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Coactosin interferes with the capping of actin filaments

Ursula Röhrig[a], Günther Gerisch[a,*], Ludmilla Morozova[a], Michael Schleicher[c], Albrecht Wegner[b]

[a]*Max-Planck-Institut für Biochemie, 82152 Martinsried, Germany*
[b]*Institut für Physiologische Chemie, Ruhr-Universität, Universitätsstraße 150, 44780 Bochum, Germany*
[c]*Institut für Zellbiologie, Ludwig-Maximilians-Universität, Schillerstraße 42, 80336 München, Germany*

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Abstract Coactosin, a 16 kDa protein associated with the actin cytoskeleton from *Dictyostelium discoideum*, was purified by an improved method, in which other components of the cytoskeleton were removed. The highly purified coactosin had no effect on the time course of actin polymerization, but when added to actin in presence of capping proteins, coactosin counteracted the capping activity of these proteins. The capping proteins cap32/34 and severin domain 1 retarded actin polymerization, on addition of coactosin to samples containing one of these capping proteins the time course of actin polymerization became close to controls without capping proteins.

Key words: Cytoskeleton; Actin polymerization; Coactosin; F-actin capping protein; cap32/34; Severin

1. Introduction

Amoeboid movement of cells in *Dictyostelium discoideum*, phagocytosis during growth and chemotactic orientation during aggregation are associated with dynamic rearrangements of the actin filament system [1]. A large number of proteins that are engaged in the regulation of the actin cytoskeleton has been isolated [2]. Coactosin is one of these proteins. It has been isolated on the basis of its coprecipitation with actin-myosin complexes from *D. discoideum* cell homogenates [3]. Coactosin shows some sequence relationships to drebrins from neurons, to ABP1p, an actin-binding protein from yeast, and to the cofilin family of actin-binding proteins [3]. Since purified coactosin has been found to have little, if any, effect on actin polymerization [3] its role in the organization of the actin system remained unclear. Therefore, we supplemented preparations of coactosin and actin with other proteins of the actin cytoskeleton in order to test whether coactosin regulates their function. It turned out that coactosin counteracts the activities of the heterodimeric capping protein cap32/34 [4,5,6] and of severin domain 1 [7]. These capping proteins inhibit actin polymerization in vitro by binding to the fast growing ('barbed') end of actin filaments.

2. Materials and methods

2.1. Preparation of coactosin

The method of coactosin preparation described by de Hostos [3] was modified in order to increase the yield and purity of the protein. Cells of *Dictyostelium discoideum* strain AX2-214 were grown axenically at 23°C to a density of 10^6 cells/ml, starved for 16 h in 17 mM K/Na-phosphate buffer, pH 6.0, and washed in this buffer. To 1 g of the cell pellet 0.7 ml of buffer A, pH 7.0 (5 mM PIPES, 5 mM EGTA, 2 mM EDTA, 3 mM dithiothreitol, 5 mM benzamidine, 0.5 mM 2-aminoethyl-*p*-benzolsulfonylfluoride, 1% of protease inhibitor mixture: 2.5 mg bestatin, 5.0 mg pepstatin, 5.0 mg antipain, 5.0 mg leupeptin in 50 ml methanol) were added. Following equilibration for 15 min at 5600 kPa with nitrogen in a Parr bomb, the suspended cells were lysed by decompression. The pH was adjusted to 6.7 and the lysate gently dispersed using a Dounce homogenizer.

After centrifugation at 100,000 × *g* for 2 h at 4°C, KCl was added to the supernatant to a final concentration of 30 mM. Contraction of the actin–myosin gel turned out to be most effective when 30 mM KCl was added. The pH was adjusted to 7.5 by dropwise addition of 2 M KOH at 0°C. The contracted pellet, that formed after 1 h incubation on ice, was collected by centrifugation at 20,000 × *g* for 15 min. The pellet was washed once by resuspension in buffer A plus 30 mM KCl, pH 7.5, followed by centrifugation, and was finally resuspended in buffer B (5 mM HEPES-NaOH, 0.2 mM dithiothreitol, 0.2 mM $CaCl_2$, 0.2 mM ATP, 1 mM benzamidine, pH 7.5) using a Dounce homogenizer with a wide plunger. It is necessary to always resuspend the pellets gently. The suspension was dialyzed against two changes of buffer B. In deviation from [3], no $MgCl_2$ was added to buffer B, in order to enhance dissociation of the actin–myosin complex during dialysis. Myosin and part of the actin was removed by centrifugation at 20,000 × *g* for 15 minutes. The supernatant was applied to a DEAE-cellulose column (2.8 × 3 cm) equilibrated with buffer B. The column was washed with buffer B plus 50 mM NaCl to remove the 30 kDa bundling protein [8,9]. A linear NaCl gradient was then applied (50–300 mM NaCl, 80 ml total volume). Coactosin eluted between 180 and 240 mM NaCl. The coactosin-containing fractions were dialyzed against buffer C (20 mM Tris-HCl, 0.5 mM EGTA, 0.2 mM dithiothreitol, 0.02% NaN_3, pH 8.0) and loaded onto a FPLC Mono-Q column (5 × 50 mm; Pharmacia) equilibrated with buffer C. Coactosin was eluted at about 230 mM NaCl in a linear gradient of 0–500 mM NaCl (15 ml total volume). The coactosin preparation obtained in that way contained an actin capping impurity that interfered with kinetic studies. This activity was also detected in Mono-Q fractions free of coactosin. By immunoblotting with mAb 135-409-16 [10] the impurity was identified as the capping protein cap32/34. Two additional purification steps were introduced in order to remove that protein: Superdex G-75 gel filtration (2.2 × 50 cm column; Pharmacia), dialysis of the eluted coactosin against buffer C, and a second Mono-Q run. The eluted coactosin was dialyzed against buffer D (2 mM Tris-HCl, 0.2 mM $CaCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, 0.02% NaN_3, pH 8.0) and stored on ice.

The yield from 10 liters of axenically grown cells was about 200 μg pure coactosin. Coactosin concentrations were determined by the method of Bradford [11] with bovine serum albumine as standard. Quantitation by amino acid analysis was in good agreement with the Bradford values.

2.2. Preparation of cap32/34 and of severin domain 1

For preparation of the heterodimeric capping protein cap32/34, the subunits were expressed separately in *E. coli* BL21 cells that contained an IPTG inducible pT7-7 vector with the proper cDNA as an insert [6]. After the subunits had been purified separately near to homogeneity [6], fractions were combined in a 1 : 1 molar ratio, brought to 6 M guanidinium hydrochloride and slowly dialyzed against buffer D. Renatured cap32/34 dimers were separated from remaining single subunits by gel filtration on a S4B-Cl column (1.5 × 70 cm) in buffer E (10 mM imidazole, 1 mM EGTA, 1 mM dithiothreitol, 0.02% NaN_3, 150 mM NaCl, 1 mM benzamidine, 0.5 mM PMSF, pH 7.6). The elution of active dimer was monitored by low-shear viscometry. Recombinant

*Corresponding author.

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domain 1 of severin was purified from *E. coli* essentially as described by Eichinger et al. [7].

2.3. Preparation of actin

Actin was prepared from rabbit skeletal muscle according to Rees and Young [12]. The protein was applied to a Sephacryl S-300 column (3.5 × 50 cm) equilibrated with buffer D. For fluoresescence assays, the actin was modified at Cys-374 with *N*-ethylmaleimide (NEM) and subsequently at Lys-373 with 4-chloro-7-nitro-2-oxa-1,3-diazol (NBD-Cl) [13]. The concentration of G-actin was determined photometrically at 290 nm using an absorption coefficient of 24,900 $M^{-1} \cdot cm^{-1}$ [14].

2.4. Fluorescence assay

Actin polymerization was measured by the 2.2–2.5-fold increase in fluorescence intensity associated with polymerization of NBD-actin [13]. NBD-actin was copolymerized with unmodified actin in a 1:20 molar ratio. This low proportion of labeled actin does not significantly alter the polymerization rate or the extent of assembly of unmodified actin [15]. The excitation wavelength was 480 nm, the fluorescence intensity was measured at 540 nm. Polymerization experiments were performed at 37°C.

3. Results

Severin fragment S1 is a bacterially expressed protein that corresponds to the N-terminal domain of severin [16], a *Dictyostelium* homologue of the mammalian F-actin fragmenting and capping proteins gelsolin [17,18] and villin [19]. Severin has a strictly Ca^{2+}-dependent severing activity [16]. Fragment S1 differs from the complete protein in two respects: it caps actin filaments not only in the presence but also in the absence of Ca^{2+}, and it has lost the capability of severing the filaments [7]. Fig. 1 shows the inhibition of actin polymerization by severin fragment S1 in the presence of EGTA and 100 mM KCl. Coactosin by itself did not significantly affect the polymerization of actin, but it strongly antagonized the capping activity of fragment S1.

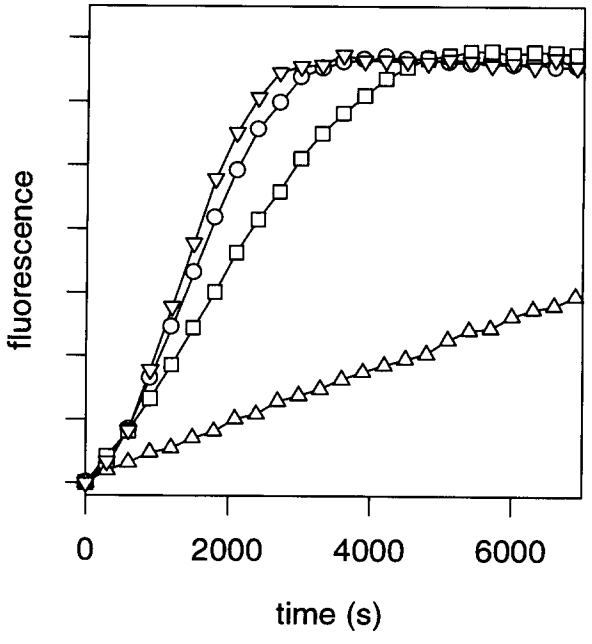

Fig. 1. Effect of coactosin on the polymerization of 2 μM actin in the presence or absence of severin fragment S1. ○ = actin; △ = actin and 0.5 μM severin fragment S1; □ actin and 0.5 μM severin fragment S1 plus 0.4 μM coactosin; ▽ = actin and 0.4 μM coactosin. All samples contained 100 mM KCl and an excess of 0.2 mM EGTA over 0.1 mM $CaCl_2$.

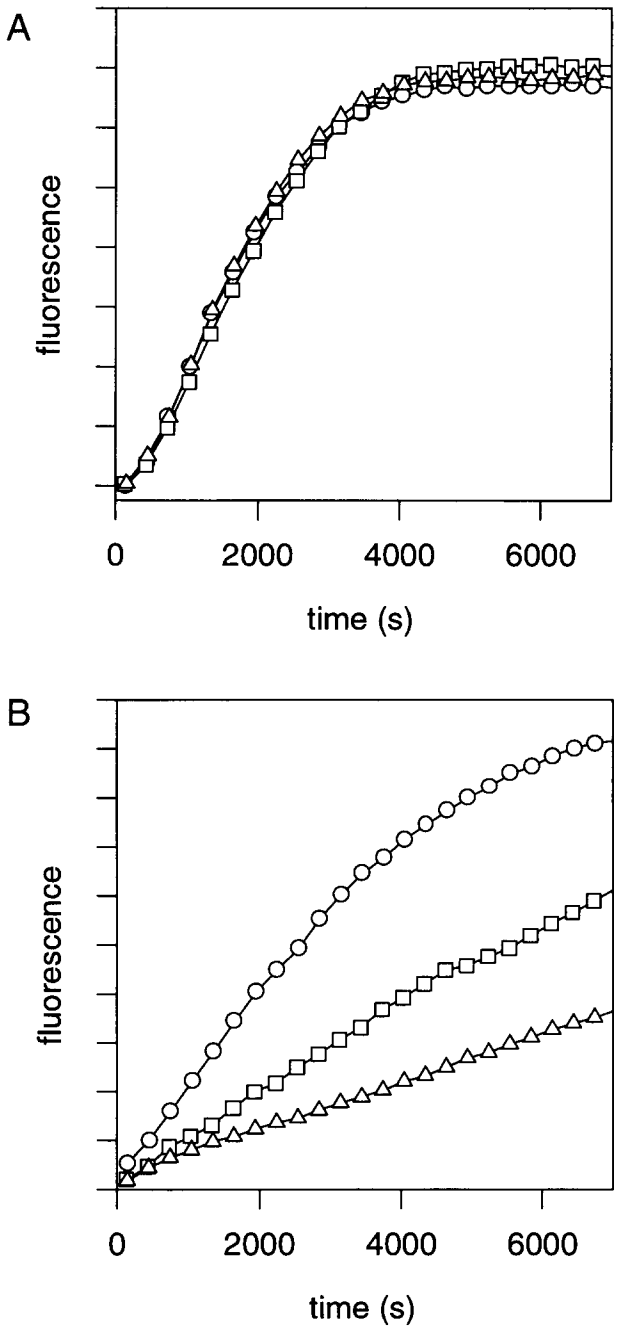

Fig. 2. (A) Time course of the polymerization of 2 μM actin in the presence of 10 mM KCl and the following concentrations of coactosin. □ = 1 μM coactosin; △ = 2 μM coactosin; ○ = control of actin. (B) Effect of 0.27 μM cap 32/34 on the polymerization of 2 μM actin with or without coactosin in the presence of 10 mM KCl. △ = cap32/34; □ = cap32/34 plus 2 μM coactosin; ○ = control of actin without additions.

The antagonistic effect of coactosin on the capping activity of fragment S1 has prompted us to test coactosin in combination with cap 32/34, a protein responsible for the major capping activity in homogenates of *D. discoideum* cells [5]. Since low KCl is optimal for the activity of cap 32/34, activity of coactosin

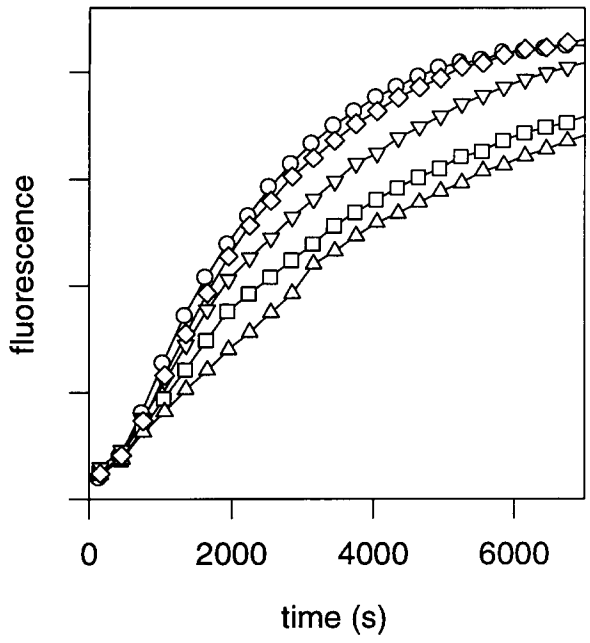

Fig. 3. Effect of 0.45 μM cap 32/34 on the polymerization of 2 μM actin with or without coactosin in the presence of 100 mM KCl. △ = cap32/34; □ = cap32/34 plus 1 μM coactosin; ▽ = cap32/34 plus 2 μM coactosin; ◇ = cap32/34 plus 4 μM coactosin; ○ = control of actin without additions.

was assayed at 10 mM KCl. Again, no effect on actin polymerization was found (Fig. 2A). Fig. 2B shows the strong capping effect of cap 32/34 at 10 mM KCl. 2 μM coactosin had a significant although incomplete inhibitory effect on the capping of actin filaments by this protein.

At 100 mM KCl, the anti-capping activity of coactosin proved to be enhanced relative to the activity of cap 32/34. Fig. 3 shows that actin polymerization was less efficiently delayed by cap 32/34, although a higher concentration of the protein was employed than in Fig. 2B. By titration with coactosin, the delay caused by cap 32/34 was gradually reduced. With 4 μM coactosin, the time course of actin polymerization was almost the same as in the control containing only actin. Under these conditions coactosin was 2-fold in molar excess of actin and 9-fold in excess of cap 32/34.

4. Discussion

In studies on the regulation of actin polymerization, usually the direct interaction of a single protein with actin is analysed. In the living cell, however, the polymerization state of actin represents the net effect of many proteins with antagonistic or synergistic activities, that are regulated in different ways and compete with each other for binding sites on monomeric or polymeric actin. In order to bridge the gap between oversimplified actin-polymerization systems that are studied in vitro and the complexity of protein–protein interactions in the cytoskeleton of living cells, it is important to combine more than two proteins in an assay. For instance, the activity of cap 32/34 of *D. discoideum* has been shown to be strongly enhanced by a chaperone of the hsc 70 family [20].

Here we found an effect of coactosin, that by itself does not detectably influence the polymerization of actin, on the activity of other proteins that are established inhibitors of actin polymerization. Coactosin thus proved to be a candidate for regulating protein–protein interactions in the actin system. The composition and structure of the coactosin-containing complexes remains to be elucidated. The anti-capping activity of coactosin in the presence of 10 and 100 mM KCl is important because of a previous report [3] according to which coactosin binds only weakly to F-actin at higher concentrations of KCl. We also found only sub-stoichiometric amounts of coactosin in relation to the number of actin monomers to co-sediment with actin filaments, both at 10 and 100 mM KCl (data not shown). This result excludes that for the anti-capping effect to occur, the actin filaments need to be decorated with coactosin along their entire length. One reasonable possibility is that coactosin binds specifically to the barbed ends of actin filaments, in a way that actin polymerization is unaffected but binding of capping proteins is sterically hindered. It is, however, not excluded that coactosin can interact with the two capping proteins, despite the fact that their primary sequences do not indicate any similarity of strucure [5,7,10]. The small yield of coactosin purifiable from *D. discoideum* in a state free of foreign activities limits a comprehensive analysis. Work is in progress to overexpress coactosin in *D. discoideum* or in *E. coli* for studies on multiple interactions of this protein with other components of the cytoskeleton.

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