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Actophorin preferentially binds monomeric ADP-Actin over ATP-bound actin: consequences for cell locomotion

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Abstract

Actophorin from *Acanthamoeba castellanii* severs actin filaments and sequesters actin monomers. Here we report that actophorin binds ADP-bound monomers with higher affinity than ATP-bound monomers. Actophorin is therefore much less efficient at severing actin filaments in the presence of ADP compared to ATP, particularly taking account of the higher critical concentration in ADP. Monomer binding is also reduced in the presence of 25 mM inorganic phosphate (which is assumed to form ADP·P_i-actin). These findings are discussed in the light of observations on the nucleotide specificity of other monomer binding proteins and related to the role of actin in lamellar protrusion and cell locomotion.

Key words: Actophorin: Actin severing protein; Actin nucleotide

1. Introduction

Microfilaments, in conjunction with other cytoskeletal proteins are responsible for maintaining the structural integrity of eukaryotic cells. Muscle contraction, cell locomotion, cytokinesis, phagocytosis and other motile processes utilize F-actin in conjunction with a wide variety of actin binding proteins. Although skeletal muscle contraction requires the presence of stable thick and thin filaments for force generation, most actin-based motility involves active turnover of filaments. Thus microfilaments undergo cycles of polymerization and depolymerization in locomoting cells: to understand motility we need to elucidate the mechanisms controlling the dynamic behaviour of actin. Among the classes of proteins involved are monomer binding proteins, which sequester much of the G-actin in cells and thereby regulate the extent of polymerization. Three main types have been identified, the profilins, thymosins and the actin depoymerising factors (ADF group) to which actophorin belongs. Actophorin was originally identified by Cooper et al. [1], and further characterized by Maciver et al. [2]. In addition to sequestering actin monomers, it severs filaments in a calcium-insensitive manner, but unlike the gelsolin family (reviewed in [3]), it does not cap the barbed ends of severed filaments.

Proteins with properties similar to actophorin include vertebrate ADF [4], which is identical to destrin [5], originally described by Nishida et al. [6], cofilin, which is related to ADF [7], depactin from echinoderms [8], and yeast cofilin [9]. The available sequence data reveal broad homologies, but surprisingly the most variant member seems to be depactin.

Despite the fact that there appears to be very little difference between the structures of monomeric actin with bound ADP or ATP [10], profilin [11,12] and thymosin β4 [13] have been shown to bind ATP-actin in preference to ADP-actin. By contrast, it is here shown that actophorin binds preferentially to ADP-actin. Some of this work has been presented previously [14].

2. Methods and materials

2.1. Buffers

Buffer G: 10 mM Tris-HCl, pH 8.0, 0.1 mM ATP (or ADP where indicated), 0.2 mM $CaCl_2$, 0.2 mM DTT and 1 mM NaN_3. 10 × KME: 0.5 M KCl, 10 mM $MgSO_4$, 10 mM EGTA. *Buffer F:* Buffer G containing 1 × KME. ATP and ADP were from Sigma (Poole, Dorset), and DTT was from Calbiochem (Nottingham, UK).

2.2. Proteins

Recombinant actophorin [15], was purified from *E. coli* by a modification of the method of Hawkins et al. for ADF [5] (see section 3). Actin was purified from rabbit skeletal muscle [16] and labeled with *N*-(1-pyrene)iodoacetamide (Molecular Probes, OR, USA) [17]. ADP-actin was made from ATP-actin by incubation with hexokinase (Sigma, Dorset) and glucose [18].

2.3. Polyacrylamide gel electrophoresis

Native gel electrophoresis was performed as described by Safer [19] with the following modifications. 10% gels were run in order to maximise separation of actin from the actin:actophorin complex. DTT was added at 2 mM to both the gel and running buffer and in some experiments ADP was substituted for ATP. The stoichiometry of the complex was determined by scanning gels which were loaded with known amounts of actin and actophorin and run under denaturing conditions. In some experiments two-dimensional gel electrophoresis was carried out: strips were cut form native gels, placed on a 13% polyacrylamide gel containing SDS, with stacking gel and overlaid with SDS-PAGE

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Abbreviations: G-actin, monomeric actin; F-actin, filamentous actin; ADF, actin depolymerizing factor; PMSF, phenylmethylsuphonylfluoride; pyrene, *N*-(1-pyrene)iodoacetamide; DTT, dithiothreitol.

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sample buffer. Electrophoresis was carried out at 200 V. Acrylamide was supplied by Severn Biotech Ltd. (Kidderminster UK).

2.4. Falling ball viscometry

Viscometry was performed by the method of Maclean-Fletcher and Pollard [20]. Actin (10 μM) was mixed with different concentrations of actophorin and volumes were made to 180 μl with buffer G. 20 μl of 10 × KME was added, the solution briefly vortexed and taken up in 100 μl capillaries which were then sealed with 'plasticene'. After approximately one hour, the viscosity was measured by timing the velocity of steel ball bearings (New England Miniature Ball-Bearing Co.) falling through the solutions.

2.5. Gel filtration experiments

Protein solutions in a total volume of 1.0 ml were gel filtered on a 38 cm × 1.4 cm S-200 column equilibrated with buffer G. Protein elution was monitored by absorbance at 290 nm, and protein concentrations determined by scanning 13% SDS-PAGE using a Molecular Dynamics 300A scanner.

2.6. Actin polymerisation assay

Pyrenyl-actin fluorescence was measured at 20°C in a Perkin Elmer LS 50B spectrofluorimeter using 0.2 × 1 cm cells and excitation and emission wavelengths of 366 and 384 nm, respectively.

3. Results

3.1. Purification of recombinant actophorin

Actophorin was expressed in *E. coli* strain BL21 (DE3) by transfection of a cDNA fragment encoding actophorin in a T7 based vector [15]. One litre of medium (2 × TY) with 0.1 mg/ml ampicillin was inoculated with several colonies of cells containing the vector from a 'streak' plate. The flask was shaken overnight at 35°C in the absence of inducer. (Experiments in which the flasks were induced by the addition of 0.1 mg/ml IPTG for 2 h resulted in only marginally increased levels of expression, so inducer was not routinely used.) Cells were harvested by centrifugation at 10,000 × *g* for 10 min and pellets frozen in liquid nitrogen for storage. Thawed pellets were solubilised in 25 ml of lysis buffer: 50 mM sucrose, 25 mM Tris-HCl pH 8.0, 1 mM PMSF, 1 mM benzamidine, 2 mM DTT. The slurry was sonicated by intermittent bursts for 1 min and re-frozen in liquid nitrogen. After thawing, insoluble material was pelleted at 10,000 × *g* for 30 min. The supernatant was dialysed overnight against 25 mM Tris-HCl, pH 8.0, 1 mM NaN_3, 1 mM PMSF, 1 mM benzamidine, 2 mM EGTA, 2 mM DTT and applied to a 6 × 1.5 cm DE53 column equilibrated with the same buffer. Actophorin was not bound to the column and hence separated from the bacterial proteins which were retained by the DE53 matrix. The flow-through was concentrated using centricon 30 filters (Amicon, Beverly, MA, USA) and gel filtered on Sephacryl S-200 (75 × 1.5 cm) in the same buffer. The resulting actophorin gave a single band on SDS PAGE even when gels were overloaded. Recombinant actophorin produced by this construct has properties that are indistinguishable from native protein isolated directly from *Acanthamoeba castellanii* [15].

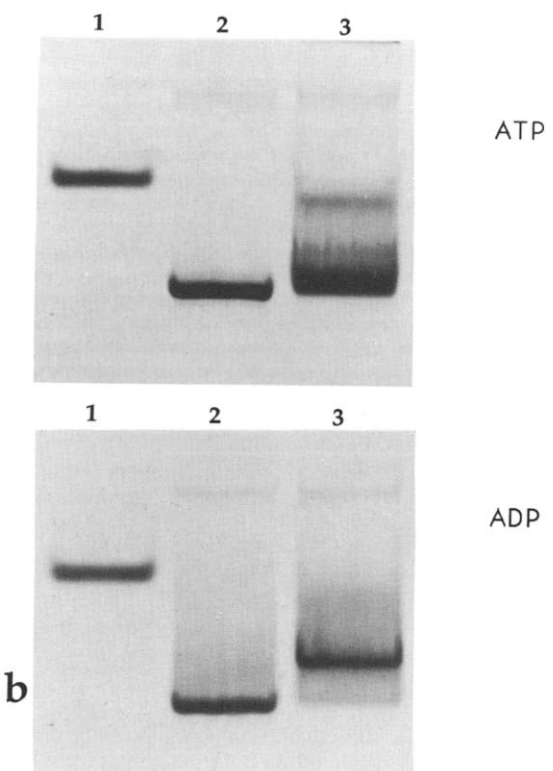

Fig. 1. Native gels demonstrating actin:actophorin complex formation. A: gel run in the presence of ATP. Lane 1 = actophorin; lane 2 = actin; lane 3 = actin and actophorin. B: gel run in the presence of ADP. Lane 1 = actophorin; lane 2 = actin; lane 3 = actin and actophorin.

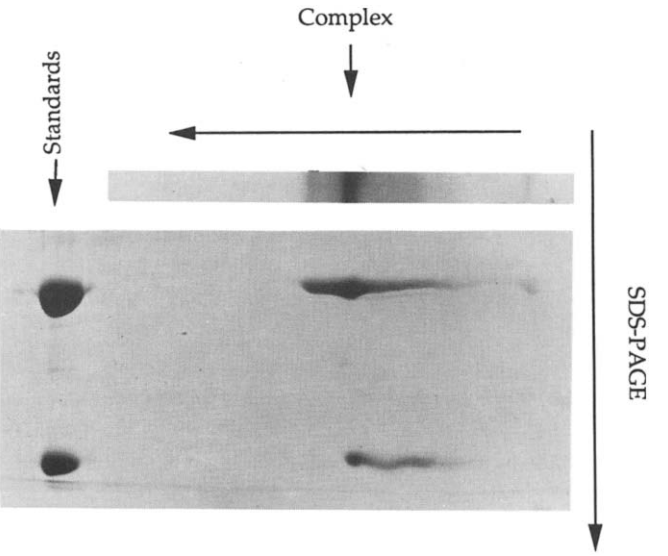

Fig. 2. 2-D electrophoresis of actin:actophorin complex. The central slice of a track similar to lane 3 in Fig. 1B was cut out prior to staining and placed across a standard 13% SDS gel. Known concentrations of actin and actophorin were run (standards) to compare relative concentrations of the two proteins.

3.2. Native gel electrophoresis

Fig. 1 shows that complex formation occurs more extensively in the presence of ADP (Fig. 1B) compared to

ATP (Fig. 1A). Pre-incubation of the proteins in F-buffer increased the extent of complex formation irrespective of the nucleotide present (not shown). Two-dimensional electrophoresis confirmed that the complex contains equimolar concentrations of actophorin and actin (Fig. 2), in agreement with previous observations using EDC crosslinking [2].

3.3. Gel filtration of actin actophorin complexes

Gel filtration also shows that actophorin has a marked preference for ADP-actin monomers. The top panel (Fig. 3A) shows the elution positions of actin and actophorin run independently. The profile was identical whether ATP or ADP was present in the elution buffer. In the presence of ATP when both proteins were mixed immediately before loading the column, the position of actophorin was shifted only partially towards the actin peak (Fig. 3B). However, in ADP, not only was the actophorin almost completely shifted to co-migrate with actin, but there was a reproducible shift in the position of the actin peak to a lower elution volume, indicating formation of a species of higher M_r. Based on the actin concentration on the column and the relative concentrations of free and bound actophorin, the K_d is estimated to be ~0.2 μM.

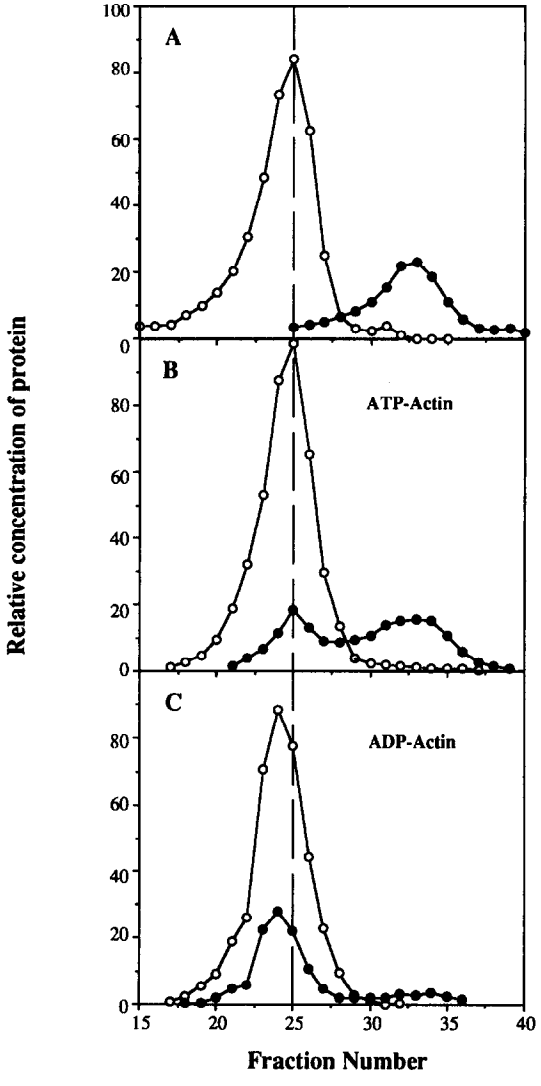

Fig. 3. S-200 gel filtration of actin actophorin complexes. A: elution of actin (○) and actophorin (●), chromatographed separately. B: mixtures of actin and actophorin in the presence of ATP. C: mixtures of actin and actophorin in the presence of ADP.

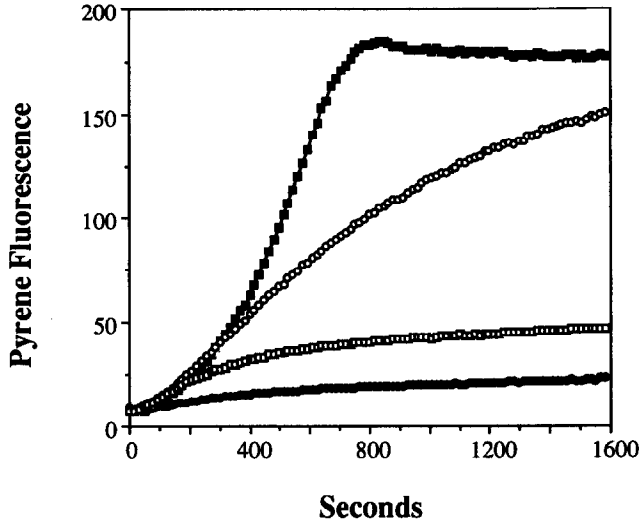

Fig. 4. Kinetics of polymerization of 10 μM ATP-actin alone (○); 10 μM ATP-actin plus 1 μM actophorin (■); 10 μM ADP-actin alone (●): 10 μM ADP-actin plus 1 μM actophorin (□).

3.4. Severing activity of actophorin

Actophorin increases the rate of spontaneous actin polymerization in the late stages by severing the growing filaments and thereby increasing the concentration of nuclei [1,2]. Although the rate of polymerization of ADP-actin is significantly lower than that of ATP-actin, due to the increased critical concentration and lower 'on' rates for the former [21], Fig. 4 shows that the rate of polymerization of ADP-actin is also enhanced by actophorin, indicating that there is sufficient free actophorin present in the mixtures containing ADP-actin for severing to occur. Severing activity can be readily detected using viscometry [1,2]. Fig. 5 shows that in the presence of ATP there is a dramatic fall in low shear viscosity at very low concentrations of actophorin. The effect was much less marked in the presence of ADP, although higher concentrations of actophorin significantly reduced the actin viscosity.

The severing activity of actophorin can be readily demonstrated in depolymerization assays. Addition of actophorin in equimolar concentration to F-actin caused an instantaneous loss of fluorescence of pyrene F-actin (Fig. 6A). Inorganic phosphate (25 mM) has been shown to inhibit severing by actophorin [2], presumably because it forms the ADP-P_i-actin species which resembles ATP-actin [22,23]. Fig. 6B shows that addition of 25 mM

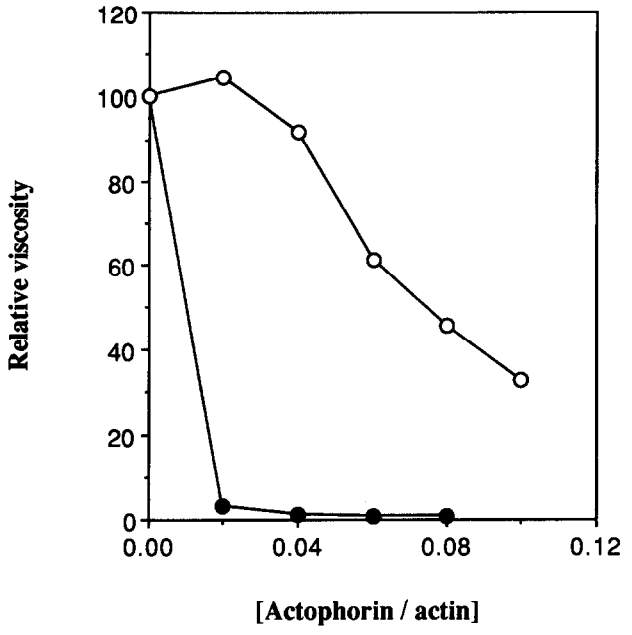

Fig. 5. Viscosity (measured as the velocity of the falling ball) of 10 μM actin and various concentrations of actophorin in the presence of ATP (●) or ADP (○) relative to that in the absence of actophorin.

phosphate reduces the rate of depolymerization and partially reverses actin monomer sequestration after filament severing, as evidenced from the fluorescence at steady state at the end of the reaction (Fig. 6A,B). The apparent rate constant for depolymerization in Fig. 6B is 0.011 s^{-1}.

4. Discussion

We have shown that actophorin binds tightly to ADP-actin monomers but very weakly to ATP-actin. The K_d estimated from Fig. 3C (~0.2 μM) is similar to that obtained previously from measurement of the effects of actophorin on the steady-state actin polymer concentration [2]. The monomer sequestering activity of actophorin will also affect its severing potential, since in the presence of ADP the formation of binary complexes will limit availability of free actophorin for filament severing. This can be seen in Figs. 4 and 5 in which the effects of actophorin on actin polymerization kinetics and on filament length (viscosity) are much more marked using ATP-actin. Since the viscosity is proportional to the fifth power of filament length [24], a small amount of severing will dramatically reduce the viscosity of F-actin.

The nucleotide-dependent specificity of binding may, at least in part, account for the filament binding and severing activity of actophorin, since severing occurs even in the presence of high concentrations of G-actin. ATP hydrolysis occurs very rapidly after subunits have been added to filaments [25], so most of the subunits in F-actin contain ADP. Because of this, it is not possible to analyse the effects of actophorin on filaments containing ATP-actin. However, inorganic phosphate binds to F-actin and changes the dynamics of the filaments towards that of ATP-actin, thereby stabilizing the filaments [22,26]. This is seen in Fig. 6. When 10 μM actophorin was added to 10 μM F-actin, the pyrene fluorescence was reduced by >90% within the mixing time (about 20 s, Fig. 6A), suggesting almost complete severing within this time. Fig. 6B shows that the rate of depolymerization of severed filaments was greatly reduced in the presence of 25 mM inorganic phosphate. The rate constant for depolymerization is 0.011/s. This reduced disassembly rate might indicate a much lower severing efficiency for filaments containing ADP·P_i-actin subunits. However, since the dissociation rate constants for ADP.Pi-actin are 12% of those for ADP-actin [26] and the rate of depolymerization is the product of severing frequency and the dissociation rate constants at the two

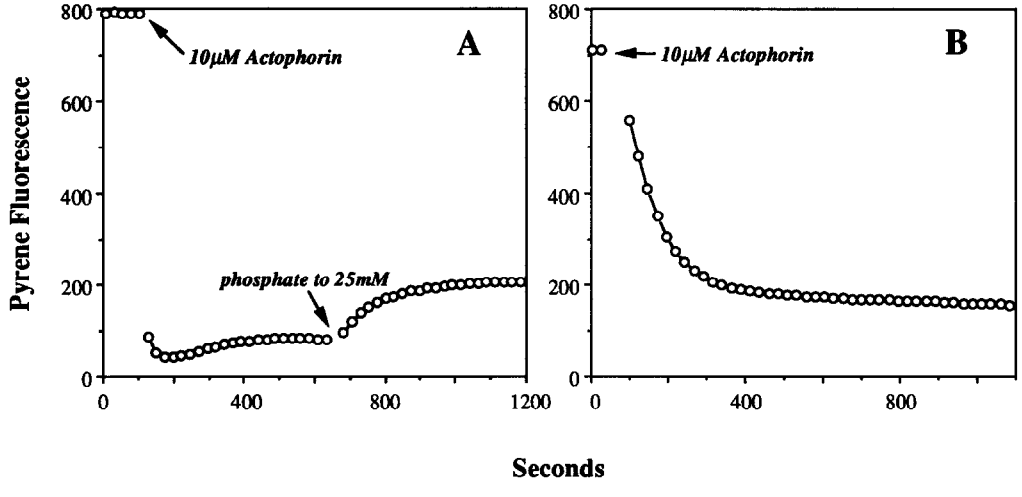

Fig. 6. The effect of inorganic phosphate on the interaction of actophorin with F-actin. A: 10 μM actophorin was added to 10 μM F-actin. 25 mM phosphate was added when the fluorescence reached steady-state. B: 10 μM actophorin added to 10 μM F-actin in the presence of 25 mM phosphate.

ends [5], the reduction of severing activity is likely to be less than 50% based on an estimated disassembly rate of ~0.15/s assuming 95% reaction in 20 s (Fig. 6A). Detailed kinetic analysis using rapid reaction methods would be required to confirm any differences in severing activity.

Fig. 6 also shows a higher steady-state fluorescence of pyrene F-actin at the end of reaction in the presence of 25 mM inorganic phosphate. This suggests a reduction in binary complex formation by ADP·P_i-actin, in comparison to ADP-actin, i.e. a higher K_d for this complex, as observed also with ATP-actin.

A number of other actin-binding protein have recently been shown to have nucleotide specificity for their interaction with actin. Thymosin β_4 has a 50-fold higher affinity for ATP-actin than ADP-actin [13]. Profilin is like thymosin β_4 in having a higher affinity for ATP-actin [12], but in addition it greatly accelerates the rate of nucleotide exchange when complexed with actin [27]. Since profilin promotes actin filament assembly in the presence of thymosin β_4 [12], it can, paradoxically, drive filament assembly using the energy of ATP hydrolysis. By contrast, gelsolin, like actophorin, binds preferentially to ADP-actin monomers [28] and, as with actophorin, ADP-actin monomers inhibit its severing activity. However, while gelsolin caps the severed filaments and thereby prevents re-annealing, actophorin dissociates from them as a binary complex with ADP-actin. Dissociation of this complex, possibly accelerated in cells by the nucleotide exchange potential of profilin, would liberate actophorin that is once again available for severing, i.e. its catalytic activity is facilitated by its nucleotide-specific actin interaction [2].

Actophorin-like proteins are found in the same cell types as the gelsolin group, are calcium independent and are enriched in the lamellae [15,29]. These proteins would be ideally suited for severing filaments into measured lengths if they were unable to interact with ATP or ADP-P_i-monomers also present in cells. An unexpected result of the severing activity of actophorin has been shown to influence the bundling activity of α-actinin [30]. This would potentially give the cell enormous scope in controlling the exact type of gel that is being created, which in turn would be expected to influence the physical properties of that particular part of the cell. Not only does the geometry of cytoplasmic gels strongly influence the rheological properties of gels [30], but the geometry is important in determining interactions with motor proteins such as the myosin I family, located at the cells leading edge [31,32].

There is considerable evidence that in many cell types, actin is constantly polymerizing at the leading edge of expanding lamellae [33–35] and depolymerising toward the cell centre [36,37]. Lamellar protrusion may be explained by 'treadmilling', a process in which ATP containing subunits join the barbed end of the filament and are dissociated at the pointed end [38], but this process is too slow to account for the flux of actin subunits in keratocytes [33,39]. This is because filaments cannot depolymerize from their pointed ends rapidly enough under conditions pertinent to cells . We postulate that the low molecular weight severing proteins of which actophorin is a member, are able to sever long filaments, but, unlike the gelsolin group, leave both ends free for monomer exchange. Thus although the severing activity of actophorin is much lower than that of gelsolin [5,40], because it does not bind to ATP-monomers, its severing action is catalytic and may thereby accelerate actin reorganisation in cells.

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