Contributions of the lower dimer to supramolecular actin patterning revealed by TIRF microscopy

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Abstract

Two distinct dimers are formed during the initial steps of actin polymerization. The first one, referred to as the 'lower dimer' (LD) was discovered many years ago by means of chemical crosslinking. Owing to its transient nature, a biological relevance had long been precluded when, using LD-specific antibodies, we detected LD-like contacts in actin assemblies that are associated with the endolysosomal compartment in a number of different cell lines. Moreover, immunofluorescence showed the presence of LD-related structures at the cell periphery of migrating fibroblasts, in the nucleus, and in association with the centrosome of interphase cells. Here, we explore contributions of the LD to the assembly of supramolecular actin structures in real time by total internal reflection fluorescence (TIRF) microscopy. Our data shows that while LD on its own cannot polymerize under filament forming conditions, it is able to incorporate into growing F-actin filaments. This incorporation of LD triggers the formation of X-shaped filament assemblies with barbed ends that are pointing in the same direction in the majority of cases. Similarly, an increased frequency of junction sites was observed when filaments were assembled in the presence of oxidized actin. This data suggests that a disulphide bridge between Cys374 residues might stabilize LD-contacts. Based on our findings, we propose two possible models for the molecular mechanism underlying the supramolecular actin patterning in LD-related structures.

Keywords: antiparallel actin dimer, lower dimer, actin patterning, TIRF microscopy

Abbreviations: LD, lower dimer; STEM, scanning transmission electron microscopy; TIRFM, total internal reflection fluorescence microscopy

Introduction

The organization of actin into higher ordered networks is key to cell shape and movement. Most cellular functions involving actin are based upon the spatially and temporally controlled rearrangement of corresponding networks. One of the best-characterized network architectures is that of the lamellipodium at the edge of a protruding cell (Bisi et al., 2013; Keren et al., 2008). Here, filamentous actin (F-actin) forms a dense, branched network of filaments that are oriented with their barbed ends towards the plasma membrane, where they elongate to push the membrane forward during actin-based motility (Mogilner, 2006; Small, 2005; Pollard and Borisy, 2003). It was recently shown that in epithelial cells competition between the dendritic F-actin network and cortical actomyosin bundles acts as a switch between the establishment of polarity and migration (Lomakin et al., 2015). Bundles of parallel actin filaments form the backbone of filopodia that protrude at the leading edge of cells (Mattila and Lappalainen, 2008). In addition, reorganization of actin suprastructures is a driving force in the endocytic pathway. For example, electron microscopy (EM) studies of endocytic budding in *Saccharomyces cerevisiae* demonstrate that the endocytic actin network remodels as budding progresses (Idrissi et al., 2012). Finally, laterally aligned antiparallel actin filaments are present in stress fibers or transverse arcs that are the site of active contraction driven by myosin motors (Hotulainen and Lappalainen, 2006; Burnette et al., 2011).

Over the past years, unconventional actin configurations with actin-actin contacts that differ from those present in canonical F-actin filaments have received increased attention (Graceffa et al., 2013; Silvan et al., 2013; Galkin et al., 2015). While some of these unconventional actin configurations have been detected in cells (Silvan et al., 2012; Miralles et al., 2003), others remain elusive in a native environment. One of the most enigmatic arrangements of actin, the lower dimer (LD), was first described over 25 years ago when chemical crosslinking of polymerizing actin revealed two dimers with different electrophoretic mobility (Millonig et al., 1988). LD, the first dimer formed, is not able to assemble into F-actin on its own, presumably because of the orientation of its subunits is incompatible with the subunit configuration present in F-actin. In 2002 Bubb et al. determined the crystal structure of the actin LD and found that in it the protomers are held together in an antiparallel arrangement (Bubb et al., 2002). Crystallization of oxidized β -actin to a resolution of 2.6 Å (Lassing et al., 2007) also revealed an antiparallel actin dimer connected by an intermolecular disulfide bond involving cysteine 374. Thiol oxidation of actin has previously been reported to produce dimers that, when incorporated

into F-actin during polymerization generated crosslinks between filaments and enhanced the elasticity of the F-actin network (Tang et al., 1999).

By means of an LD-specific antibody, we have demonstrated the existence of LD-like contacts in association with the endolysosomal compartment and underneath the plasma membrane in fixed cells (Silvan et al., 2012). Interactions of the actin polymerization machinery with membrane binding and membrane trafficking proteins, such as ARF1, are emerging and reveal the existence of an 'endocytic wiring system' that spatially restricts branched actin elongation (Bisi et al., 2013). However, in most instances, the precise organization of actin filaments at specific cell sites and their rearrangement during cell function is poorly understood. For example, the organization of dendritic actin networks in the lamellipodium has been questioned (Higgs, 2011; Small, 2010; Urban et al., 2010), although today it is widely accepted that the Arp2/3 complex is responsible of the formation of branched structures at these sites. *In vitro*, the Arp2/3 complex predominantly generates side branches on pre-formed filaments, with a characteristic angle between daughter and mother filament of 70° (Mullins et al., 1997). Interestingly, branched filaments were also observed in the absence Arp2/3 when actin was polymerized in the presence of covalently crosslinked LD (Steinmetz et al., 1997; Schoenenberger et al., 2002). Moreover, the correlation of ragged filament morphology and the formation of LD at early stages of polymerization, proffered the idea that the LD might play a role in the formation of filament junctions. However, the structural organization of LD-related junction sites and their effect on the spatial arrangement of actin networks remained unknown.

In the present study, we used TIRF microscopy to examine the incorporation of chemically crosslinked LD into filaments in real time. Our data show that the LD mediates contacts between filaments that are predominantly oriented with their barbed ends in the same direction. Oxidized actin samples produced similar links between filaments. Based on these *in vitro* polymerization experiments, we propose two molecular models for the LD-mediated organization of supramolecular actin-filament assemblies.

Materials and methods

Purification of monomeric G-actin and crosslinked LD

Skeletal muscle actin was isolated from rabbit acetone powder (Innovative Research, IR41995) as previously described (Steinmetz et al., 1997). To obtain monomeric G-actin, actin

preparations were centrifuged in a swing-out rotor for 2h at 150'000 x g at 4°C. The upper two thirds were loaded onto a Superdex™ 200 prep grade HiLoad™ 26/60 gel filtration chromatography column (GE Healthcare) and eluted at a flow rate of 1 ml/min in buffer A (2.5 mM imidazole, 0.2 mM CaCl₂, 0.2 mM ATP, 0.005% NaN₃, pH 7.2-7.4) at 4°C. Fractions representing the tail of the actin peak were collected. Monomeric G-actin was generally stored for up to 2 weeks on ice in buffer A with 0.1 mM dithiothreitol (DTT).

Freshly isolated monomeric G-actin was crosslinked under conditions that promote LD formation. Briefly, N,N'-1,4-phenylenebismaleimide (1,4-PBM) was dissolved in dimethylformamide (DMF) at 5 mM, and diluted prior to use in sodium borate buffer (1:400, 20 mM Na₂B₄O₇, 20mM CaCl₂, pH 9.5). Polymerization of monomeric actin at a concentration of 1 mg/ml was initiated by the addition of CaCl₂ to a final concentration of 20 mM. Crosslinking was achieved by the immediate addition of an equal volume of freshly diluted 1,4-PBM. The reaction was quenched after 10 min at room temperature (RT) by the addition of excess β-mercaptoethanol. Next, the sample was dialyzed for 24 h at 4 °C against buffer A to remove excess crosslinker and β-mercaptoethanol. LD was isolated by three consecutive gel filtration chromatography runs on a Superdex™ 200 prep grade HiLoad™ 26/60 column (GE Healthcare).

The concentration of G-actin and LD was determined by measuring the absorbance at 290 nm using an extinction coefficient of 0.63 mL mg⁻¹ cm⁻¹ for G-actin. The purity of the different samples was examined by SDS-PAGE. Oxidation of monomeric actin was performed by dialyzing the protein against buffer A containing 0.1-0.5% H₂O₂ for 12 h at 4°C.

The Arp2/3 complex was purified from *Acanthameba castellani* as described (Qu et al., 2015), and the activating peptide VCA from N-WASP (comprising residues 392 to 501) was expressed in *E. coli* and purified; both were kindly supplied by Prof. D.L. Barber, Prof. L.L. LeClaire and Dr. A. Schoenichen (University of California, San Francisco, USA).

High speed pelleting assay of G-actin/crosslinked LD copolymers

Purified G-actin and crosslinked LD were mixed at different ratios and polymerization was induced at RT by the addition of 2 mM MgCl₂ and 50 mM KCl and a two-fold molar excess of phalloidin over actin where indicated. After 2 h, the total polymerization reaction (T) was centrifuged at 100,000 x g at 4°C for 1 h. The supernatant (S) was carefully removed and the

pellet (P) was washed in one volume of polymerization buffer F (buffer G containing 2 mM MgCl_2 /50 mM KCl). After washing, the pellet was gently resuspended in one volume of buffer F and incubated for at least 1 h at 4°C.

Total internal reflection fluorescence microscopy (TIRFM) chamber preparation

Coverslips (Menzel Gläser #1) were sequentially cleaned with ethanol, milliQ water, Hellmanex II (Fluka, #61257) and milliQ water. They were then coated with polyethylene glycol to prevent actin sticking to the glass. Briefly, clean coverslips were air dried and subsequently incubated with PEG-silane (MPEG-SIL-5000, 0.1 mg/mL in 96% ethanol) for 12 h. Coated coverslips were rinsed in milliQ water, gently air-dried and stored at 4°C until further use. A sample flow chamber with the approximate dimensions 100 μm x 20 mm x 20 mm was built by mounting a second coverslip using double-sided adhesive tape.

Imaging

In order to avoid overcrowding of polymerization products, the optimal starting concentration of G-actin was empirically determined for each experiment. Polymerization experiments were carried out using 10 to 15% Alexa Fluor® 488-labeled G-actin (A488-rabbit muscle actin, Invitrogen). For dual color experiments, rhodamine-labelled actin was added (Cytoskeleton, AR05). Polymerization was started by the addition of an equal volume of TIRF polymerization buffer (20 mM imidazole, 100 mM KCl, 2mM MgCl_2 , 2 mM EGTA, 0.4 mM ATP, 100 mM DTT (omitted in the oxidation experiments), 2% methylcellulose, 10 $\mu\text{g}/\text{ml}$ glucose oxidase, 50 $\mu\text{g}/\text{ml}$ catalase, 250 $\mu\text{g}/\text{ml}$ glucose, pH 7.2-7.4). Immediately after the addition of the TIRF polymerization buffer, the sample was applied to the flow chamber mounted on a DMI 6000B inverted microscope (Leica) equipped with 488 nm and 561 nm solid state lasers and emission bandpass filters for GFP (525 +- 25 nm) and Cy3 (610 +- 33 nm). Images were recorded every five seconds using an EMCCD camera (Hamamatsu C9100-02), with the excitation laser being shuttered between images to minimize photo-bleaching. Exposure time and gains were independently set for each experiment (exposure times ranged from 100 to 400ms). For the dual color and oxidation experiments, the laser power had to be reduced to prevent actin severing.

Scanning transmission electron microscopy analysis (STEM) of LD copolymerized with G-actin

Copolymerization of crosslinked LD with native G-actin was induced by the addition of salt to a final concentration of 100 mM KCl in the presence of a 2:1 M ratio of phalloidin over actin. After

2 h at RT, the solution was centrifuged at 100,000 x g for 1 h at 4°C. The pellet was washed with buffer F (buffer A containing 100mM KCl) and subsequently resuspended in buffer F and incubated for an additional hour at 4°C. Aliquots of the F-actin solution were adsorbed for 1 minute to a freshly glow-discharged thin carbon film that spanned a thick fenestrated carbon layer covering a gold-plated copper microscopy grid. Specimens were washed on four droplets of quartz double-distilled water and stained with 0.75 % (w/v) uranyl formate. Digital dark-field images were recorded using a Vacuum Generators HB5 STEM interfaced to a modular computer system (Tietz Video and Image Processing Systems GmbH, D-8035 Gauting). The microscope was operated at 100 kV and a nominal magnification of 500,000x, using doses that ranged between 8400 and 10,000 electrons/nm².

Modelling of LD-mediated filament assemblies

Filament assemblies were assembled *in silico* by superimposition based on secondary structure matching. For the junction mediated by a single LD, two strands of the tilted state of Actin, T1 (pdb 3J8J) were aligned based on a superposition of their central subunits onto either of the protomers of a lower dimer structure (pdb 1LCU). For the junction mediated by two LDs, a dimer of LDs was modeled based on superpositioning of one protomer of each LD (pdb 1LCU) onto either protomer of a standard actin dimer (pdb 2A5X). Interfilament angles were calculated by (i) fitting a line through each of the two linked filaments (ii) calculating the shortest vector between the two lines (iii) calculation of the dihedral angle between the two lines fitted to the filaments via the shortest connecting line.

Results

The ragged appearance of 'young' filaments, which correlates with the transient formation of LD at early stages of actin polymerization *in vitro*, smoothens over time (Steinmetz et al., 1997). Correspondingly, at steady state scanning transmission electron microscopy (STEM) of polymerized actin revealed the well-known filaments with a highly ordered structure (Figure 1, left panel). In contrast, when mixtures of stably crosslinked LD and G-actin were co-polymerized in the presence of phalloidin, STEM analysis of negatively stained filaments revealed lateral protrusions at steady state (Figure 1, right panel). Higher magnification (shown for the boxed areas) showed that the protrusions extend to approximately half of the filament diameter. Analysis of 66 individual protrusions showed that the average protrusion corresponds well with the dimensions of an actin subunit (Supplementary Figure 1C). Conceivably, the protrusion

represents the free actin subunit of the incorporated LD that is jutting out from the filament. LD incorporation was further corroborated by high speed centrifugation of actin polymerized in the presence of different amounts of LD (Supplementary Figure 1A and B). This data showed that LD was compatible with filament assembly provided that at least 60% of the subunits were monomeric actin.

Figure 1. Scanning transmission electron microscopy images of negatively stained filaments obtained from the polymerization of monomeric actin (A) and from the copolymerization of crosslinked LD with monomeric actin (B). Boxed areas are shown at 2-fold magnification. LD incorporation results in lateral protrusions that are absent in conventional filaments obtained from pure monomeric actin. The scale bar represents 20 nm.

To gain insight into the role of LD in the assembly process and in the dynamics of supramolecular actin structures, we imaged the polymerization of G-actin and LD in real time by TIRFM. For these experiments, actin mixtures including 15% of Alexa Fluor-488-conjugated G-actin were induced to polymerize by the addition of 1 mM MgCl_2 /50 mM KCl. In the absence of LD, time-lapse TIRFM revealed the elongation of individual linear actin filaments that moved independently and did not interact with each other (Figure 2, top row). Under the same polymerization conditions, the presence of LD resulted in elongating filaments that frequently formed X-shaped structures (Figure 2, bottom row; supplementary videos 1-4). In some instances, X-shaped assemblies appeared to emanate from Y-shaped branching structures. At higher ratios of LD to actin in the initial mixture, X-shaped junctions became more frequent, which confirms that the LD configuration is essential for the establishment of a connection between two filaments. Although the junction sites persisted over the time frames imaged, the angle between the filaments forming the junction fluctuated. Because actin polymerization is sensitive to laser illumination, we did not succeed in localizing single LD molecules that were labeled with a different fluorescent dye.

Figure 2. Time-lapse TIRF microscopy of the polymerization of G-actin in the absence and presence of crosslinked LD. G-actin alone polymerized into single, linear filaments that moved independently from each other (top row). LD incorporation into growing filaments induced the formation of stable junctions between filaments. The resulting filament assemblies were mostly X-shaped structures (bottom row). The scale bar represents 30 μm .

Arp2/3 is a multiprotein complex that regulates the formation of dendritic actin networks by nucleating 'daughter' filaments on the sides of pre-existing ones (Volkman et al., 2001). TIRFM images of G-actin polymerized in the presence of activated Arp2/3 confirm the frequent formation of side branches (Figure 3, top row). On the contrary, the presence of LD in polymerization reactions triggered junction sites involving filaments that continued beyond the branching point (Figure 3, bottom row). As a result, X-shaped filament assemblies were formed. Occasional Y-shaped structures suggested the possibility of side-polymerization induced by LD. We cannot completely rule out such a branching mechanism because it was not possible to distinguish *bona fide* LD-mediated branching from a "secondary" branch, i.e., the side-binding of a small F-actin oligomer with an LD incorporated at one end. Pyrene actin fluorescence assays in the presence of LD with or without activated Arp2/3 complex (Supplementary Figure 2) showed that LD does not significantly affect the bulk polymerization dynamics of actin, either alone or in the presence of activated Arp2/3 complex.

Figure 3. Arp2/3 and LD mediate distinct branching phenotypes. The VCA-activated Arp2/3 complex generated side branches with a characteristic angle of around 70° between the mother and daughter filaments (top row). In LD-mediated branching (bottom row), both filaments extended beyond the junction site (arrowheads) to give a typical X-shaped structure. The scale bar represents $5\ \mu\text{m}$.

Precise knowledge of the relative polarity of intersecting filaments is crucial to understanding the significance of the various F-actin arrangements. Because the addition of subunits to actin filaments occurs primarily at the barbed end, we added rhodamine-labeled monomers to samples that already had LD-induced junctions. In experiments that required two excitation wavelengths, the laser power had to be reduced to avoid actin filament fragmentation and bleaching of the fluorescent signals. As illustrated in Figure 4A, TIRFM revealed that the majority of the LD-related structures were composed of two filaments with an acute angle between their fast-growing barbed ends (Figure 4A, supplementary video 5). Nevertheless, intersections where the angle between the filaments was larger than 90° were occasionally observed (Figure 4B, supplementary video 6).

Figure 4. Dual color TIRF microscopy reveals the orientation of fast growing ends in LD-mediated branching structures. Rhodamine-G-actin added to F-actin pre-polymerized in the presence of Alexa-488-actin and crosslinked LD labels the fast growing ends. (A) In most X-structures, barbed ends (arrowheads) of both filaments pointed in the same direction with respect to the narrow aspect of the junction. (B) Occasionally, angles larger than 100° were observed between the fast growing ends of connected filaments. The scale bar represents $2\ \mu\text{m}$.

To unravel possible mechanisms that might promote and stabilize LD-like contacts *in vivo*, we analyzed the effect of an oxidized actin species on polymerization, since actin oxidation has been reported to produce dimers that incorporate into F-actin during polymerization (Tang et al., 1999). For this purpose, we dialyzed a G-actin sample overnight against buffer A containing 0.5 - 1% H₂O₂. Together with the absence of DTT, the presence of oxidizing agents in the actin mixture augmented bleaching of the fluorophores. Thus, TIRFM experiments had to be carried out with reduced laser power. Two distinct effects of oxidized actin on polymerization reactions were observed (Figure 5). First, bundles composed of two or more laterally aligned filaments formed abundantly (Figure 5B-C; supplementary video 7). Interestingly, the filaments within these structures retained a certain extent of freedom and were frequently observed gliding along each other. Another, with respect to the functionality of LD more significant effect was the formation of stable filament intersections similar to those obtained in the presence of crosslinked LD (Figure 5A; supplementary video 8).

Figure 5. Effects of oxidized actin on filament assembly. A sample containing 1 μ M G-actin, 1 μ M oxidized actin and 10% Alexa488-labeled actin was induced to polymerize, and imaged by TIRFM. (A) Single frame at steady state with individual filaments and an X-shaped assembly of two connected filaments (arrow). (B) Oxidized actin promoted the lateral alignment (arrowheads) of two or more filaments. (C) Schematic representation of an LD-like contact (yellow) formed between two subunits of neighboring filaments with opposite polarity. The scale bar represents 2 μ m.

Our TIRFM results suggested that LD-related patterning includes filaments that extend beyond the junction site in both directions and have the same polarity with respect to the acute angle between them. These experimental observations gave rise to two molecular models for LD-mediated filament assembly (Figure 6A and B). In both cases, the models were built on the basis of the tilted state of actin (T1-actin) described by Galkin et al., where superimposing chain A of the antiparallel actin dimer 1LCU (Lassing et al., 2007) upon a protomer in T1-actin revealed that there are no significant clashes between the protruding subunit and the protomers of the filament (Galkin et al., 2015).

In the first model (Figure 6A), a single antiparallel actin dimer (1LCU in red and blue; Bubb et al., 2002) shared between two actin filaments in the T1 state (Galkin et al., 2015) mediates the contact in the X-shaped filament assembly. This interaction results in an interfilament angle of approximately -131° . Based on the antiparallel orientation of the subunits in the LD, the connected filaments have opposite polarity, which is inconsistent with their function to push the membrane forward at the leading edge of a moving cell. Figure 6B shows a different model where the filaments have the same polarity. Here, the contact between apposing filaments is mediated by two LDs that establish a parallel, filament-like actin-actin contact via their protruding subunits. In addition to the LD-related ragged and branched filaments described previously (Steinmetz et al., 1997; Schoenenberger et al., 2002; Galinska-Rakoczy et al., 2009), STEM analysis of copolymerized LD and G-actin revealed the presence of X-shaped filament structures (Figure 6C). However, we cannot determine the polarity or rule out a random overlay of two filaments without further analysis.

Figure 6. Molecular models for LD-mediated filament assemblies. (A) Front and side views of a filament junction mediated by a single LD. The direct antiparallel interaction results in filaments pointing in nearly opposite directions at an interfilament angle of -131° . Filament junctions were assembled by superpositioning of actin T1 state filaments (Galkin et al., 2015), antiparallel (1LCU; Bubb et al., 2002) and parallel actin dimer (2A5X; Kudryashov et al., 2005) structures. The antiparallel dimers are shown with one protomer colored in blue, the other in red. (B) Front and side views of a filament junction mediated by two LDs. Each LD is incorporated with one subunit in apposing T1-actin filaments. The second, protruding subunit interacts through a conventional filament contact with the protruding subunit of the apposing filament. The junction yields an angle of 112° between filaments of the same polarity. (C) STEM micrographs of higher-order filament assemblies obtained from the copolymerization of crosslinked LD with monomeric G-actin. Scale bar represents 20 nm.

Discussion

Lower dimer incorporation is associated with distinct filament assemblies

Correlative analysis of ultrastructural actin filament morphology and chemical crosslinking suggests a role for LD in the supramolecular patterning of actin (Steinmetz et al., 1997; Schoenenberger et al., 2002), although the antiparallel orientation of actin subunits is difficult to reconcile with the canonical F-actin filament structure (Bubb et al., 2002; Millonig et al., 1988). Because EM documents specific stages of actin assembly, we performed TIRFM to gain insight on the assembly dynamics of samples containing monomeric actin, LD and fluorescently labeled actin. The resulting time-lapse videos reveal the ability of the LD to mediate the formation of junctions between filaments.

The constant movement of intersecting filaments relative to the junction site, which results in considerable changes in the relative angle between filaments, indicates that the interfilament contact is highly flexible. Although this flexibility may be in part attributed to the 1,4-PBM linker, for which an average distance of approximately $11\text{\AA}$ between crosslinked amino acid residues has been reported (Green et al., 2001), corresponding structures that were equally flexible were also observed with oxidized actin in the absence of crosslinker. Analysis of LD-effects on filament morphology by EM suggested that the LD promotes side branching (Silván et al., 2012; Steinmetz et al., 1997; Schoenenberger et al., 2011), whereas the TIRF studies presented here reveal that LD incorporation is associated with the formation of X-shaped filament assemblies. This might reflect the difference between examining the dynamics of LD patterning versus visualizing the effects of stabilized LD incorporation. It is possible that X-shaped filament structures (Figure 6C) were simply overlooked in the earlier EM studies as they were considered fortuitous crossings of filaments. While live TIRF microscopy enables to discriminate

between true interfilamentous associations and random contacts, the high variability of the process renders quantification of the assembly process unfeasible. Slight changes in temperature, protein integrity or laser intensity, for example, completely alter the efficiency of forming X-shaped structures. Moreover, the previously mentioned high flexibility of the structures in solution makes angle measurements rather ineffective.

Structural differences between Arp2/3 nucleation and LD incorporation

TIRF time-lapse images reveal distinct morphologies for LD and Arp2/3-induced actin assemblies. The Arp2/3 complex is an actin nucleator known to generate distinctive Y-branched networks of actin filaments by producing new (daughter) filaments from the sides of preexisting (mother) filaments (Figure 3, top row; Blanchoin et al., 2000; Amann et al., 2001). Although Smith et al. provide evidence that Arp2/3 binding only rarely leads to branch formation *in vitro* (Smith et al., 2013), the fast growing ends of 'mother' and 'daughter' filaments have the same orientation with an interfilament angle of around 70° (Mullins et al., 1997). Such an arrangement of barbed ends allows a protrusive force to be generated, as observed for the dense, nearly two-dimensional dendritic meshwork of the lamellipodium (Chhabra and Higgs, 2007), for *in vitro* reconstituted *L. monocytogenes*-actin tails (Mogilner, 2006; Loisel et al., 1999), and at sites of clathrin-mediated endocytosis (Collins et al., 2011). The most obvious difference between Arp2/3- and LD-mediated filament junctions is that in the case of the LD-mediated connection, branching rarely yields an end-to-side structure but rather an X-shaped assembly of filaments that extend beyond the contact point (Supplementary videos 1 and 2). Occasionally, one of the filaments involved associates with the other close to its end, resulting in structures that are similar to Arp2/3-induced side branches (Supplementary videos 3 and 4). It is known that depending on the type of nucleation promoting factor (NPF) that activates the Arp2/3 complex, the architecture of the network is largely modified. For example, Helgeson et al. have recently shown by single molecule TIRFM that cortactin, a weak NPF, bundles branching filaments (Helgeson et al., 2014). Considering the LD contacts observed in filament bundles (Silvan et al., 2012), it is noteworthy that in cortactin-mediated bundling filaments exhibited parallel as well as antiparallel orientations. Likewise, the orientation of the filaments making up X-shaped LD-mediated assemblies is fundamental to understanding the possible biological function of LD patterning. Two-color TIRF microscopy time-lapse series revealed the formation of LD-structures with acute and obtuse angles between the barbed ends (Figure 4, supplementary videos 5 and 6). This observation suggests that connected filaments have the same or opposite polarity although the former was predominant.

Models that describe how an LD-like contact mediates branching off a daughter filament with opposite polarity, have been proposed by Reutzel et al. and our own laboratory (Silvan et al., 2012, Reutzel et al., 2004). To better understand the arrangement of filaments in X-shaped LD assemblies, we have modeled two branching structures where filaments extend beyond the contact point. The models account for an antiparallel (Figure 6A) or a parallel filament orientation (Figure 6B). For modeling, we used the T1-actin filament structure reported by Galkin and coworkers where actin protomers are tilted by 15° with respect to the filament axis (Galkin et al., 2015). Moreover, filaments in the T1 state are enriched at early stages of polymerization (Reutzel et al., 2004) when LD accumulation occurs (Millonig et al., 1988), which in turn has been associated with ragged filament morphology and increased filament branching (Schoenenberger et al., 2002; Steinmetz et al., 1997). Galkin et al. showed that when one subunit of an antiparallel actin dimer structure is superimposed on a T1-actin protomer the protruding subunit does not clash with the filament (Galkin et al., 2015). In contrast, superimposing the LD chain A on canonical F-actin filaments resulted in extensive steric clashes that are also evident in our previous model of LD-mediated branching (Silvan et al., 2012). Moreover, the 1-LD model (Figure 6A) illustrates that a second T1-actin can be built around the other LD subunit. In both our models, filaments are shown in the T1 state along the entire filament length although a local tilt of actin protomers would be sufficient to allow the establishment of an LD contact between filaments without significant clashes.

In cells, the LD configuration was detected in the lamellipodium and in the proximity of endolysosomal vesicles by means of an LD-specific antibody (Silvan et al., 2012). The localization of Arp2/3 in similar locations might indicate LD as an actin configuration adopted by actin subunits in Arp2/3-induced branches. However, experiments in which crosslinked LD and Arp2/3 were incorporated to the polymerization mixture did not result in significant differences under the conditions used. It is, however, possible that the LD-contacts represent an actin configuration that precedes Arp2/3 binding and thereby supports Arp2/3 nucleation (Qu et al., 2015).

Actin oxidation as a bundling and branching trigger

The C-terminal region of actin, in particular the cysteine residue at position 374 (Cys374) is highly conserved. In the LD configuration, the Cys374 residues of the two subunits are close enough to establish a disulfide bond. Consistently, a disulfide bond between neighboring actin

molecules was described for a crystallized antiparallel dimer, although it was not considered to be biologically relevant (Lassing et al., 2007). Under oxidizing conditions, SDS-PAGE revealed the formation of a covalently-linked actin dimer with an electrophoretic mobility comparable to that of crosslinked LD, i.e., it ran faster than the filament dimer (Tang et al., 1999). It is conceivable that the 'oxidized dimers' have a configuration similar to that of LD, although in our experiments the LD configuration is stabilized by 1,4-PBM crosslinking and therefore the distance between the subunits is larger than that in the dimers formed by sulfhydryl oxidation (Green et al., 2001). It has been shown that the formation of intermolecular Cys374 disulfide bridges *in vitro* occurs through the random collisional diffusion of actin (Graceffa et al., 2013). Oxidation has been proposed to regulate the activity of a number of cellular proteins (Brandes et al., 2009), including actin (Tang et al., 1999; Hung et al., 2011). Not only the reductive nature of the cytoplasm but also actin-binding proteins (ABPs) that bind and sequester monomeric actin, makes it unlikely that disulfide-stabilized antiparallel dimers are unspecifically formed in cells. However, it was shown that the two actin subunits in complex with gelsolin are in the LD configuration (Hesterkamp et al., 1993). Conceivably, specific ABPs might regulate sulfhydryl oxidation leading to LD formation. For instance, debrin has been shown to regulate the formation of inter- and intrastrand disulphide bridges in actin filaments, resulting in the stabilization of the structures formed (Mikati et al., 2013). Nevertheless, the ABP or ABP complex involved in the stabilization of LD in cells remains unknown.

The importance of a free thiol in Cys374 for actin assembly is not absolutely clear. While modification of Cys374 with tetramethylrhodamine (TMR) is known to impair actin polymerization (Pelikan Cochaudron et al., 2006), covalent coupling of pyrene is commonly used in kinetic assays and believed to have little impact on polymerization (Kouyama et al., 1981). Interestingly, S-(cysteine-374)glutathionyl actin formed filaments, but the resulting networks showed impaired mechanical response to stress and were easily disrupted (Stournaras et al., 1990). The modulation of actin patterning by oxidized actin was observed previously (Tang et al., 1999). In their study, Tang and coworkers described that at steady state, filaments polymerized from oxidized actin yield a significant number of end-to-side-associated actin filament pairs. However, there is no mention of filament crossings. Our real-time observations of polymerization in the presence of oxidized actin revealed the formation of X-shaped filament assemblies similar to those mediated by crosslinked LD. This finding supports the notion that oxidation may be conducive to an LD configuration similar to that observed in the LD-samples (Figure 5A and Supplementary video 2). Interestingly, electron tomography has

demonstrated an abundant existence of overlapping filaments as well as filament pairs in the lamellipodia of different cells (Urban et al., 2010). Since LD contacts were detected at this location (Silvan et al., 2012), we surmise that local oxidation associated with LD formation and X-shaped filament assembly, may represent an additional mechanism involved in patterning the lamellipodial actin network.

Conclusions

Based on the unconventional arrangement of subunits and its transient nature, the biological relevance of the LD has remained obscure for decades. The detection of LD in cells and in filament bundles (Silvan et al., 2012), as well as insights into structural polymorphism of the actin filament (Galkin et al., 2015), manifest that this unconventional actin species cannot be an *in vitro* artifact. TIRFM allowed us to analyze the incorporation of crosslinked LD into polymerizing actin in real time and unveil a role of the LD in the patterning of supramolecular actin structures. Corresponding supramolecular patterning was observed in the presence of oxidized actin, indicating that disulfide bridges might stabilize actin molecules in an LD-like configuration. In the light of these results, we believe LD contacts might contribute to the functionality of the lamellipodium and other actin machineries of the cell.

Author contributions

US, JH and HGM carried out and analyzed TIRFM experiments; PR and SAM performed STEM imaging and analysis; TM, US, CAS were involved in modeling; US, UA and CAS designed the study and discussed data; US and CAS wrote the manuscript with contributions from all authors.

Acknowledgements

We would like to thank the German Society for Cell Biology (DGZ) and the organizers of the Summer School on Actin Dynamics of the year 2011, Prof. Dr. Eugen Kerkhoff (University of Regensburg, Germany) and Prof. Dr. Klemens Rottner (University of Braunschweig, Germany). We as well thank Prof. Roderick Lim (Biozentrum, University of Basel, Switzerland) for using the TIRF microscopy system and Prof. Brigitte M. Jockusch for fruitful discussions. We gratefully acknowledge the experimental support by Ulrich Schröder, computational support by Biozentrum Research IT/Adam Mazur, and Prof. D.L. Barber, Prof. L.L. LeClaire and Dr. A. Schoenichen (University of California, San Francisco, USA) for supplying the VCA plasmid. This work was supported by Swiss National Science Foundation Grant 3100-063956 (to CAS) and the Maurice E. Müller Foundation of Switzerland. The STEM microscopy received additional

funding by Swiss National Foundation Grant 3100A0-108299 to Andreas Engel and by the Swiss systems biology initiative SystemsX.ch grant CINA to Andreas Engel and Henning Stahlberg, both of whom we thank for their interest in this project.

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