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ORIGINAL PAPER

The regulatory action of α-actinin on actin filaments is enhanced by cofilin

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Abstract We have used fluorescence recovery after photobleaching to study the effect of muscle α-actinin on the structure of actin filaments in dilute solutions. Unexpectedly we found that α-actinin partitioned filaments into two types: those with a high mobility and those with low mobility. We have determined that the high mobility (smaller sized) population is too large to be simple monomeric actin:α-actinin complexes. Although it is known that cofilin encourages the transformation of α-actinin:actin gels into large meshworks of inter-digitating actin filament bundles (Maciver et al. 1991), we have found that the presence of cofilin also increases the cross-linking of actin filaments by α-actinin and hypothesize that this is due to cofilin's ability to alter the filament twist. This effectively makes more potential α-actinin binding sites per unit of actin filament. As expected from previous work, this effect was more marked at pH 6.5 than at pH 8.0. Both effects are likely to operate in cells to deny other actin-binding proteins access to binding these particular filaments and may explain how very different actin cytoskeletal structures may co-exist in the same cell at the same time.

Keywords Actin · Cofilin · α-actinin · FRAP · Cytoskeleton

Introduction

The actin cytoskeleton is responsible for a wide range of motile events in the cell, many of the cell's structural properties, and locomotion of the cell. The actin cytoskeleton forms a variety of physical structures to accomplish these tasks and sometimes forms very different structures in the same cell, at the same time. These actin-rich structures are formed under the direction of about 100 different types of actin-binding proteins (dos Remedios et al. 2003). Much is known about how these individual proteins bind actin and modify the behaviour of actin filaments, but far less is known about how these different actin-binding proteins behave as they act together on actin. The few studies that have been undertaken in this direction have often produced unexpected and counterintuitive results (Esue et al. 2009; Maciver et al. 1991).

Two of the most abundant and widely expressed actin-binding proteins in typical eukaryotic cells are the cofilins and the α-actinins. The cofilin family severs and depolymerises actin filaments (Chen et al. 2004). This role is usually combined with the action of other cytoskeletal proteins (e.g., profilin), working coordinately in dynamic cell processes. α-Actinin and its multiple isoforms crosslink the actin filaments into isotropic gels, crosslink them into bundles or crosslink them so that they produce a three dimensional network of interconnected bundles in the presence of cofilin (Maciver et al. 1991; Wachsstock et al. 1993). Many studies have been carried out on the physical actions of this family of proteins, and the molecular mechanism of its role in maintaining nets of filaments has

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been very well described, including details in the anchoring of the actin-interacting domain of α-actinin to the actin filaments (Sjöblom et al. 2008). In the present article we explore the possible mutual dependence of the action of cofilin and α-actinin on actin filaments. For this purpose, we reconstruct actin cytoskeletons from isolated proteins in order to study their properties through their hydrodynamic properties. Fluorescence techniques are used and combined in this study, in particular fluorescence recovery after photobleaching (FRAP). This technique is especially suited for this purpose since it allows the simultaneous observation of diverse populations of species with different motilities, as well as their relative changes in controlled conditions (Bonet et al. 2000). One difficulty associated with this study lies in the high affinity of the interaction of α-actinin with actin filaments, making the polymerisation process of actin a very fast and therefore intractable process. For this reason most polymerisation experiments are carried out under "slow" polymerisation conditions, that is, polymerisation is initiated at relatively low salt concentrations ($[Mg^{2+}] < 1$ mM). Under these conditions it has been possible to show that cofilin, in spite of its severing/depolarising effect on actin filaments, enhances the compacting role of α-actinin on actin filaments. A possible model is proposed to explain this apparent paradox. Our findings may explain a number of observations made by others on the nature of the actin cytoskeleton in cells, but these studies have tended to use static techniques such as electron microscopy and fluorescence microscopy. Here we use photobleach techniques to explore the dynamics of actin in the presence of actin binding proteins in unfixed samples.

Materials and methods

Samples

G-actin from rabbit muscle acetone powder was obtained and purified by the method of Pardee and Spudich (1982) and stored at 0°C as F-actin pellets. Fluorescent derivative of actin, designated as IAF-actin, was obtained by labelling actin with the photobleachable molecule 5-iodoactamidofluorescein (5-IAF) using the method of Wang and Taylor (1980) (labelling molecules were from Molecular Probes, Leiden, the Netherlands). In preparation for the experiments, actin was dialysed against either buffer G (2 mM Tris/HCl, 0.2 mM $CaCl_2$, 0.2 mM ATP and 0.5 mM 2-mercaptoethanol) at pH 8.0, or buffer G′ (2 mM Mes, 0.2 mM $CaCl_2$, 0.2 mM ATP and 0.5 mM 2-mercaptoethanol) at pH 6.5. α-Actinin from rabbit muscle was purchased from Cytoskeleton and used without further purification. α-Actinin was used by directly dissolving the lyophilised powder in the appropriate buffer. Both actin and α-actinin were used in buffer G (pH8.0) or buffer G′ (pH 6.5). Complexes were formed by direct mixing of different amounts of α-actinin (0–20 μM) with G-actin, keeping the actin concentration constant at 20 μM. Since α-actinin undergoes conformational changes as a function of ionic strength (Kuroda et al. 1994), Mg^{2+} concentrations were kept in the range between 0.65 and 1.0 mM. These values allow the time course of polymerisation to be within the temporal resolution of the FRAP apparatus. It is known (Travé et al. 1995) that muscle isoforms of α-actinin are not calcium-dependent, so the use of buffers containing Ca^{2+} ions will not influence the results.

The isotropic filament nets show mechanical properties similar to elastic solids (Nossal 1988). When the filaments align, their behaviour changes to that of a less rigid viscous fluid because the filament bundles behave as long rigid cylinders, sliding among them (Jockusch and Isenberg 1981). The tendency of α-actinin to organise filament bundles depends on filament length (Maciver et al. 1991). In the present study we have chosen protein and salt concentrations such as to allow α-actinin to organise filaments in parallel bundles (Wachsstock et al. 1993) and nets of such bundles (Tseng and Wirtz 2001).

Human non-muscle cofilin was produced in *Escherichia coli* [BL21 (DE3)] and purified by a method similar to that used for human ADF (Hawkins et al. 1993). Briefly, cofilin was purified from the bacterial lysate by passage through a DE52 column at pH 8.0 and eluting the protein from a CM-11 column with KCl. The protein was pure as judged by SDS/PAGE. The concentration of actin and cofilin was determined spectrophotometrically: $A_{280} = 1.0\ cm^{-1}$ is equivalent to 74 μM cofilin and $A_{290} = 1.0\ cm^{-1}$ is equivalent to 38 μM actin. Both actin and cofilin were used in buffer G (pH 8.0) or buffer G′ (pH 6.5). Complexes of actin with cofilin and/or α-actinin were formed by direct mixing of different amounts of either cofilin or α-actinin with G-actin, keeping the actin concentration constant at 10 μM. "Slow" polymerisation of the complexes was achieved by adding $MgCl_2$ (0.65–0.8 mM). All measurements were performed at steady-state, at least 1 h after the addition of the salt to ensure full polymerisation.

FRAP

The FRAP apparatus used in our experiments is similar to that described elsewhere, with a few modifications (see Mozo-Villarias and Ware 1984, 1985 and references therein). In short, a laser light beam from an Ar^+ laser ($\lambda_{em} = 488$ nm) attenuated 10^3 times by a neutral density filter (Edmund Scientific, Barrington, NJ, USA) is shone into a Zeiss Axiovert inverted fluorescence microscope and used as the monitor beam to record fluorescence recovery

from the samples. During photobleach the neutral density filter (mounted on a step motor) is removed for a short period of time (0.3 to 1 s) during which the incident beam bleaches the sample, which is confined in a cuvette (VitroCom, NJ, USA) with dimensions 20 × 2 × 0.2 mm.

The sample is illuminated through the striped pattern of a Ronchi Ruling (Edmund Scientific), leaving a pattern of alternating bleached and non-bleached stripes. Immediately after this flash, recording of the fluorescence recovery in the photobleached stripes proceeds, as the pattern on the sample is moved transversally and uniformly relative to that of the Ronchi Ruling. This movement generates an exponentially decaying oscillating photocurrent in the photomultiplier from the variable contrast generated between the two patterns. The decay time depends on the spacing of the Ronchi Ruling as observed at a given magnification of the microscope (wave vector K) and the diffusion coefficient, D, of the samples under observation according to the expression: $F(t) = F(0) \times \exp(-K^2Dt)$, where $F(0)$ and $F(t)$ are the fluorescence intensities monitored at time 0 and time t after the flash respectively. By changing the Ronchi Ruling spacing and the microscope objective, one can change the K value, which allows the observation of diffusion coefficients of very different orders of magnitude. Our apparatus allows the detection of diffusion coefficients ranging from 10^{-6} to 10^{-11} cm^2/s. In a normal experiment, using a low value of K (Ronchi Ruling of 50 lines cm^{-1}, ×10 objective, K in the range 300–400 cm^{-1}), we measured the diffusion coefficient of high-mobility species, D_{HM} (G-actin or small oligomers, on the order of 10^{-7}–10^{-8} cm^2/s), and the fraction of low-mobility samples present in the solution, f_{LM} (of the total actin present). Using high values of K (Ronchi Ruling of up to 200 lines cm^{-1}, ×40 objective), in the range of 3,000–4,000 cm^{-1}, we measured D_{LM}, the average diffusion coefficient of the low-mobility species present in the sample ranging from 10^{-9} to 10^{-11} cm^2/s.

In all FRAP experiments, labelled actin made up 1% of the total actin (<0.2 μM) before complexes were made with cofilin and/or α-actinin and the addition of salts to initiate polymerisation. All measurements were performed at 20°C. For every single experimental session involving polymerisation of actin, measurements of the diffusion coefficient of monomeric actin were systematically carried out, prior to any actin polymerisation. Using the adequate "window" (combination of Ronchi Ruling spacing and objective) as described above, the diffusion coefficient of G-actin was measured in order to prevent the presence of filaments or small oligomers, which would be indicative of defective depolymerisation of actin prior to any polymerisation experiment. G-actin diffusion coefficient determinations yielded expected values (see Fig. 1). The process of fitting FRAP data of G-actin samples to two exponentials should converge to values with non-significant differences among them.

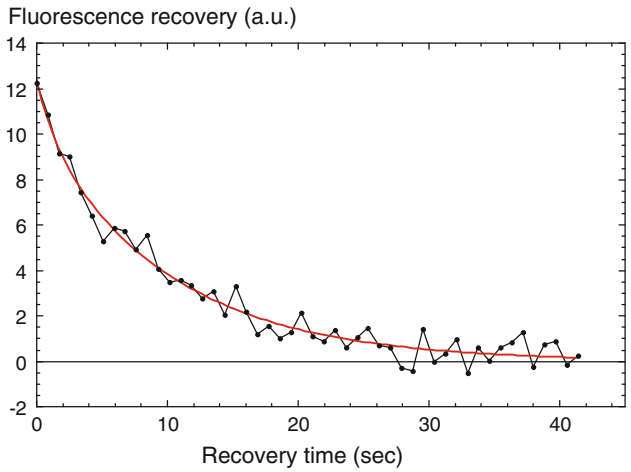

Fig. 1 Fluorescence recovery of monomeric actin. *Red line* represents fitting to a single exponential corresponding to a diffusion coefficient of 7.34 × 10^{-7} cm^2/s

Fluorescence microscopy

Aliquots of samples prepared for FRAP experiments were mounted for simultaneous observation in a Nikon E-600 Eclipse fluorescence microscope. Most pictures of the samples were taken using ×20 and ×40 objectives. Individual actin filaments at these magnifications are not observable directly but as a green dim fluorescent background. Only aggregated structures (see "Results and discussion" section), obtained by the interaction of actin and α-actinin, were observable and recorded.

Results and discussion

The cross-linking effect of α-actinin on actin filaments

Fluorescence recovery was used to determine the diffusion coefficient of monomeric actin (Fig. 1) and this value (7.34 × 10^{-7} cm^2 s^{-1}) was in good agreement with that measured previously (Bonet et al. 2000; Mozo-Villarias and Ware 1984). In order to investigate the cross-linking effect of α-actinin on actin filaments in terms of their hydrodynamic properties, 10 μM actin was polymerised in the presence of α-actinin with concentrations ranging from 0 to 10 μM by adjusting the ionic strength to 0.65 mM Mg^{2+}. An example of the kinetics of this process is shown in Fig. 2, in which the fluorescence recovery at the end of the polymerisation process is plotted against the recovery time. It can be seen that by increasing the concentration of α-actinin present in the sample, the high

mobility fraction increases (Fig. 2). The results of fitting these recovery curves to two exponentials, one of high mobility (HM) and the other of low mobility (LM), are shown in Fig. 3 and Table 1. Errors associated with values of f_{HM}, D_{HM}, f_{LM} and D_{LM} obtained in these fittings are presented in the Electronic Supplementary Material. Figure 3 shows the increase in the high mobility fraction (f_{HM}) as the content of α-actinin increases at the end of the polymerisation process. Table 1 shows these values plotted in Fig. 3. It can be seen that the high mobility fraction increases as the α-actinin:actin molar ratio increases. It should be noted that the appearance of two clear populations in mobility does not necessarily mean that there are two populations of filament sizes, but rather a division between a high mobility component, relatively free to diffuse, and a component of cross-linked filaments of relatively very slow mobility. Fluorescence images (Fig. 4) of these species show these cross-linked aggregates. Single filaments cannot be seen at these magnifications.

A theoretical model of length distribution of actin filaments in these experimental conditions can be made with an exponential distribution of sizes (Edelstein-Keshet and Ermentrout 1998). This theoretical model has been tested in the presence and absence of proteins that sever or cap the actin filament (Littlefield and Fowler 1998; Piekenbrock and Sackmann 1992; Xu et al. 2000). The critical concentration of monomers is about 1 μM, and their diffusion coefficient is on the order of $\approx 7 \times 10^{-7}$ cm^2/s. A simple estimation shows that the average separation of monomers is on the order of 200–500 μm and that the corresponding average diffusion time is about 30–50 μs. On the other hand, the typical time for association/dissociation of monomers in polymers lies in the 0.1–1 s range. These differences are an order of magnitude, making the process of monomer interchange a much slower process than their diffusion. This in turn means that polymerisation is a spatially homogeneous process. Under these conditions the process can be considered as a "zero order process". Such processes yield exponential length distributions (Evans 2000) that yield fluorescence recovery curves such as that shown in blue in Fig. 2. α-Actinin stabilises the actin polymer, at an interaction energy on the order of (2 ± 1) kT (Miyata et al. 1996), by linking two consecutive monomers (McGough et al. 1994). As a consequence, monomer interchange is no longer a zero-order process and the size distribution is not exponential. A situation like this gives rise to fluorescence recovery curves such as the one depicted in red in Fig. 2. These observations agree with computer simulations carried out by Biron and Moses (2004). This deviation from the "standard" behaviour of

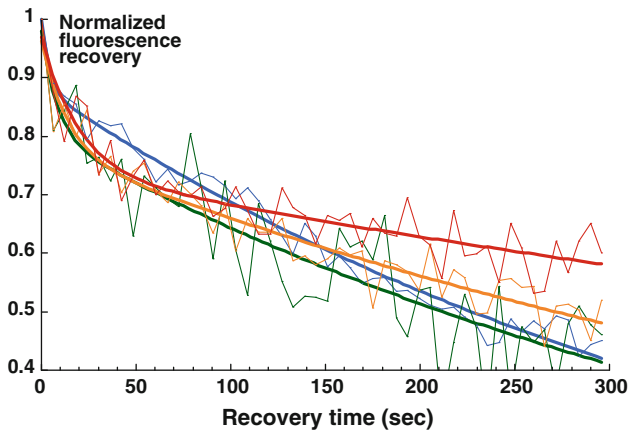

Fig. 2 Fluorescence recovery curves (normalised to their initial values) taken at the end of the polymerisation process (about 1 h after adding Mg^{2+} up to 0.65 mM). [actin] = 10 μM; [α-actinin] = 0 μM (*blue line*), 2.5 μM (*green*), 7.5 μM (*yellow*), 10 μM (*red*). *Smoothed lines* are the result of fitting these curves to two exponentials (see text). Note that α-actinin causes a decrease in diffusion of some filaments but an increase in others

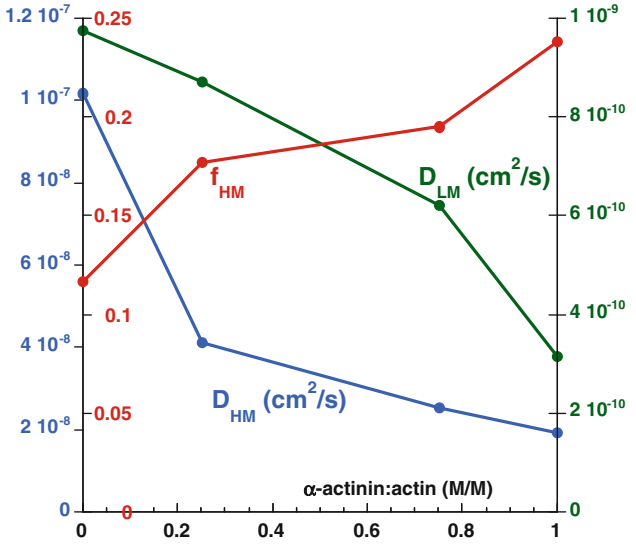

Fig. 3 *Red line* represent the increase in high mobility fraction f_{HM} of α-actinin:actin complexes formed as α-actinin is increased. The average diffusion coefficients of the high mobility fraction, D_{HM} (*blue*), and low mobility fraction, D_{LM} (*green*), are also shown. The abscissa shows the molar ratio of α-actinin over actin

Table 1 The high mobility fraction (f_{HM}) as a function of the α-actinin:actin molar ratio

α-actinin:actin	f_{HM}	D_{HM} (cm^2/s)	D_{LM} (cm^2/s)
0.0	0.12	1.01×10^{-7}	9.76×10^{-10}
0.25	0.18	4.11×10^{-8}	8.70×10^{-10}
0.75	0.195	2.52×10^{-8}	6.21×10^{-10}
1.0	0.24	1.93×10^{-8}	3.15×10^{-10}

The f_{HM} increases with the ratio but the diffusion constants are too large to be monomeric actin in a simple complex with α-actinin (see ESM). Experimental details same as for Fig. 3 and ESM

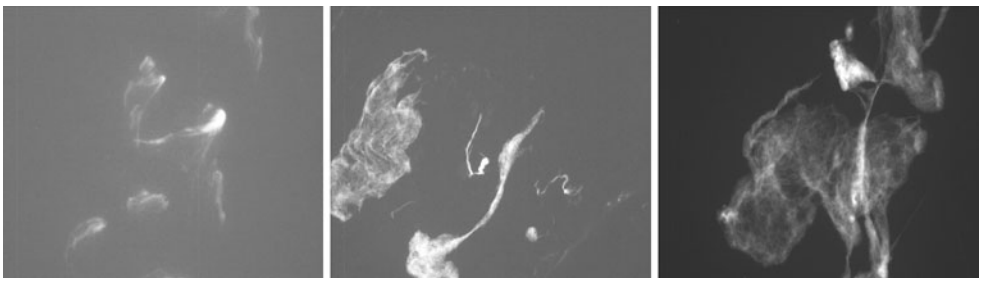

Fig. 4 F-actin structures cross-linked with different amounts of α-actinin. From *left to right* α-actinin:actin = 0.6, 0.8, 1.0, obtained by fluorescence microscopy, using a ×20 objective. These pictures reveal the formation of big agglomerates due to the presence of α-actinin. These structures are formed by aggregates of increased size and density as the dose of α-actinin increases. These structures are shown over a continuous background where monomers and individual filaments are present (obviously invisible at these magnifications)

the polymers when decorated with α-actinin can explain this "separation" of populations. Theoretical models use Brownian movement as translation and rotation simulation of filaments (Doi and Edwards 1986). In order to facilitate cross-linking, two filaments must orient in parallel directions (Yu and Carlsson 2004). When this is accomplished by rotational diffusion, the filament is subjected to a shear stress and this mechanism may filtrate filament length before its alignment.

Regulatory effect of α-actinin on F-actin

Polymerisation of actin with $Mg^{2+} \geq 1$ mM is a fast process and relatively difficult to follow by FRAP techniques (at our current actin concentrations), when time resolution is required. Figure 4 shows that the effect of cross-linking is not evident when both ionic strength and α-actinin concentration are reduced. Figure 5 shows the evolution of the average diffusion coefficient of polymerised actin. It is observed that the time evolution of the mobility of F-actin (as polymerisation progresses) is practically coincident with that of the actin:α-actinin complex. It should be clear then that the cross-linking effect may be driven by three different factors:

(1) Cross-linking depends intrinsically on ionic strength. (2) The action of α-actinin with actin is a cooperative effect, above a critical concentration. (3) Low filament population is due to low ionic strength. The first hypothesis is certainly plausible, since the interaction of actin and α-actinin takes place in a very hydrophobic domain of α-actinin (residues 120–134) (Hemmings et al. 1992; Borrego-Diaz et al. 2006). Increasing ionic strength should favour driving hydrophobic domains together. However, it does not agree with the fact that in co-sedimentation experiments, the independence of cross-linking effect and salt concentration has been proven (Hemmings et al. 1992; Kuhlman et al. 1992). On the other hand, the modulation effect of the salt is that of a change in the length of the central dominion of α-actinin (Kuroda et al. 1994), leaving unaltered those domains responsible for the interaction with F-actin. Figure 3 shows that the dependence of the low mobility fraction on α-actinin concentration is not sigmoidal, rejecting the possibility that the action of α-actinin with actin is a cooperative effect, despite the fact that at ratios of actin:α-actinin below 0.08, this behaviour is present (Meyer and Aebi 1990). α-Actinin interacts with F-actin by contacting adjacent actin monomers (McGough et al. 1994), thus preventing the interaction with monomeric actin. Moreover, working in conditions in which $[Mg^{2+}] > 0.1$ mM should assure the interaction of actin and α-actinin (Meyer and Aebi 1990). In addition, low ionic strength ends up with a low polymer population, as well as a shortening of the filaments (Table 2). With these facts in mind, the graphs in Fig. 5 most likely show that the lack of effect is mostly due to the absence of the adequate polymers for α-actinin to bind. Figure 6 shows how, by maintaining the relative proportions of both proteins and slightly increasing the ionic

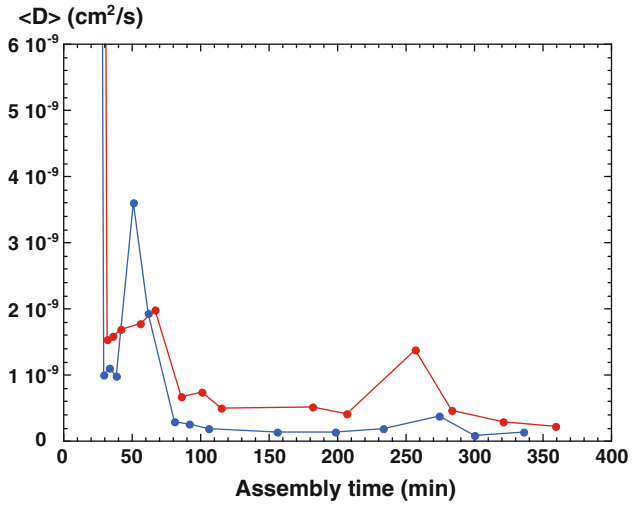

Fig. 5 Time evolution of the average diffusion coefficient of actin (*blue*) or α-actinin:actin (*red*) samples, polymerised with $[Mg^{2+}] = 0.6$ mM. No actin filament cross-linking is evident under these conditions

Table 2 Ionic strength influence on the steady state parameters of an actin solution

$[Mg^{2+}]$ (mM)	D (cm^2/s)	f_{LM}	Actin (μM)	Time (min)
0.5	5.52×10^{-10}	0.48	20	>120
0.7	4.35×10^{-10}	0.66	20	>120
0.8	3.78×10^{-10}	0.73	20	>120

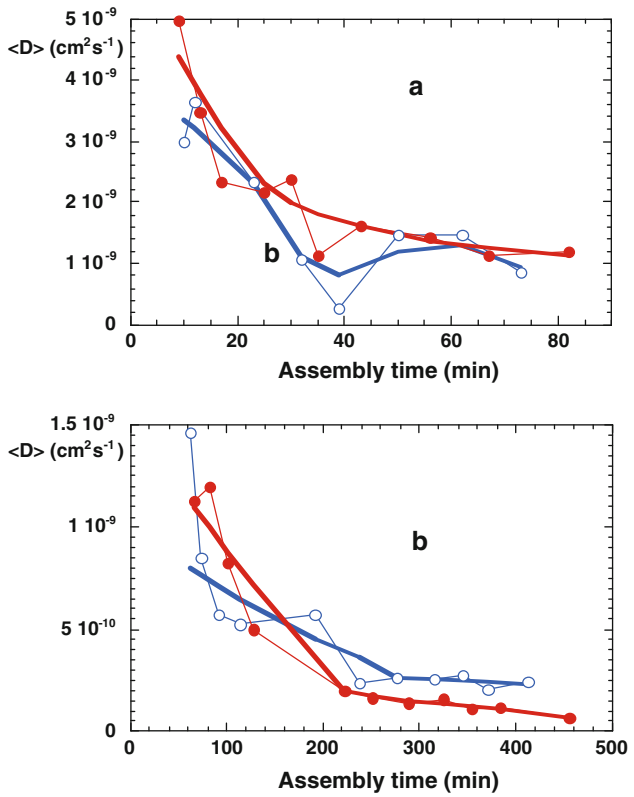

Fig. 6 **a** Time evolution of the average diffusion coefficient actin (*blue*) or α-actinin:actin (*red*) samples, polymerised with $[Mg^{2+}] = 0.75$ mM. *Smoothed lines* represent a weighted fit of the experimental points. Crossing of the curves begins to be seen. **b** Same as **a** with an expanded time scale

strength, the cross-linking proceeds during a period (30–60 min) in which full polymerisation of the actin should have taken place in high ionic strength conditions. Here, full polymerisation is reached in about 3 h.

As far as is known, only the polyphosphoinositol lipids are capable of regulating the affinity of muscle α-actinin for actin filaments (Fraley et al. 2003). Our results show that ionic strength, by controlling polymer population, is able to control the interaction with α-actinin. Consequently, ionic strength, by modulating the speed of filament formation, also regulates the extension of the cross-linking leading to a degree of more or less filament compactness (Wachsstock et al. 1993). It was observed that after 1 h, the mobility of the samples stays practically constant and the steady state is

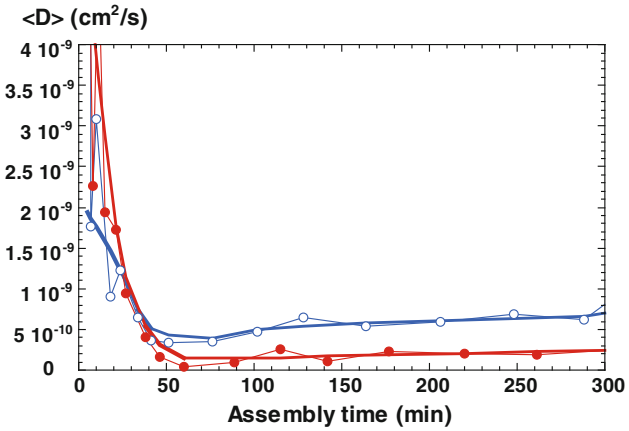

Fig. 7 Time evolution of polymer mobility under quasi-physiological conditions ($[Mg^{2+}] = 0.85$ mM). *Blue* Actin, *red* α-actinin:actin

reached. As long as there is ATP present in the solution, filaments will maintain this steady-state in a quasi-indefinite way, similarly to the case of polyelectrolytes, as has been theoretically predicted. Theoretical models for polyelectrolytes (Oosawa and Asakura 1975; Gennes 1991) applied in similar conditions as those set in our experiments, yield stabilisation times on the order of 80 h (Fig. 7).

Within this experimental context, the rate of monomer exchange in the filaments (treadmilling) is quite slow (Coluccio and Tilney 1983). Also, filament accumulation (Fig. 8) provides closed compartments reducing the diffusion of monomers and filaments (Luby-Phelps et al. 1987; Mastro et al. 1984), and those filaments "fixed" in bundles by the action of α-actinin have much less possibility of interchange with filaments that are still free (Wachsstock et al. 1993). The main implication is that once the environmental conditions are set, the final state remains invariable with time, with no significant direct consumption of energy. As a consequence, the active regulation of the cytoskeleton will be established by the direct action on the factors able to take the system to another point of operation. In this sense, the cytoskeleton acts as a dissipative system, able to transit between two metastable states (Nicolis and Prigogine 1981).

Another interesting aspect lies in the "temporality" of α-actinin and actin filaments, as revealed in Figs. 5 and 6. Initially, actin:α-actinin complexes show higher mobility than free actin filaments. At a given moment, both curves cross each other and actin:α-actinin complexes become slower. This intrinsic property of the interaction between actin and α-actinin depends on the speed of filament formation, regulated by the ionic strength as discussed above.

Since α-actinin interacts with filaments and not with monomers (McGough et al. 1994), the initially formed filaments will occasionally incorporate α-actinin molecules reversibly. It is known that α-actinin makes the average

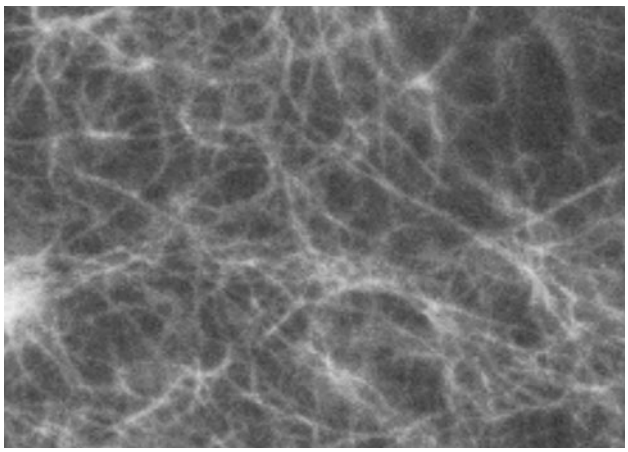

Fig. 8 Actin filament cross-linked as bundles by α-actinin, observed under the fluorescence microscope (magnification ×40, see the "Materials and methods" section). α-Actinin present at 5 μM, actin at 10 μM

length of F-actin uniform (Biron and Moses 2004) and also that α-actinin stabilises the filaments providing extra energy, 2kT, to the polymer (Miyata et al. 1996). The flexural rigidity of actin filaments is defined (Gittes et al. 1993) as:

$$U = \frac{1}{2}\mathrm{EI}\int\left(\frac{d\Theta}{ds} - \frac{d\Theta^0}{ds}\right)^2 ds$$

where E is the Young modulus, which is a function of the binding energy of the monomers conforming the filament and $\frac{d\Theta}{ds}$ is the curvature. The filament, thus decorated with α-actinin, will show a higher local rigidity and its persistent length $L_\mathrm{p} = \frac{\mathrm{EI}}{k_\mathrm{B}T}$ will also be higher. Since cross-linking is highly dependent on filament alignment (Yu and Carlsson 2004), it will take place as soon as two filament segments encounter each other in parallel orientations and with a sufficient number of α-actinin molecules bound. Due to geometrical constraints, the paired filament-filament presents a higher moment of inertia that that of a single filament and thus a higher flexural rigidity. This fact can facilitate the interaction with a third filament sufficiently decorated with α-actinin and thus repeat the circle of filament linking. In summary, polymers, initially without α-actinin, start growing faster since the interaction of α-actinin with the filament is a second-order process and it takes place when the polymer is already formed. Later in the process, cross-linking generates large accumulations of filaments, which give rise to the very low mobility as compared to actin filaments that are not cross-linked.

Summarising these experiments, we can reach the following conclusions:

1. α-Actinin separates filaments into two distinct populations of sizes. (The smaller sized population is too large to be monomeric actin:α-actinin complexes).
2. α-Actinin restricts the growth of actin filaments as it interacts with filaments with a given minimum length.
3. α-Actinin displays a selective action on the filament population: it acts to homogenise the size of those filaments that remain free of cross-linking.
4. α-Actinin, at the beginning of the polymerisation process, promotes the formation of a relatively high mobility fraction.

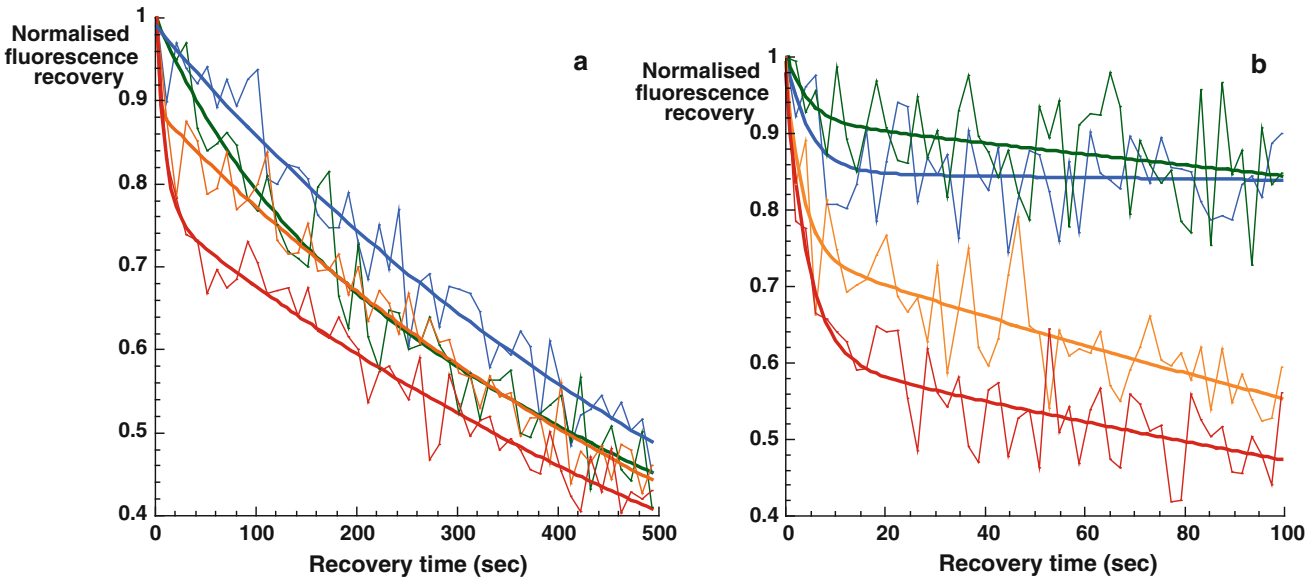

Fig. 9a, b Polymerisation of actin complexes. *A* Actin alone (*blue*), *B* cofilin:actin complex (*green*), *C* α-actinin:actin complex (*yellow*), and *D* cofilin:α-actinin:actin complex (*red*), in a window in which fast and slow components can be observed at 45 min after addition of 0.8 mM Mg^{2+}. **a** pH 8.0, **b** pH 6.5

Table 3 Diffusion coefficients of the high and low mobilities of the four actin complexes and their respective proportions, at both pH 8.0 and pH 6.5

Sample[a]	pH 8.0				pH 6.5			
	f_{HM}	D_{HM}	f_{LM}	D_{LM}	f_{HM}	D_{HM}	f_{LM}	D_{LM}
A	0.0	–	1.0	9.77×10^{-10}	0.16	8.40×10^{-8}	0.84	4.02×10^{-11}
B	0.17	7.17×10^{-9}	0.83	8.41×10^{-10}	0.09	9.40×10^{-9}	0.91	3.22×10^{-10}
C	0.11	3.47×10^{-7}	0.89	9.66×10^{-10}	0.74	1.20×10^{-7}	0.26	1.16×10^{-9}
D	0.23	7.88×10^{-8}	0.77	8.75×10^{-10}	0.60	9.00×10^{-8}	0.40	9.80×10^{-10}

[a] *A* Actin alone, *B* cofilin:actin, *C* α-actinin:actin, and *D* cofilin:α-actinin:actin

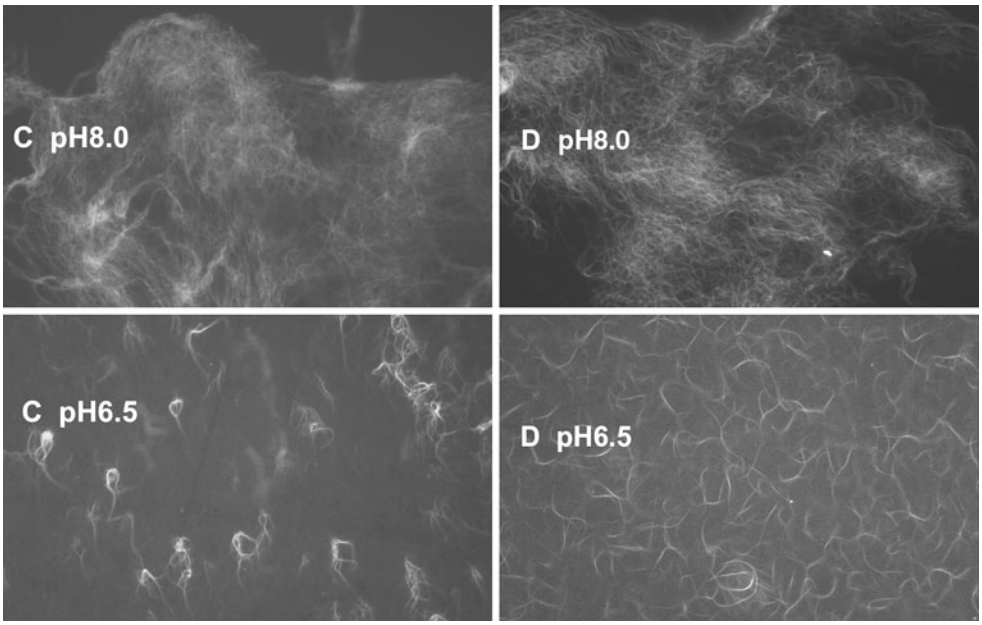

Fig. 10 Fluorescence images of samples C (α-actinin:actin complex) and D (cofilin:α-actinin:actin complex) at both pH 8.0 and pH 6.5. All images were taken with ×40 objective. As can be seen, at pH 6.5, all species are, in general, less extensive than at pH 8.0, and sample D seems to be more compact than sample C

5. α-Actinin organises actin filaments in superstructures in a dose-dependent manner.

Enhancement of cross-linking capacity: interaction with cofilin

In this section, we discuss the mutual influence between a cross-linking and bundling protein, such as α-actinin, and proteins that accelerate cytoskeleton dynamics, such as cofilin, by depolymerising (Carlier et al. 1997; Maciver 1998) and/or severing filaments, increasing the mobility of the actin species present (Bonet et al. 2000) or transforming the latticework of actin filaments cross-linked by α-actinin into bundles (Aizawa et al. 1996). In a previous article (Bonet et al. 2000), we showed that as cofilin is increased in a polymerising actin solution, the mobility of the filaments increases, with this effect being more apparent at pH 6.5 than at pH 8.0. Taking into account the K_d and the strong cooperativity of the interaction, as cofilin increases, this effect would reach saturation when the concentration of cofilin present is about 1.16 times that of actin (McGough et al. 1997). In conditions similar to those used in our previous work ([actin] = 20 μM) this saturation effect would be reached at a concentration of cofilin of 23 μM. We showed in our previous work (Bonet et al. 2000) that this is true at pH 6.5 but not at pH 8.0. Here we have carried out actin polymerisation experiments for four kinds of species: actin alone (A), cofilin:actin complexes (B), α-actinin:actin complexes (C) and cofilin:α-actinin:actin complexes (D), at both pH 8.0 and pH 6.5, and at relatively low Mg^{2+} concentrations ("slow polymerisations").

Figure 9 shows the fluorescence recovery time of those four complexes at both pH 8.0 and pH 6.5 about 45 min after addition of Mg^{2+} to 0.8 mM (near the end of full polymerisation). In both cases, it can be seen that in the

absence of α-actinin, cofilin provokes the increase in the mobility of actin polymers (Bonet et al. 2000), at both pHs. In Fig. 9a, diffusion coefficients of fast components are on the order of that of G-actin, (7–8) × 10^{-8} cm^2/s, whereas in Fig. 9b fast components appear to be slower and at a higher proportion, revealing the presence of a mixture of G-actin and small actin oligomers. Table 3 summarises the result of fitting of curves in Fig. 9 to two exponentials. It should be noted that, at both pH 8.0 and pH 6.5, cofilin causes α-actinin:actin complexes to slow down, as values in row D in Table 3 are significantly lower than those in row C. This effect is more pronounced at pH 6.5, since by adding cofilin to the complex, the contribution of the slower component increases (from 26 to 40%, see Table 3). This results in the compaction of these complexes, displacing the composition of the actin cytoskeleton towards bigger macrostructures (Fig. 10).

It is possible overall to distinguish three collectives within the filament population: (1) Massive, immobile structures (Fig. 4a–c), where, due to the high concentration of filaments, the diffusion of molecules from the exterior is difficult (Luby-Phelps et al. 1987; Mastro et al. 1984) as the packing competition phenomena for available space (Minton 1981, 1983) or molecular confinement (Minton 1992) may appear. Being three dimensional structures, most filaments remain unavailable from the exterior. Mobile filaments make up the other collectives, grouped as (2) "fast" and (3) "slow" fractions.

It has been shown that cofilin promotes the reordering of big structures such as those of Fig. 4 in bundles of filaments. Cofilin's severing capacity also contributes to reorganising the cytoskeleton (Aizawa et al. 1996; Maciver et al. 1991). It has also been observed that over-expression of cofilin in *Dictyostelium* increases the amount of F-actin, while maintaining intact the concentration of monomeric actin. This fact suggests that the severing of polymers is a preferred mechanism rather than depolymerisation (Aizawa et al. 1996). In spite of the fact that actin presents relatively close binding sites for cofilin and α-actinin (McGough et al. 1994, 1997) and that in certain situations there may be some allosteric effects, their biochemical activity, in conditions similar to those described here, does not seem to be affected by their simultaneous presence (Yonezawa et al. 1988). In addition, their K_d constants are in the same order of magnitude (0.1–0.15 μM). Cofilin locally alters the orientation angle of successive actin subunits in the filament and thus alters the filament twist. This shortens the filament period (Fig. 11b) (McGough et al. 1997). The effective length of α-actinin is approximately that of the actin filament period and also similar to their separating distance along the filament (Meyer and Aebi 1990). Consequently, cofilin increases the angular displacement per monomer, reducing the filament period and also increasing

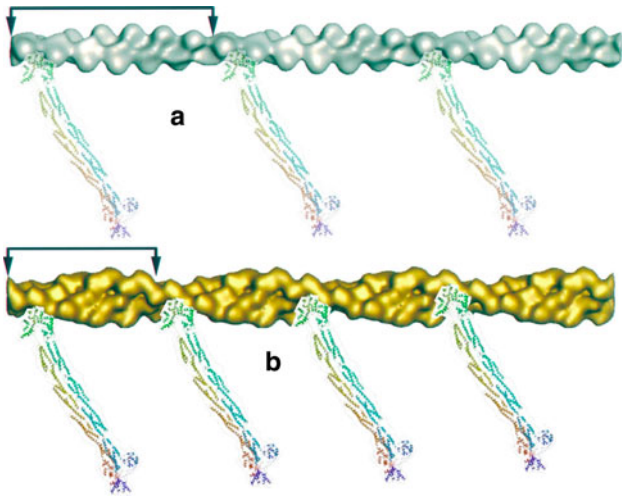

Fig. 11a, b Cartoon representation of the Lorenz-Holmes model of actin filament in their cofilin bound and unbound state (after McGough et al. 1997), showing the increased density of α-actinin binding sites per millimeter of filament in the presence of cofilin. **a** Actin filament saturated with α-actinin–actin interacting domains. These domains are spaced about the axial twist of the filament and this makes the α-actinins lie approximately in the same plane. **b** Actin filaments decorated with cofilin. Since these filaments have a shorter repeat distance, there are more interactions with α-actinins per unit length of actin filament. This accounts for the more compact actin structures

the number of α-actinin molecules per unit length (Fig. 11). As a consequence the filament cross-linking probability increases; this would be the origin of the reduction in mobility of filaments in Fig. 9. In summary, cofilin generates shorter filaments and at the same time provides them with an enhanced capacity for cross-linking (accounting for the observed "fast" fraction). The rest of the actin is not appreciably affected by the presence of cofilin.

Cofilin and α-actinin are found in the same cell compartment at the same time in a number of situations meaning that the effects shown in this report may well occur in living cells. Cofilin is concentrated toward the rear of expanding lammelipods (Svitkina and Borisy 1999) in regions where small perpendicularly oriented actin bundles knowns as "arcs" form. These are rich in α-actinin and so may well form by mechanisms suggested in this study. In support of this contention it is observed that *Dictyostelium* cells forced to overexpress cofilin respond by making bundles of filaments (Aizawa et al. 1996). In this system cofilin and α-actinin are also localised to the contractile ring which again may well form by cofilin and α-actinin cooperation (Aizawa et al. 1997).

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