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Slow down of actin depolymerization by cross-linking molecules

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ABSTRACT

The ability to control the assembly and disassembly dynamics of actin filaments is an essential property of the cellular cytoskeleton. While many different proteins are known which accelerate the polymerization of monomers into filaments or promote their disintegration, much less is known on mechanisms which guarantee the kinetic stability of the cytoskeletal filaments. Previous studies indicate that cross-linking molecules might fulfill these stabilizing tasks, which in addition facilitates their ability to regulate the organization of cytoskeletal structures *in vivo*. The effect of depolymerization factors on such structures or the mechanism which leads finally to their disintegration remain unknown. Here, we use multiple depolymerization methods in order to directly demonstrate that cross-linking and bundling proteins effectively suppress the actin depolymerization in a concentration dependent manner. Even the actin depolymerizing factor cofilin is not sufficient to facilitate a fast disintegration of highly cross-linked actin networks unless molecular motors are used simultaneously. The drastic modification of actin kinetics by cross-linking molecules can be expected to have wide-ranging implications for our understanding of the cytoskeleton, where cross-linking molecules are omnipresent and essential.

1. Introduction

The requirements on the eukaryotic cytoskeleton are of high complexity and include even conflicting demands: while a dynamic character of cytoskeletal structures is essential for the motility of cells, their ability for morphological reorganizations and cell division, also stability is needed to guarantee the integrity of cells. For the former, the dynamic properties of microtubules (Mitchison and Kirschner, 1984; Margolis and Wilson, 1998; Shaw et al., 2003; Howard and Hyman, 2003) and actin filaments (Stossel, 1993; Welch et al., 1997) are of particular importance. The polymerization and depolymerization properties of actin filaments both *in vivo* (Welch et al., 1997; Watanabe and Mitchison, 2002) and *in vitro* (Pollard, 1986; Wendel and Dancker, 1986; Korn et al., 1987; Kuhn and Pollard, 2005; Fujiwara et al., 2007; Kueh et al., 2008; Li et al., 2009; Kueh and Mitchison, 2009) have been studied extensively.

It is well known, that cells make use of actin binding proteins (ABPs) in order to accelerate the intrinsic dynamics of actin filaments. While nucleation factors like the ARP2/3 complex (Mullins et al., 1998) induce the formation of new filaments, capping proteins and actin depolymerizing factors like cofilin result in a significant increase of depolymerization dynamics (Carlier, 1998; De La Cruz, 2009). Yet, some cross-linking proteins have been reported to have a stabilizing effect on individual filaments (Zigmond et al., 1992; Cano et al., 1992; Loomis et al., 2003; Lebart et al., 2004). This seems to be harnessed by cells for the regulation of bundle size *in vivo*: the lack of espin results in inhibition of stereociliary growth followed by progressive degeneration of the hair bundle (Rzadzinska et al., 2005). Also varying expression levels of fascin in drosophila bristle cells resulted in changes of filament turn-over accounting for distinct phenotypes (Tilney et al., 2003). An overexpression of α-actinin has been shown to cause accumulation of actin filaments and inhibition of actin dynamics (Mukhina et al., 2007). As such a stabilization of filamentous structures could be an important aspect for cells, it is crucial that filaments are protected against depolymerization factors such as cofilin. Yet, it is still necessary to enable a controlled depolymerization of bundles and filaments, which suggests that additional mechanisms are needed to enable a disintegration of these actin structures. Despite the importance of the regulation of actin dynamics for many cytoskeletal processes, quantitative investigations of their mediation by cross-linking molecules remain scarce.

Here we show that cross-linking proteins suppress actin depolymerization in a concentration dependent manner. This is a generic effect for all actin binding proteins which simultaneously bind to two F-actin subunits. Moreover, cross-linking proteins even protect actin filaments from disintegration by cofilin. Molecular motors can overcome the stabilization effect: even extremely stable actin bundle structures can be disintegrated by the concerted action of cofilin and myosin II.

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2. Materials and methods

G-actin is obtained from rabbit skeletal muscle by a modified protocol of Spudich et al. (1971), where an additional gel filtration (Sephacryl S-300 HR) as well as an additional polymerization–depolymerization step is done. The actin is stored in lyophilized form at −21 °C (Spudich et al., 1971). The G-actin solution is prepared by dissolving lyophilized actin in deionized water and dialyzing against G-buffer (2 mM Tris, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.2 mM DTT and 0.005% NaN_3, pH 8) at 4 °C. The G-actin solution is kept at 4 °C and used within 10 days. Polymerization is initiated by adding 10% volume 10× F-buffer (20 mM Tris, 5 mM ATP, 20 mM $MgCl_2$, 2 mM $CaCl_2$, 1 M KCl, 2 mM DTT, pH 7.5). In the case of cross-linked networks, actin is polymerized in the presence of the cross-linking molecules. For actin/rigor-HMM networks, F-buffer without ATP is used. For the actin/filamin networks polymerized in the presence of myosin II a different 10× polymerization buffer (100 mM Imidazol, 30 mM $MgCl_2$, 2 mM $CaCl_2$, 0.05% NaN_3, pH 7.2) is used. For fluorescence microscopy, actin is labelled with the amine-reactive dye Alexa Fluor 555 carboxylic acid succinimidyl ester (Invitrogen A20009). For this purpose, G-actin is dialyzed against Borat-buffer (50 mM boric acid, 0.2 mM $CaCl_2$, 0.2 mM ATP, pH 8). After polymerization induced by addition of 10% of polymerization buffer (100 mM Imidazol, 10 mM ATP, 30 mM $MgCl_2$, 2 mM $CaCl_2$, 0.05% NaN_3, pH 7.2) the dye (dissolved in DMSO) is added in 1–2-fold molar excess. After centrifugation the pellet is resuspended in G-buffer and dialyzed against G-buffer. The sample is clarified by centrifugation and the supernatant is stored in lyophilized form at −21 °C. A degree of labelling ≈20% is achieved and used for all microscopy experiments. In the case of the LatB and cofilin depolymerization assays applied to filamentous networks a 1% fraction of labelled reporter filaments is mixed with unlabelled F-actin after full polymerization. This is necessary as at a concentration of 3 μM labelled actin, individual actin filaments are hardly detectable. For pyrene assay experiments, actin is labelled with pyrene by a modification of the method of Kouyama and Mihashi (1981) as described in Cooper et al. (1983). Muscle filamin is isolated from chicken gizzard and further purified as reported in Shizuta et al. (1976). Recombinant human fascin is prepared by a modification of the method of Ono et al. (1997) as described by Vignjevic et al. (2003). HMM is prepared from myosin II by chymotrypsin digestion and tested using motility assays as in Uhde et al. (2004). Tropomyosin troponin is prepared from the residue of rabbit muscle acetone powder left after the actin extraction (Spudich et al., 1971) and separated into tropomyosin and troponin by hydroxyl apatite column chromatography (Eisenberg and Kielley, 1974). *Dictyostelium discoideum* cofilin is expressed as a GST fusion protein in *Escherichia coli* DH5alpha cells. The GST tag is removed by cleavage with the factor Xa and cofilin is stored in 10 mM Tris, 0.2 mM $CaCl_2$, 0.2 mM DTT, pH 8. Latrunculin B (Sigma–Aldrich) dissolved in DMSO (5 mM) is stored at −20 °C. Prior to use, it is diluted to 500 μM in G-buffer without ATP.

A total actin concentration $c_a = 3$ μM and a degree of labelling ≈11% is used for the pyrene actin assays as described in Cooper et al. (1983) using a spectrofluorometer FP-6500 (JASCO). Upon full polymerization, 50 μM LatB is added and thoroughly mixed using a pipette. Note that this assay is not applicable for actin/filamin networks, where an addition of LatB by pipetting is not possible without destroying the network. A normalized fluorescence intensity $I_n = \frac{I-I_0}{I_1-I_0}$ is calculated. Here I_0 is the fluorescence intensity prior to polymerization and I_1 is the fluorescence intensity directly after addition of LatB.

Diffusion chambers (Fig. 2) are used for addition of LatB, cofilin or ATP to actin networks without mechanical disruption: the actin network is fully polymerized between a cover slip and a 0.1 μm pore size Durapore PVDF filter membrane (Sigma–Aldrich). Upon polymerization, LatB, cofilin or ATP is added via the membrane. This is essential for actin/filamin networks, where due to the non-equilibrium network character (Schmoller et al., 2008) a mechanical disruption results in an irreversible change of the network architecture. Therefore, most depolymerization assays are not applicable to these networks as they would require pipetting.

Fluorescence microscopy data are acquired on a Zeiss Axiovert 200 inverted microscope with a 100× oil immersion objective with a numerical aperture of 1.4. For actin/filamin bundle networks a 25× oil immersion objective with a numerical aperture of 0.8 is used. Time lap videos were taken at each time point in order to allow for a distinction between filaments and bundles.

For quantitative image analysis a gaussian blur is applied, background subtraction is done with ImageJ and a threshold which is adjusted for each time point is used to create a binary image. For filamentous networks the number of pixels which are part of filaments is counted and averaged for minimal 30 images. For bundle networks the fluorescence intensity of the bundle structures which are determined by the binarization is measured to account for bundle thickness. Again, it is averaged over at least 30 images. The time courses of depolymerization are normalized on the initial values.

3. Results

3.1. A pyrene assay reveals that fascin and HMM slow down LatB induced depolymerization of actin

Actin depolymerization can be induced by addition of the depolymerization factor latrunculin B (LatB), which sequesters monomeric actin and thus causes a depolymerization at both the barbed and pointed ends. A standard pyrene assay, where the normalized fluorescence intensity $I(t)$ is a measure for the degree of polymerization, allows monitoring of actin depolymerization induced by LatB and is a well established method to study actin depolymerization kinetics (Cooper et al., 1983). In the case of pure actin solutions, the depolymerization induced by addition of 50 μM LatB (Fig. 1A) has been suggested to follow a double exponential decay (Wendel and Dancker, 1986; Kueh et al., 2008). Interestingly, the depolymerization kinetics are drastically modified in the presence of the actin binding protein fascin, which bundles actin into individual polar bundles (Courson and Rock, 2010). As depicted in Fig. 1B, fascin is slowing down the depolymerization process in a concentration dependent manner: while at a molar ratio between fascin and actin $R_{fas} = c_{fas}/c_a = 0.01$ no effect is resolvable, the time $t_{\frac{1}{2}}$ at which the normalized fluorescence is halved, $I(t_{\frac{1}{2}}) = \frac{1}{2}I(0)$, increases about a factor of 10 for $R_{fas} = 1$. In order to test whether bundling accounts for this stabilization, the effect of the ideal cross-linker heavy meromyosin (HMM) in the rigor state is investigated. Rigor-HMM can form cross-links between filamentous actin and can also decorate individual filaments (Tharmann et al., 2007). As shown in Fig. 1A, rigor-HMM completely inhibits actin depolymerization at $R_{HMM} = 1$, even though it does not cause any bundling at all (Tharmann et al., 2007).

3.2. Using a diffusion chamber it is shown that various actin binding proteins slow down LatB induced depolymerization

A pyrene assay cannot be used to study the effect of all ABPs as it is sensitive to quenching effects and pipetting the polymerized solution is mandatory. This is a problem for networks which – such as actin/filamin bundle networks – are kinetically trapped and show syneresis effects (Schmoller et al., 2008). Using a diffusion chamber (Fig. 2) allows to add LatB to an actin network without disrupting the network mechanically. Fluorescence microscopy experiments confirm the effect of 50 μM LatB on the network architecture: while pure