked actin to polymerize could be derived from an inability to form the proper monomer-monomer interaction necessary for nucleus formation, filament stabilization, or both. However, it is possible that interactions weakened by C180-C269 cross-linking could be assisted by proteins or drugs that promote inter-protomer interactions, overcoming the energetic barriers that might otherwise preclude them from occurring. Previous attempts to rescue polymerization using the filament stabilizer phalloidin and myosin subfragment 1 (S1) were unsuccessful.¹¹ This result suggested that either the cross-linked actin itself could not nucleate or the nucleus, if formed, could not convert into a nascent filamentous structure that would interact properly with phalloidin and elongate.

In this paper, to gain more insight into the role of the hydrophobic loop in these two steps of filament formation, we examined the effect of cross-linking the loop on the ability of the actin specifically to form a nucleus and then elongate in the presence of agents that promote one or the other of these two processes. Based on recent findings, we used cofilin to promote nucleation. This protein originally was described as an actin-filament depolymerizing/severing/destabilizing protein.^{13,14} However, recent works showed that at high concentrations, cofilin can also nucleate filament formation¹⁶⁻¹⁸ presumably by acting as a scaffold for two monomers which it orients into a longitudinal pair of protomers as would be found in an actin helix.¹⁹ Nucleation would be completed by addition of a third monomer or an additional cofilin-actin complex. As a stabilizer of nascent filaments that might form, we utilized phalloidin. To study elongation directly, we bypassed the need for the cross-linked actin to nucleate by using gelsolin-stabilized WT actin seeds. Our results showed that the cross-linked actin could form nuclei and stable filaments, but only in the presence of reagents that would compensate for normal interactions impaired by the formation of the intra-monomer disulfide bond. The cross-linked actin also exhibited a polymerization-induced hydrolysis of ATP typical of WT actin.

Results

Cofilin and phalloidin co-rescue the polymerization of (LC)₂ CA cross-linked yeast actin

By forming a disulfide bridge between cysteines at position 180 and 269 of (LC)₂CA actin, we could lock the 262-274 hydrophobic loop to the side of the actin monomer preventing actin polymerization. Phalloidin itself could not rescue the polymerization of this cross-linked protein.¹¹ To determine if this result arose because of failure of the actin to form filament nuclei, or to elongate the nuclei, we examined the polymerization of cross-linked actin in the presence of cofilin +/- phalloidin. We chose cofilin because of its recently

demonstrated ability to act as a filament nucleator at high cofilin concentrations. As monitored by light scattering, neither phalloidin (as shown before¹¹) nor cofilin alone (Figure 1A, curve 1) could induce polymerization. In contrast, a combination of cofilin and phalloidin caused an increase in light scattering suggestive of actin polymerization (Figure 1A, curve 2). Electron micrographs of aliquots of the polymerization reactions (after 30 and 60 minutes) confirmed this conclusion. Images of the cross-linked actin polymerized in the presence of cofilin alone revealed mainly nonspecific aggregates of variable shape and size (Figure 1B). In contrast, together, cofilin and phalloidin rescued partially the ability of the cross-linked actin to form filaments in the presence of $MgCl_2$ (Figure 1C). The filaments formed with cofilin and phalloidin were determined to be F-actin, and not some form of nonspecific aggregate, by several means. First, power spectra were calculated from these filaments, and they showed the characteristic layer lines associated with F-actin (data not shown). Second, two-dimensional averages of segments cut from these filaments showed a clear F-actin structure (Fig 1C, inset). And third, three-dimensional reconstructions from these filaments showed an F-actin structure (see below). These results suggested that although the cross-linked actin itself was incapable of forming nuclei, cofilin could provide a scaffold that would bind two monomers in a conformation compatible with nucleation. Phalloidin would then stabilize the elongating oligomers resulting in stable filament formation.

The effect of WT actin seeds on the polymerizability of cross-linked (LC)₂CA actin

If the cross-linked-actin could undergo phalloidin-assisted elongation from a preexisting nucleus, preformed WT actin seeds should substitute for the requirement for cofilin. To test this prediction and the assumption that cofilin served as a nucleator in this system, we omitted the cofilin, but added 50 nM WT F-actin seeds formed from mixing gelsolin and WT actin (as described in Materials and Methods). The addition of $MgCl_2$ (indicated by the long arrow in Figure 2A) to the mixture of cross-linked G-actin and WT actin-GS seeds (Figure 2A, curve 2) resulted in a very small and slow increase in the light scattering signal, suggesting the onset of actin nucleation and polymerization. Subsequent addition of phalloidin (indicated by the short arrow in Figure 2A) to this solution accelerated dramatically the increase in light scattering, suggesting explosive elongation of WT actin-GS seeds by the cross-linked actin, followed by a much slower polymerization step (Figure 2A, curve 2). No such increase was observed with the actin seeds alone (Figure 2A, curve 1), nor with the addition of cofilin to the mixture of actin seeds and cross-linked G-actin (Figure 2A, curve 3) prior to the addition of phalloidin. Filament formation in this experiment was confirmed by EM observation of aliquots withdrawn from the polymerization reaction monitored in the light scattering measurements (Figure 2B). These filaments appeared shorter and less smooth than control filaments (data not shown) and were partially kinked. The need for phalloidin addition indicates that with the cross-linked actin, the stabilization normally afforded by lateral protomer-protomer interactions is not achieved, and to maintain filament elongation and integrity additional stabilization is required. Such stabilization of inter-strand contacts and support of filament elongation is not provided by cofilin. These observations with the seeds further indicate that the primary role of cofilin in the polymerization of cross-linked actin is nucleation.

Cofilin promotes conversion of amorphous (LC)₂CA actin aggregates into filaments

We showed previously¹² that Cu²⁺-catalyzed disulfide cross-linking of uncross-linked (LC)₂CA F-actin stabilized by rhodamine phalloidin resulted in conversion of the filaments to amorphous aggregates accompanied by the partial displacement of phalloidin from actin. We wished to determine if addition of cofilin to these aggregates could convert them into nuclei capable of inducing filament formation. To this end, DTT-free (LC)₂CA F-actin (10 μM; stabilized with a phalloidin/rhodamine phalloidin mixture) was disulfide cross-linked at room temperature in a spectrofluorometer cell, using 10 μM CuSO₄ to catalyze the air oxidation reaction. The release of rhodamine phalloidin (Figure 3A) and the C180-C269 cross-linking (Figure 3A, inset) were virtually completed in 30-40 min. EM images confirmed the destruction of filaments and the presence of aggregates in the aliquot of this reaction (Figure 3B).

The addition of cofilin at this point (indicated by an arrow in Figure 3A), caused a further incremental release of rhodamine phalloidin from actin. Strikingly, electron micrographs of this solution revealed that cofilin converts actin aggregates into filaments (Figure 3C). This may be achieved in two possible ways: either via nuclei formation from cofilin and cross-linked monomers and/or alteration of actin aggregates by cofilin. The small additional displacement of rhodamine phalloidin by cofilin suggests that it may act here by binding to actin aggregates, thereby converting them into elongation-capable filament seeds. Such a conversion may be due to cofilin bridging between two longitudinal actin protomers in the aggregates, thus stabilizing a structure that is present in cofilin decorated F-actin.¹⁹ The displacement of the phalloidin would result because of the known inability of actin filaments to accommodate phalloidin and cofilin binding simultaneously.²⁹⁻³²

Effect of hydrophobic loop immobilization on actin ATPase activity

Monomeric actin is a poor ATPase. However, polymerization greatly enhances this activity which is important, because the nucleotide hydrolytic cycle is a major factor in the regulation of filament stability. Although loop mobility is necessary for actin polymerization in the absence of cofilin and phalloidin, it is not clear whether such mobility was also necessary for the conformational changes needed for ATPase activation. Given the allosteric interactions that have previously been observed³³ between the hydrophobic loop and the DNase I-binding loop in G-actin, it is not inconceivable that cross-linking of this loop could have an effect on the filament ATPase activity.

To address this question, we used a real-time assay for phosphate release to determine the effect of loop immobilization on ATPase activity. Figure 4 shows that following addition of salt to induce polymerization, Pi release occurs in both WT and the cross-linked (LC)₂CA actin. The curve for WT actin is distinctly biphasic: a first phase that corresponds to the polymerization reaction and a second phase that corresponds to continued treadmilling of the monomers once polymerization steady-state has been achieved. In contrast, the phosphate release observed with the cross-linked actin is basically linear, consistent with salt-induced activation of the ATPase which continues due to the failure of the actin monomers to form stable filaments. A control experiment in the presence of the magnesium (0.2 mM) concentration needed for the PNP assay showed negligible production of Pi over

the course of the experiment. Under these conditions, actin does not polymerize. Thus, although immobilization of the hydrophobic loop via disulfide cross-linking prevents formation of stable filaments, it does not inhibit the conformational changes needed to catalyze ATP hydrolysis.

EM reconstruction of cofilin-phalloidin-F-actin points to conformational changes in F-actin structure

Our previous results indicate that cofilin bridges between longitudinal phalloidin-free protomers, while phalloidin stabilizes lateral and also longitudinal contacts at the interfaces of the cofilin-free actin protomers. To assess better the structural effects of cofilin and phalloidin on the architecture of the filaments formed from the cross-linked actin, we employed single particle iterative helical real space reconstruction (IHRSR) of the filament electron micrographs.²⁷

We used WT yeast actin polymerized in the presence of equimolar phalloidin and 2.0 mM MgCl_2 as a control. No “tilted” actin was found in either the control or in the cofilin-phalloidin cross-linked filaments (Figure 5). EM reconstruction revealed two structural states of F-actin in both the disulfide cross-linked actin-cofilin-phalloidin complex (b, d) and in control (a, c) sets: the closed cleft (shown in grey) and the open cleft (yellow) state. A similar degree of connectivity between the strands (indicated by arrows) is seen in both sets, despite the fact that the hydrophobic loop cannot be responsible for this in (b, d), suggesting that phalloidin is accounting for part of this connectivity. The cofilin-decorated segments were sorted based on the extent of cofilin decoration. We found that the low occupancy filaments have subdomain 2 ordered (f, black arrow) and have poor connectivity between the strands (f, blue arrow) when compared with the more extensively decorated filaments (e). The difference in connectivity is shown in magenta in (g), a difference map. The dense contact between opposite strands in the extensively decorated F-actin suggests cofilin-phalloidin-induced conformational changes that support the interstrand interactions.

Earlier, we have shown that the change of twist caused by ADF ($\sim 5^\circ$ per subunit) can propagate far along the filament, and even poorly occupied segments have a substantial change in twist.²⁸ EM reconstruction revealed a weak ridge of density at the primary cofilin-binding site. Both low occupancy and/or disorder of the cofilin bound to F-actin may explain such a low cofilin density in our 3D map. A small change of twist within these filaments ($\sim 1^\circ$ per subunit, from 166° to 165°) might result from both cofilin and phalloidin and their competing effects on F-actin structure.

Discussion

The focus of this study was to examine the necessity of a conformationally flexible subdomain 3/4 hydrophobic loop to the capacity of actin to both nucleate filament formation and to then undergo subsequent elongation. The ability to lock the loop to the surface of the monomer by disulfide cross-linking L180C/L269C/C374A mutant actin afforded us the possibility to address these questions.

Previously, we showed that the C180-C269 cross-linked species could not polymerize by itself.¹¹ Furthermore, phalloidin could not stabilize the monomer-monomer contacts sufficiently to overcome the impaired interactions resulting from the inability of the loop to alter its conformation during the polymerization process. Our demonstration that addition of cofilin to the phalloidin solution allows limited polymerization indicates that the cross-linking prevents formation of the monomer-monomer interfaces required for nucleation. Thus, the binding sites for phalloidin never formed in the absence of cofilin. By providing a scaffolding for the cross-linked actin, cofilin restores the nucleation interface, allowing subsequent elongation and phalloidin binding. The inability of the cofilin to rescue polymerization alone shows that subsequent monomer addition does not result in stable-enough monomer-monomer interactions to keep the filament intact. Thus, not surprisingly, the inter-strand lateral interactions impaired by the loop immobilization are partially restored by phalloidin, but not by cofilin.

The cross-linking of phalloidin-stabilized F-actin, in the absence of cofilin, led to the formation of monomers and aggregates^{6,11,12} not a homogeneous solution of monomers. Addition of cofilin to these aggregates restored some filaments, albeit short and less uniform. These results lead to two conclusions. First, cofilin can provide enough of a scaffold to force this altered actin conformation back to a more normal nucleus-like state. Second, cross-linking does not prevent monomer-monomer association under filament forming conditions. However, this interaction - though perhaps approaching normal monomer-monomer contacts - is not compatible with an F-actin helical structure. If one assumes that in the cross-linked state there is an equilibrium which favors an alternative intermonomer association over that needed for helical polymerization, then phalloidin appears to pull that equilibrium toward the F-actin. The stabilization afforded by phalloidin, however, is not sufficient to restore the monomer-monomer contacts to their original strength, resulting in the short filaments we observe.

Our phosphate release experiment showed that while loop mobility is needed for filament formation, it is not required for salt-dependent activation of actin-catalyzed hydrolysis of ATP. However, it is not possible to discern if the catalysis is carried out by activated monomers or as a result of conformational changes brought about by monomer-monomer contacts in an abortive attempt to form a filament. We favor the latter possibility based on the similar initial rates of ATP hydrolysis by WT and cross-linked actin. The results further suggest that the inability to form stable filaments may derive from a significantly increased critical concentration caused by the conversion of ATP-cross-linked actin to ADP-cross-linked actin with its concomitant conformational change.

A puzzling observation is the lack of significant change in the twist in those filament segments that have high cofilin occupancy, which is normally a hallmark of this protein-protein interaction.^{5,6,14} The reported inability of cofilin alone to promote filament formation from the cross-linked actin indicates that even filaments densely coated with cofilin must also contain phalloidin as a stabilizer. Although it is thought that they cannot co-exist at the same site on a filament because of their opposing structural effects, they could exist in independent patches along the filament. If so, the opposing twists in the filament caused by these two reagents might cancel each other out, leading to the observed

filament fragments with little if any abnormal twist present, cofilin-actin and phalloidin-actin interactions weaker than normal, and a resulting decrease in the density of interstrand contacts leading to the observed reduced filament stability.

In summary, our results show that a change in conformation of the hydrophobic loop is necessary for formation of the normal monomer-monomer interfaces that allow both filament nucleation and elongation resulting in the creation of a stable filament. In the absence of this loop rearrangement, some of the normal conformational changes that lead from G- to F-actin might occur, resulting in the ability of monomers to associate with one another but in a nonproductive fashion. Addition of a nucleating scaffold and a reagent that subsequently stabilizes the weakened interactions that form in attempts to elongate the nuclei pull enough of these structures to a more normal state, allowing limited formation of weakened filament structures.

Materials and Methods

Preparation of proteins

WT yeast cofilin was expressed in *Escherichia coli* BL21 DE3 cells under control of the T 7 promoter from the plasmid pBAT4 plasmid (kindly provided by Dr. Steven Almo, Department of Biochemistry, Albert Einstein College of Medicine, New York, NY) and then purified as described previously.^{20,21} Gelsolin was purified from bovine plasma according to Kurokawa et al.²² Myosin subfragment 1 (S1) was prepared by chymotryptic digestion of myosin by the method of Weeds and Pope.²³

The preparation of yeast (*Saccharomyces cerevisiae*) actin mutant (LC)₂CA was described previously.¹¹ Yeast actin (WT and the mutant) was purified by affinity chromatography on a DNase I column, as described before.²⁴ Purified G-actin was stored in 10 mM Tris HCl (pH 8.0), 0.2 mM CaCl₂, 0.2 mM ATP, and 1.0 mM DTT on ice and was used within three days from the completion of the preparation. To keep the cysteines in reduced conditions, freshly prepared DDT (0.5 mM final concentration) was added daily. F-actin was prepared by polymerizing G-actin with 3.0 mM MgCl₂ and an equimolar amount of phalloidin. The concentration of actin was measured by the Bradford protein assay using skeletal rabbit muscle actin as a standard.

Cross-linking of actin and SDS PAGE analysis

Prior to the cross-linking reaction, DTT was removed from G-actin by passing the actin solution over Sephadex G-50 spin columns equilibrated with 10 mM MOPS buffer (pH 7.2), 0.2 mM CaCl₂, and 0.2 mM ATP. Cysteines 180 and 269 on G- and F-actin were cross-linked on ice or at room temperature for the indicated times (1-35 minutes), via disulfide bond formation, using 5-10 μ M CuSO₄ to catalyze air oxidation. Cross-linking of G- and F-actin resulted in its complete transformation into species with higher electrophoretic mobility on SDS PAGE.

Actin polymerization assays

Actin polymerization was monitored *via* light scattering measurements, at room temperature, in a PTI fluorometer (Photon Technology Industries Co., South Brunswick, NJ) set at 325 nm for both the excitation and emission wavelengths. The polymerization of 10 μ M mutant G-actin was induced by adding to it 3.0 mM $MgCl_2$ and either equimolar phalloidin and cofilin (when needed) or WT actin-gelsolin seeds (1:10 mole ratio of gelsolin:actin). The actin seeds were prepared by polymerizing WT actin with 2.0 mM $MgCl_2$ in the presence of gelsolin (GS), at 1:10 (gelsolin:actin) mole ratio, in 10 mM MOPS buffer (pH 7.2), 0.2 mM $CaCl_2$, and 0.2 mM ATP. The WT yeast actin-GS seeds were used in polymerization assays immediately after their preparation.

Rhodamine phalloidin release by disulfide cross-linking (C180-C269)

Rhodamine phalloidin release from $(LC)_2CA$ F-actin was monitored in a PTI fluorometer *via* a decrease in rhodamine fluorescence at 575 nm. The cross-linking was initiated by the addition of 10 μ M $CuSO_4$ to 15 μ M F-actin.

Assessment of release of phosphate from WT and cross-linked actin

To 10 μ M actin, either WT or cross-linked $(LC)_2CA$ was added 2 mM $MgCl_2$ and 50 mM KCl, which usually induces polymerization. The real-time release of inorganic phosphate from actin, produced by ATP hydrolysis, was quantitated by the Enzchek phosphate assay (Molecular Probes, Inc.), according to the manufacturer's instructions, as previously reported.²⁵ This assay is based on the reaction of Pi with methylthioguanine in the presence of purine nucleoside phosphorylase. Phosphorolysis of the N-glycosidic bond causes a change in absorbance of the base which is followed as a function of time at 360 nm. All phosphate release experiments were performed at least three times with different actin preparations. Phosphate standard curves were generated for each assay.

Electron Microscopy

For EM observations, samples of actin freshly polymerized with 3.0 mM $MgCl_2$ and equimolar amounts of phalloidin, either uncross-linked (with 2.0 mM DTT) or cross-linked and then polymerized with equimolar cofilin and/or phalloidin, were diluted with the polymerizing buffer to 2.5 μ M immediately before spreading on the carbon-coated grids. Electron microscopy grids were prepared according to the previously reported protocol.²⁶ The grids with negatively stained actin samples were examined in a Hitachi H-7000 electron microscope with nominal magnification of 31,200X.

Image reconstruction

Image reconstruction was done using IHRSR method.²⁷ We extracted 1,405 segments of $(LC)_2CA$ F-actin stabilized with phalloidin and cofilin, each ~416Å long. After 60 cycles of IHRSR refinement this set yielded a stable solution of ~165deg./27Å. The final reconstruction has a weak ridge of density at the primary cofilin binding site. The sorting of segments by the occupancy was done as described by Galkin et al.²⁸ The SPIDER software package was used for image processing.

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Abbreviations

(LC)₂CA	yeast actin mutant (L180C/L269C/C374A)
DNase I	deoxyribonuclease I
F-actin	filamentous actin
G-actin	monomer actin
S1	myosin subfragment 1
GS	gelsolin
ABP	actin binding protein
EM	electron microscopy
IHRSR	iterative helical real space reconstruction

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Figure 1.

Cofilin and phalloidin co-rescue the polymerization of (LC)₂CA disulfide cross-linked G-actin. A- Light scattering from the actin polymerization solution. Actin (10 μ M) polymerization was monitored via light scattering measurements in a PTI fluorometer set at 325 nm for both excitation and emission wavelengths. Curve 1, disulfide cross-linked G-actin polymerized in the presence of cofilin (10 μ M); curve 2, disulfide cross-linked G-actin polymerized in the presence of cofilin and phalloidin. The addition of phalloidin is indicated by an arrow, the addition of Mg is shown by an arrowhead. B- Representative electron micrographs of the cross-linked G-actin polymerized in the presence of cofilin (B) and cofilin and phalloidin (C). In the presence of cofilin and phalloidin, some aggregates are still found, but F-actin like filaments are also seen. An average of 438 segments cut from these filaments (C, inset) clearly shows an F-actin structure. D,E - Power spectra of the cross-linked and uncross-linked actin filaments. (D) - The power spectrum from 1,433 short segments of control F-actin, where each segment is 150 pixels long (630 $\text{\AA}$). The power spectrum of each segment has been calculated, and these were added together. This shows the $n=2$ layer line near the equator, and the $n=-1$ layer line arising from the 59 $\text{\AA}$ pitch left-handed F-actin helix. (E) - power spectrum from 1,251 segments of the same length from the cross-linked filaments, showing the same features. The spectrum is noisier due to the greater background of unpolymerized G-actin.

B

Figure 2.

Polymerization of the $(LC)_2CA$ disulfide cross-linked G-actin in the presence of WT actin-gelsolin seeds and in the presence or absence of cofilin and phalloidin. A - Light scattering traces of polymerization reaction. 1 – control, WT actin-GS seeds (50 nM) alone are not polymerized by 3.0 mM $MgCl_2$ and 10 μM phalloidin. 2 - disulfide cross-linked G-actin polymerized with WT actin-GS seeds (50 nM) in the absence of cofilin (10 μM). 3 - disulfide cross-linked G-actin polymerized with WT actin-GS seeds in the presence of cofilin. The polymerization was initiated by adding 3.0 mM $MgCl_2$ (indicated by a long arrow) to 13 μM disulfide cross-linked G-actin containing 50 nM WT actin-GS seeds, followed by the addition of equimolar phalloidin (indicated by a short arrow). B - Representative electron micrograph of the cross-linked G-actin polymerized in the presence of WT actin-GS seeds without cofilin (from an aliquot of the reaction shown by curve 2).

Figure 3.

Cofilin promotes the conversion of (LC)₂CA aggregates into filaments. A- Rhodamine phalloidin release from F-actin and from actin aggregates, as monitored *via* a decrease in rhodamine fluorescence at 575 nm. To catalyze the disulfide cross-linking (C180-C269), 10 μ M CuSO₄ was added to F-actin (15 μ M) stabilized with a phalloidin/rhodamine phalloidin mixture. Additions of cofilin and CuSO₄ are indicated by arrows. *Inset*- SDS PAGE evidence for the complete disulfide cross-linking of (LC)₂CA actin after a 35 min copper-catalyzed oxidation reaction and after addition of cofilin. 1, uncross-linked actin; 2, cross-

linked actin (after oxidation for 35 min); 3, cross-linked actin (35 min oxidation reaction) followed by the addition of cofilin.

B- Representative electron micrograph of the cross-linked F-actin after a 35 min oxidation reaction. C- The same actin as in B, after the addition of equimolar cofilin. The bar corresponds to 1000 Å.

Figure 4.

Phosphate release from wild-type and cross-linked (LC)₂CA actin.

Polymerization of actin (10 μM) was initiated by the addition of 2 mM MgCl₂ and 50 mM KCl and, formation of WT F-actin was followed as a function of time by the increase in light scattering (solid line). In a separate experiment, phosphate release from both WT and (LC)₂CA actins was followed using the Enzchek assay as described in Methods. A control for salt-dependent phosphate release was also performed in which the actins were observed in G buffer containing the 0.2 mM MgCl₂ necessary for the polynucleotide phosphorylase used in the assay. Under these control conditions, neither actin polymerized (data not shown). The left vertical axis denotes phosphate release. The right axis denotes change in light scattering for the WT actin polymerization curve (large filled circles). Phosphate release curves are as follows: Filled small circles: WT actin + salt; Filled triangles: cross-linked actin + salt; Open circles: WT actin, no salt; Open triangles: cross-linked actin, no salt. These results are representative examples of experiments that were repeated with at least three different actin preparations.

Figure 5.

Three-dimensional (3D) EM reconstructions of phalloidin-WT yeast actin and cofilin-phalloidin (LC)₂CA F-actin. Two structural states of F-actin in both the phalloidin-cofilin-complex (b, d) and in control wild type yeast actin polymerized in the presence of phalloidin and 2mM MgCl₂ (a, c): closed cleft (grey) and open cleft (yellow). A similar degree of connectivity between the strands is indicated by arrows. The low occupancy filaments have subdomain 2 ordered (f, black arrow) and have poor connectivity between the strands (f, blue arrow) when compared with the more extensively decorated filaments (e). The difference in connectivity is shown in magenta in (g), a difference map.