'Cap 90', a 90-kDa Ca^{2+}-dependent F-actin-capping protein from vertebrate brain

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Received 21 September 1983

A Ca^{2+}-dependent actin filament-capping protein of 90 kDa was purified from bovine brain using a new and rapid isolation procedure. This basically includes affinity purification on DNase-I agarose. The protein caps the fast-growing end of actin filaments but has no fragmenting or severing activity. Using Triton X-100-extracted cytoskeletons, capping and severing activities of actin-binding proteins become clearly distinguishable from each other.

Actin *Capping protein* *Ca^{2+}* *Bovine brain* *Cytoskeleton* *Triton model*

1. INTRODUCTION

Actin filament capping proteins from various cells and tissues (review [1–3]) are currently investigated as potential cytoskeleton-associated proteins which influence the kinetics of actin polymerization and hence are of importance for regulating filament length and network formation during motile events within cells [4]. Capping proteins bind to only one end of the actin filament [5]. If this happens to be the 'barbed', fast growing end of the actin filament, they block actin monomer addition at this end and induce a rapid depolymerization at the 'pointed', slow-growing end, due to the different critical concentrations for both ends [6]. For neuronal tissue and especially for axons and dendrites all attempts to demonstrate the organization of actin filaments have been unsuccessful so far. This may be at least in part due to the fact that in these cells, like in red blood cells, actin exists only as short filaments or even as oligomers which are difficult to visualize. Our search for capping proteins in the nervous system led to the discovery of a first capping protein, which was isolated from bovine brain cortex [7]. This protein has a native M_r-value of 63 kDa, consists of two polypeptides of 36 kDa and 31 kDa and caps the fast-growing end of actin filaments in a Ca_2^+-insensitive manner. Recently, the isolation of a 90-kDa Ca^{2+}-dependent actin fragmenting protein from neuronal tissue was reported [8]. This protein, however, in its native form could only be isolated as a 1:1 complex with another protein of 42 kDa. We have now developed a rapid purification procedure which allows the isolation of the 90-kDa protein in its native, pure form. In addition, we present evidence that this protein is not an actin-fragmenting protein but is a second actin filament-capping protein in bovine brain which binds to filament ends in a Ca^{2+}-dependent manner.

Abbreviations: DNase-I, deoxyribonuclease I; EGTA, ethyleneglycol bis (β-aminoethylether)-*N,N,N',N'*-tetraacetic acid; PMSF, phenylmethylsulfonylfluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; S-1, myosin subfragment-1

2. MATERIALS AND METHODS

2.1. *Proteins*

The 90-kDa capping protein was purified as described in section 3. A 90-kDa actin filament-severing protein from smooth muscle was kindly

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

supplied by Dr H. Hinssen (Salzburg). Rabbit skeletal muscle actin was prepared from acetone powder [9] and column purified on Sephadex G-150 [10]. Protein concentrations were determined as in [11]. SDS–PAGE was performed as in [12] or was carried out according to [13].

2.2. *Viscometry*

The low shear viscometry of actin solutions (0.5 mg/ml) was measured using the falling ball viscometer (100 μl capillary) of [14] as in [5].

2.3. *Electron microscopy*

Protein samples were spread on parlodion-coated, carbon-shadowed and glow-discharged electron microscope grids, which were pretreated with 0.02% (w/v) cytochrome *c* in 0.1% (v/v) isoamylalcohol prior to negative staining with uranyl formate (0.75%, w/v; pH 4.25) and examination in a Zeiss EM 10 microscope.

2.4. *Triton X-100 cell models*

Chicken heart fibroblasts were grown on glass coverslips. The cells were rinsed in Ca^{2+}–Mg^{2+}-free Hanks buffer and extracted for 2 min with 0.1% Triton in Hanks buffer. The permeabilized cells were then incubated for 5 min with either 40 μg/ml of a 90-kDa severing protein from smooth muscle in 10 mM imidazole (pH 7.2), 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, or 66 μg/ml of the 90-kDa capping protein purified from bovine brain in the same buffer. Control cells were incubated with a buffer containing no additional protein. The cells were then rinsed in Hanks buffer, fixed for 15 min in 1% glutaraldehyde in the same buffer and after a brief wash stained with rhodamine-coupled phalloidin, which specifically stains actin filaments [15]. After removal of the staining solution and a subsequent wash the cells were embedded in gelvatol, viewed in a fluorescence microscope and photographed on Kodak Tri-X film.

3. RESULTS

3.1. *The purification of the 90-kDa capping protein from bovine brain*

The fractionation on DEAE–cellulose of an extract from bovine brain cortex was performed essentially as in [7]. The fractions of the DEAE column which reduced the viscosity of actin solutions and eluted with a step-gradient between 75 mM and 150 mM NaCl were pooled, dialyzed against IDE-buffer (20 mM imidazole, pH 7.2, 1 mM EGTA, 0.5 mM DTT, 0.25 mM PMSF) and

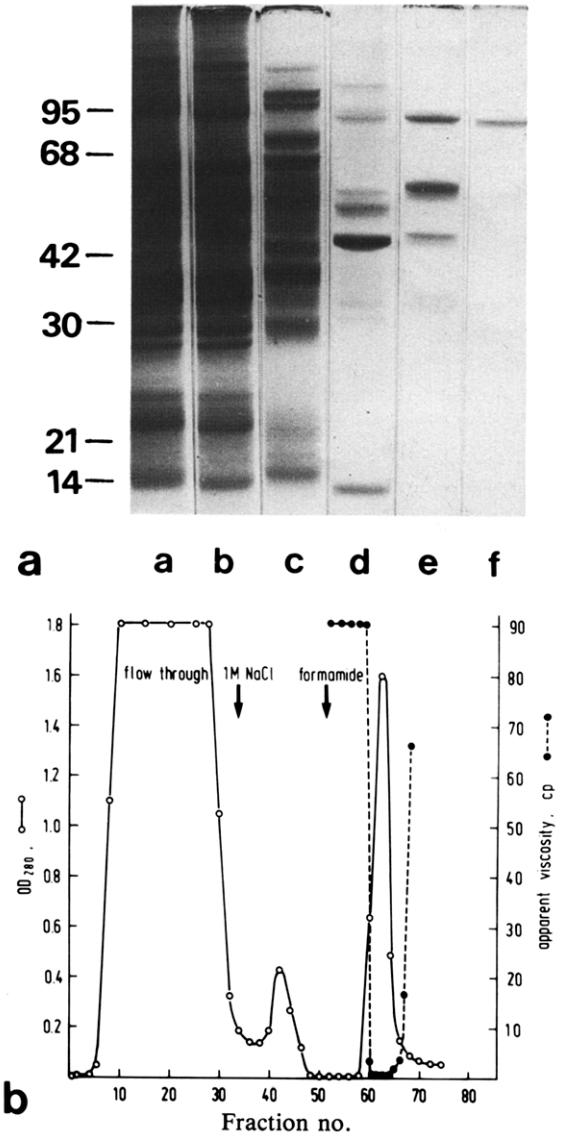

Fig.1. Purification of 'cap 90'. (a) 7.5% SDS gels of the 75–150 mM DEAE eluate loaded onto DNase-I (lane a); DNase-I flow-through fractions (lane b); 1 M NaCl eluate (lane c); 8.8 M formamide eluate (lane d); active peak fractions from DE-53 (lane e); G-150 Sephadex fractions (lane f). (b) Elution profile of the DNase-I affinity column. The Ca^{2+}-dependent viscosity reducing activity (●——●) resides only in the fractions eluting with 8.8 M formamide.

directly applied to a DNase-I column (1.5 × 6 cm of affi-gel 10, BioRad, to which 200 mg of DNase-I (Sigma D4763) had been covalently coupled) equilibrated with: IDE buffer containing 5 mM $CaCl_2$. When the flow-through fractions, a high salt eluate (1 M NaCl) and the fractions eluting with 8.8 M formamide in IDE [16] were assayed by viscometry, the activity which reduces the viscosity of F-actin in a Ca^{2+}-dependent manner resides only in fractions which eluate with 8.8 M formamide (fig.1b). The corresponding gels of the purification steps using DNase-I affinity chromatography are shown in fig.1a, lane a–d. Following dialysis against IDE-buffer, the active fractions are rechromatographed on DE-53 (fig.1a, lane e). The active fractions from the DE-53 column contained a contaminating protein and proteins of 90 kDa and 42 kDa, the latter being comprised of the described 1:1 90 kDa–42 kDa complex and a free form of 'cap 90'. The pure, native 'cap 90' can be obtained by a final gel-filtration step on Sephadex G-150 (fig.1a, lane f).

3.2. *'Cap 90' caps but does not fragment actin filaments*

As shown in viscometric assays (fig.2), 'cap 90' reduces the low shear viscosity of F-actin in substoichiometric amounts when Ca^{2+} (0.2 mM) is present. In the absence of Ca^{2+} (1 mM EGTA) 'cap 90' is inactive. Since the decrease in viscosity could be due to either fragmenting or capping actin filaments, actin was polymerized for 10 min under exactly the same conditions as used for viscometric assays in the presence of increasing molar ratios of 'cap 90' and viewed in the electron microscope. When compared with controls (fig.3a) actin

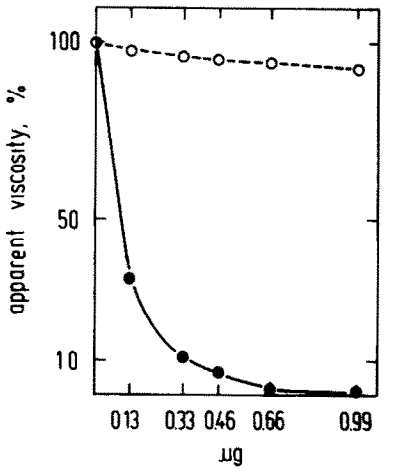

Fig.2. Ca^{2+}-dependency of the low shear reducing activity of 'cap 90'. The purified protein substoichiometrically reduces the viscosity of 0.5 mg/ml F-actin in a concentration-dependent manner when Ca^{2+} (0.2 mM) is present (●—●). The protein is completely inactive in 1 mM EGTA (○—○).

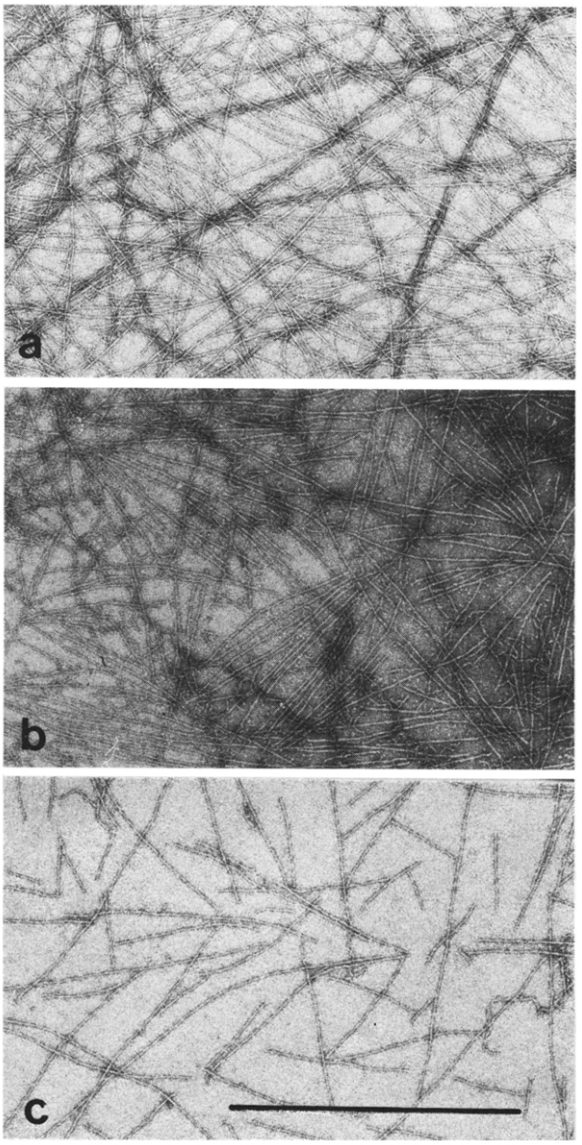

Fig.3. Effect of 'cap 90' on the length of actin filaments. Control, F-actin 0.5 mg/ml (a); 'cap 90' and actin copolymerized for 10 min at a molar ratio of 1:10 (b) and 1:5 (c). Magnification × 31000; the bar represents 1 µm.

filaments were not appreciably shorter, unless the 'cap 90' concentration reached molar ratios of 1:10 (fig.3b) or 1:5 (fig.3c), indicating that 'cap 90' does not belong to the class of severing proteins which fragment actin filaments very efficiently within seconds at even very low concentrations (molar ratio 1:500–1:1000).

To further substantiate these results, the action of 'cap 90' on Triton-extracted fibroblast cytoskeletons was compared side by side with a true 90-kDa actin filament-fragmenting protein purified from smooth muscle (fig.4). When compared with buffer-treated control cells (fig.4a) the incubation of Triton-extracted cell models with a 90-kDa actin-fragmenting protein leads to a severing of actin filaments and hence to a massive destruction of the actin cytoskeleton within 5 min (fig.4b), whereas the 'cap 90', as a true capping but not fragmenting protein, has no effect even after a prolonged incubation time (fig.4c). The binding of 'cap 90' to only the 'barbed' end of actin filaments could directly be demonstrated (fig.5).

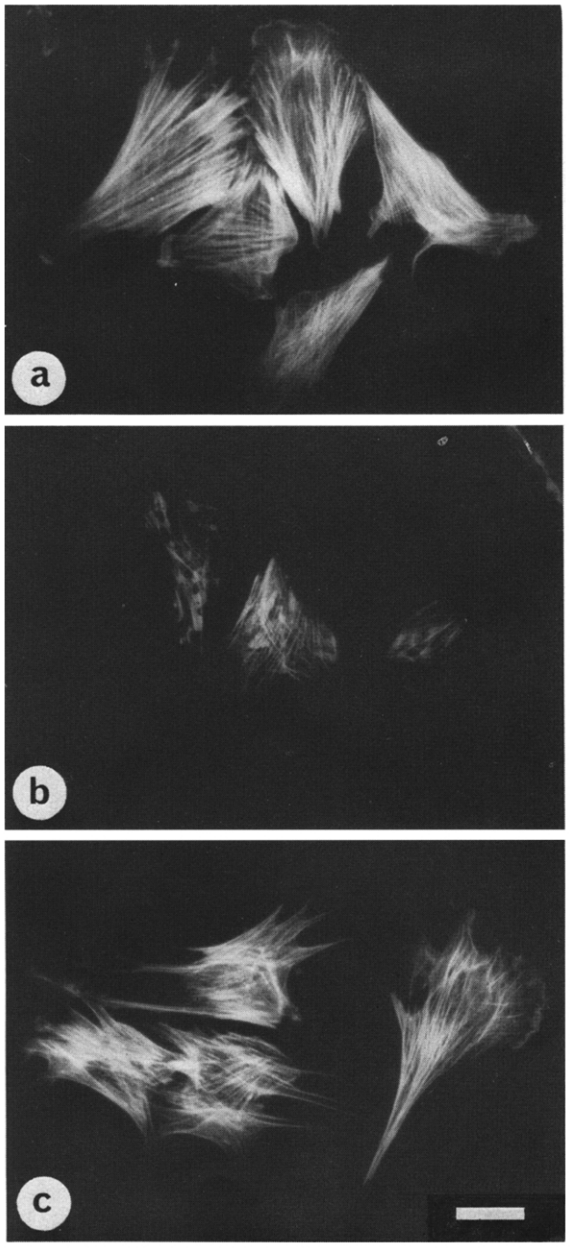

Fig.4. Triton-extracted cytoskeletons stained specifically for F-actin with rhodamine conjugated phalloidin after buffer treatment; control (a); 5 min incubation with an actin filament-fragmenting protein (40 μg/ml) (b); and 'cap 90' (66 μg/ml) (c). Magnification × 120, the bar indicates 100 μm.

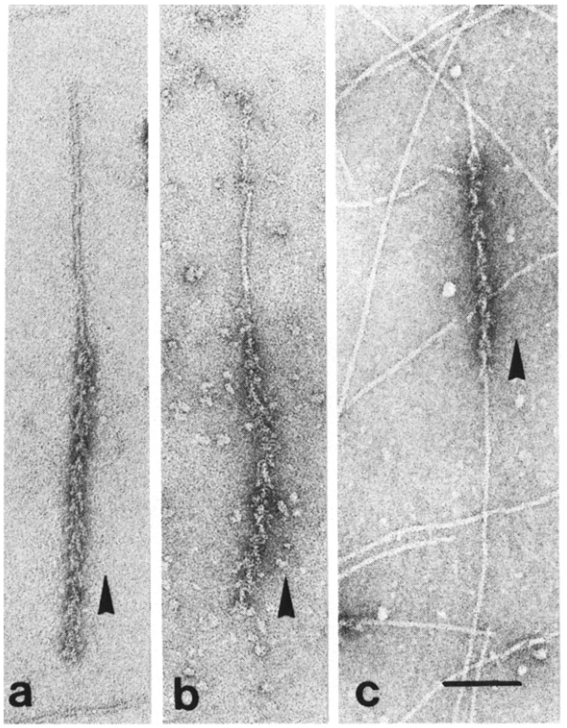

Fig.5. Binding of 'cap 90' to the ends of actin filament fragments decorated with S-1. (a,b) The growth of actin filaments at the 'barbed' ends is blocked in the presence of 'cap 90'; (c) control: actin filament fragments grow bidirectionally in the absence of 'cap 90'. Magnification × 200000; the bar represents 0.05 μm.

4. DISCUSSION

We have shown here that 'cap 90', a Ca^{2+}-dependent F-actin-capping protein from bovine brain, can be relatively rapidly obtained in its pure, native form by using DNase-I affinity chromatography, ion-exchange chromatography and gel filtration. Probably, the 'cap 90' binds to DNase-I as a 1:1 complex with actin [8], from which the pure 'cap 90' is released during elution from the DNase-I column with 8.8 M formamide.

By two different experimental approaches we found that 'cap 90' is not an actin filament fragmenting protein: Only very high molar ratios of 'cap 90' to actin lead to a pronounced decrease in filament length, since then:

(i) nucleation of filament growth is enhanced, resulting in a rapid depletion of actin monomers, whereas
(ii) filaments with a 'barbed', capped end will simultaneously depolymerize by a net loss of actin monomers at their slow growing ends [16].

The nevertheless rapid decrease of the low shear viscosity of F-actin at even much lower concentrations of 'cap 90' may reflect slight changes in filament lengths, too small to be detected by electron microscopy, as well as the loss of end-to-side interactions between filaments [5,17] which apparently influence the viscosity of actin networks considerably.

In order to discriminate clearly between actin filament capping and actin filament fragmenting activity, we have designed an easy experimental test: severing or fragmenting proteins, which by their high affinity for actin monomers attack single filaments along their entire length, are still able to act efficiently on Triton-extracted cytoskeletons, leading to the observed destruction of actin meshworks and stress-fibers. True capping proteins, however, once bound to the fast-growing end of actin filaments will not induce any depolymerization, unless actin monomers can disassemble at the opposite filament end. Since in the Triton model a dissociation of actin monomers from a filament no longer occurs, capping proteins, though probably bound to the filament end, have no effect on the actin cytoskeleton, whereas they potently disassemble actin filament-containing structures when microinjected into living cells [4].

ACKNOWLEDGEMENTS

We thank Professor Theodor Wieland for a gift of rhodamine-coupled phalloidin. Dr Horst Hinssen showed his results on a 90 kDa protein from smooth muscle (Hinssen, H., Sobieszek, A. and Small, J.V. submitted). We thank Dr Jim Bee for culturing heart fibroblasts and Dr A. Sobieszek for the gift of S-1. G.I. is recipient of a grant from the Deutsche Forschungsgemeinschaft, Is 25/3-1.

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