Modulation of gelsolin-induced actin-filament severing by caldesmon and tropomyosin and the effect of these proteins on the actin activation of myosin Mg^{2+} -ATPase activity

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We have investigated the cumulative effects of three smooth-muscle actin-binding proteins, gelsolin, caldesmon and tropomyosin, on actin activation of myosin Mg^{2+} -ATPase activity under low-ionic-strength conditions. A combination of tropomyosin (at a stoichiometric ratio to actin) and gelsolin (at a molar ratio to actin of up to 1:100) showed essentially additive stimulatory effects that were counteracted by caldesmon. Suppression of the gelsolin-induced activation of the ATPase by caldesmon was higher in the presence of tropomyosin although it was not complete even at stoichiometric amounts of both proteins to actin. Since activation of actin-activated ATPase activity of myosin by gelsolin is related to its severing action, it is concluded that caldesmon and tropomyosin cannot fully

protect actin filaments against the severing activity of gelsolin. Direct analysis of the actin-severing activity of gelsolin by a fluorimetric assay using pyrene-labelled actin confirmed this conclusion. Tropomyosin and caldesmon in saturating amounts relative to actin inhibited the activity of gelsolin by between 21 and 40 % and 25 and 48 % respectively, depending on the molar ratio of gelsolin to actin. The inhibitory effect was increased with a combination of both (up to 67 %) although it was evident that even under these conditions the actin filaments were not fully protected from being severed by gelsolin. These findings were corroborated by electron-microscopic investigation of actin filaments with or without tropomyosin and caldesmon after the addition of gelsolin.

INTRODUCTION

Actin filaments are present in both muscle and non-muscle cells and play an important role in motility phenomena and maintenance of the three-dimensional integrity of the cytoskeleton. Their state is affected by a number of actin-binding proteins [1,2]. Among them is gelsolin, which severs actin filaments and can thereby reduce the polymer size of F-actin at substoichiometric amounts [3]. After the severing process, gelsolin remains bound to one of the fragments and caps its fast polymerizing end [3,4]. Gelsolin is Ca^{2+} -regulated, and, in addition to its severing effect, it also promotes the nucleation step of actin polymerization, possibly by forming ternary complexes with G-actin molecules [3,4]. In smooth muscle, gelsolin is present in relatively high concentrations comparable with those of non-muscle cells rather than skeletal-muscle cells [5].

Tropomyosin and caldesmon, on the other hand, are major components of smooth muscle and non-muscle thin filaments. Both are rod-shaped molecules aligned laterally along the whole length of the actin filaments [6,7] and therefore they probably have a stabilizing effect on the structure of the actin polymer.

Considering the possible functions of these actin-binding proteins *in vivo*, it is of interest how they may affect each other with respect to their interaction with actin. Since all three proteins, gelsolin, caldesmon and tropomyosin, modulate the interaction of actin with myosin, they may directly influence contractile events. It has been shown previously that gelsolin has a stimulatory effect on actomyosin ATPase activity [8] as does tropomyosin [9], whereas caldesmon strongly inhibits it both in the presence and absence of tropomyosin [10–12].

In addition, these two types of actin-binding proteins might be involved in regulation of cytoskeletal architecture. It has been shown by Ishikawa and others [13] that the binding of tropo-

myosin and caldesmon affects the susceptibility of actin filaments to the severing action of gelsolin. High- and low-molecular-mass isoforms of tropomyosin isolated from cultured rat cells had a differential effect of protecting actin filaments from fragmentation by gelsolin. As judged from the average length of filaments treated with gelsolin in the presence of the two isoforms of tropomyosin, high-molecular-mass tropomyosin protected actin filaments to a much greater extent than low-molecular-mass tropomyosin. Non-muscle caldesmon, when added to filaments together with tropomyosin, potentiated its protective effect and, in consequence, full protection was reported in the case of high-molecular-mass tropomyosin. In the presence of the low-molecular-mass tropomyosin isoform and non-muscle caldesmon, actin filaments treated with gelsolin remained significantly longer but did not reach the length of untreated control filaments.

In this paper we have examined the effect of gelsolin on binary complexes of F-actin with caldesmon and F-actin with tropomyosin as well as on the ternary complex of F-actin with tropomyosin and caldesmon with regard to their influence on the Mg^{2+} -ATPase activity of myosin assayed in the presence or absence of gelsolin. To explain the observed effects we have re-investigated the gelsolin-induced fragmentation of actin filaments either alone or with bound tropomyosin, caldesmon or both these proteins together.

A preliminary report of this work has been presented [14].

MATERIALS AND METHODS

Preparation of proteins

Actin was purified from rabbit skeletal-muscle acetone powder as described by Spudich and Watt [15] with an additional gel

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filtration on Sephadex G-150 [16]. For fluorimetric measurements, actin was labelled with *N*-(1-pyrenyl)iodoacetamide by the method of Kouyama and Mihashi [17]. Pyrene-actin was purified by the procedure of Cooper and Pollard [18]. Myosin was obtained from rabbit skeletal muscle as described by Stepkowski et al. [19]. Caldesmon and tropomyosin were prepared from chicken gizzard smooth muscle by the method of Bretscher [20]. Gelsolin was purified from pig stomach smooth muscle [5]. The purity and integrity of the prepared proteins were assessed by SDS/PAGE.

Protein concentration

Protein concentration was determined either by a microbiuret method [21] or spectrophotometrically using absorption coefficient values of 0.63 for actin at 290 nm [22], 0.40 for caldesmon at 276 nm [23], 0.19 for smooth-muscle tropomyosin at 278 nm [24], 0.54 for myosin at 279 nm [25], each at 1 mg/ml and with a 1 cm light path. The relative molecular masses of actin, gelsolin, caldesmon, tropomyosin and myosin were taken to be 42.3, 80, 89, 68 and 470 kDa respectively.

ATPase assays

Actin-activated Mg^{2+} -ATPase activity of skeletal-muscle myosin was determined by mixing 120 μ g/ml myosin and 30 μ g/ml actin or complexes of actin, with either caldesmon or tropomyosin or caldesmon with tropomyosin or gelsolin in a medium containing 2 mM $MgCl_2$, 50 mM KCl, 0.2 mM $CaCl_2$ (or alternatively 1 mM EGTA), and 10 mM imidazole buffer, pH 7.0. The molar ratios of caldesmon and gelsolin to actin are specified in the legends to the Figures. The molar ratio of tropomyosin to actin was 1:6 in all assays. Gelsolin was usually added to actin filaments alone or complexed for 15 min with caldesmon in the absence or presence of tropomyosin with gentle mixing on a Vortex mixer, and the mixture was incubated for 2 min at 25 °C to allow severing. In some experiments gelsolin was added to actin before the polymerization process and then polymerized for 2 h with 100 mM KCl. In this case, caldesmon without or with tropomyosin was added to preformed actin filaments. Myosin was added immediately before the start of the reaction with 1 mM ATP. After incubation for 5 min at 25 °C, the reaction was terminated by addition of 1% SDS, and the phosphate concentration was determined by the method of Fiske and SubbaRow [26].

Fluorimetric determination of actin-filament-severing activity of gelsolin

The actin-filament-severing activity of gelsolin in the presence and absence of caldesmon, tropomyosin or both proteins together was determined fluorimetrically using pyrene-labelled skeletal-muscle actin by a modification of an assay originally described by Bryan and Coluccio [27]. All actin mixtures contained 10% pyrene-actin. Actin (10 μ M) in 10 mM imidazole buffer, pH 7.2, containing 1 mM ATP was polymerized in the presence of 33 nM gelsolin (molar ratio to actin 1:300) by addition of 100 mM KCl, 2 mM $MgCl_2$ and 0.3 mM $CaCl_2$ to prepare precapped actin filaments. After incubation for 2 h at 25 °C for polymerization, either tropomyosin or caldesmon or both were added at molar ratios to actin of 1:5 and 1:3 respectively, and the mixtures were again incubated for 15 min at 25 °C. These (control) samples were then diluted to 200 nM actin, and to measure the depolymerization of actin, the time course of the decrease in fluorescence intensity was monitored with a Shimadzu 5001 PC spectrofluorimeter. To determine the actin-filament-

severing activity of gelsolin in the presence and absence of tropomyosin and caldesmon, the undiluted samples (described above) were mixed with gelsolin at molar ratios to actin between 1:200 and 1:10, and after 120 s of incubation, diluted and immediately measured as above. The initial rates of fluorescence decrease were determined graphically and used to calculate severing activities and inhibition.

Sedimentation assays

Samples containing 1 mg/ml actin and the specified amounts of gelsolin, caldesmon and tropomyosin were centrifuged in an airfuge for 15 min at 207 kPa. Under these conditions only a small amount of actin-gelsolin was found in the pellet, whereas nearly all the F-actin (approx. 90%) was sedimented. After centrifugation, the supernatants were carefully removed and the pellets subjected to SDS/PAGE and densitometric analysis.

Electron microscopy

Samples used in sedimentation assays containing actin filaments or complexes of actin-binding proteins with actin were adjusted to 0.1 mg/ml actin and negatively stained with uranyl acetate using carbon-coated formvar films which were glow-discharged immediately before use. The grids were rinsed with 50 μ g/ml bacitracin before application of sample to improve spreading. The samples were examined with a Zeiss EM9 electron microscope at 60 kV accelerating voltage.

SDS/PAGE and densitometry of polyacrylamide gels

For electrophoresis, the Laemmli buffer system [28] was used with 10% acrylamide and 0.27% bisacrylamide. Gels were stained with Coomassie Blue R 250, and after destaining evaluated by densitometric scanning using a Hewlett-Packard Scanjet II P desktop scanner together with a personal computer and the Quantiscan program (Biosoft, Cambridge, U.K.).

RESULTS

Effect of gelsolin, caldesmon and tropomyosin on the actin stimulation of Mg^{2+} -ATPase activity of myosin

It has previously been reported [8] that gelsolin, because it shortens actin filaments, significantly stimulates actin-activated myosin Mg^{2+} -ATPase activity under conditions of low ionic strength and in substoichiometric amounts to actin. This stimulation of the ATPase was observed only in the presence of Ca^{2+} (Table 1), when gelsolin binds tightly to actin ($K_a \approx 10^{11} M^{-1}$) and severs actin filaments [29]. In reconstituted systems, neither tropomyosin nor caldesmon nor both together significantly affected the Ca^{2+} -sensitivity of gelsolin although the control ATPase activities were different in all cases.

As shown in Table 1, the extent of gelsolin stimulation of actomyosin ATPase depended on whether it was added to G-actin and took part in its transformation to F-actin or was added to preformed actin filaments. As actin filaments formed in the presence of gelsolin were apparently shorter, owing to its promoting effect on actin nucleation [3,4], their activating effect on myosin Mg^{2+} -ATPase was higher (126% of stimulation). Addition of gelsolin to F-actin (polymerized in the absence of gelsolin) resulted in much lower stimulation of myosin Mg^{2+} -ATPase (80%), indicating that the severing activity of gelsolin may be less effective in producing short filaments than its nucleation activity.

As shown previously [9], smooth-muscle tropomyosin also stimulated actin-activated myosin Mg^{2+} -ATPase, and its effect

Table 1 Individual and cumulative effects of caldesmon, tropomyosin and gelsolin on actin-activated Mg^{2+} -ATPase activity of myosin

ATPase activity was assayed as described in the Materials and methods at molar ratios of caldesmon, tropomyosin and gelsolin to actin of 1:10, 1:6 and 1:100 respectively. Gelsolin was added to either preformed actin filaments or G-actin before polymerization. Control ATPase activity of reconstituted actomyosin (without any proteins added) was $0.42 \mu\text{mol}$ of P/min/produced per mg of myosin. Standard deviations were calculated from three to five experiments.

Protein(s) added	Actomyosin ATPase activity (% of control)		
	F-actin		G-actin (+ Ca^{2+})
	– Ca^{2+}	+ Ca^{2+}	
Gelsolin	102 ± 2	180 ± 8	226 ± 9
Tropomyosin	174 ± 12	174 ± 13	175 ± 12
Tropomyosin + gelsolin	170 ± 8	238 ± 9	243 ± 10
Caldesmon	40 ± 6	40 ± 7	41 ± 5
Caldesmon + gelsolin	41 ± 5	81 ± 17	105 ± 17
Caldesmon + tropomyosin	30 ± 8	31 ± 7	30 ± 8
Caldesmon + tropomyosin + gelsolin	31 ± 6	54 ± 23	53 ± 20

was additive to the effect of gelsolin provided that the latter was added to F-actin–tropomyosin complexes (Table 1). On the other hand, the effect of tropomyosin on the ATPase activity of actomyosin reconstituted with F-actin polymerized in the presence of gelsolin was very small (7.5%), possibly because of a reduction in tropomyosin binding to very short filaments. The effect of gelsolin on caldesmon-inhibited actomyosin ATPase measured both in the presence and absence of tropomyosin was also dependent on the length of actin filaments; the inhibition was lower when the filaments were shorter.

Titration of caldesmon-inhibited actomyosin ATPase (at a molar ratio of 1:20 to actin) with gelsolin both in the absence and presence of tropomyosin resulted in a decrease in the inhibitory effect of caldesmon (Figure 1). A similar effect of gelsolin, although less pronounced, was observed at a saturating level of caldesmon (molar ratio to actin of approx. 1:10). The maximum effect of gelsolin on the actomyosin ATPase activity was observed at a gelsolin/actin ratio of 1:100 (mol/mol), independent of the absence or presence of tropomyosin, caldesmon or both proteins together added up to stoichiometric ratios to actin (i.e. 1 caldesmon:2 tropomyosins:14 actin monomers [30]). However, gelsolin-induced stimulation of actomyosin ATPase can be almost completely overcome by the excess of caldesmon (at a molar ratio of 1:2.5 to actin). This indicates that caldesmon alone and together with tropomyosin inhibits actin fragmentation by gelsolin and the degree of the inhibition depends on the ratio of caldesmon to actin.

On the other hand, titration of gelsolin-stimulated actomyosin ATPase activity with caldesmon in either the absence or presence of tropomyosin caused a gradual decrease in gelsolin influence (Figure 2A). The effect was somewhat stronger in the presence than in the absence of tropomyosin. To evaluate the net effect of tropomyosin on the inhibition of actin filament fragmentation by gelsolin, the data were also expressed as a percentage of the changes in actomyosin ATPase evoked by tropomyosin in the absence and presence of gelsolin. As shown in Figure 2(B), gelsolin diminished the potentiation effect of tropomyosin alone or of tropomyosin together with substoichiometric amounts of caldesmon. However, the stimulatory effect of tropomyosin on caldesmon-induced inhibition of the ATPase at saturation levels of caldesmon was not appreciably affected by gelsolin. This indicates that tropomyosin alone is not sufficient to protect actin

Figure 1 Effect of gelsolin on caldesmon-inhibited actomyosin ATPase activity in the absence and presence of tropomyosin

Caldesmon was added at a molar ratio to actin of 1:20 (○, ●), 1:10 (△, ▲) and 1:2.5 (□, ■) in the absence (open symbols) and presence (closed symbols) of tropomyosin; ▽, actomyosin alone; ▴, actomyosin and tropomyosin. ATPase activity was assayed in the presence of Ca^{2+} as described in the Materials and methods section. The data represent means $\pm$ S.D. of at least four experiments.

filaments against the severing activity of gelsolin and that caldesmon, which strengthens the binding of tropomyosin to actin [11], significantly increases the protective effect.

Influence of caldesmon and tropomyosin on the modulation of actin filament length by gelsolin

When gelsolin-precapped actin filaments (containing 10% pyrene-actin) are severed by gelsolin, the extent of severing can be monitored from the decrease in the rate of fluorescence after dilution below the critical concentration of actin. The additional filaments generated by the action of gelsolin led to a net increase in the release of actin monomers from the ends of the filaments (expressed as depolymerization rate in Table 2). The inhibition of gelsolin activity by caldesmon and tropomyosin was calculated from the ratio of depolymerization rates measured in the presence and absence of the respective protein. For F-actin alone it may be assumed that, with the gelsolin to actin ratio of 1:300 used for precapping, the hypothetical average precapped filament was approximately 300 monomers long. Hence when gelsolin was added to these filaments at a molar ratio of e.g. 1:10, it may be expected that 30 cuts are made per average filament provided that every gelsolin molecule cuts. This would lead to an increase in depolymerization rate of a factor of 30. It is seen from Table 2 that the actual increase was much lower and the efficiency of severing apparently lower since only a 7-fold rate increase was measured at this molar ratio. The severing efficiency decreased with increasing gelsolin concentration. To calculate the inhibition of severing by tropomyosin and caldesmon, the depolymerization rates observed without severing had to be taken into account: whereas the depolymerization rates in the presence of tropomyosin and caldesmon alone were almost identical with those of

Figure 2 Effect of caldesmon on gelsolin-stimulated actomyosin ATPase activity in the absence and presence of tropomyosin

(A) Gelsolin was added at a molar ratio to actin of 1:100 in the absence (○) and presence (●) of tropomyosin; ▽, actomyosin alone; ▼, actomyosin and tropomyosin. ATPase activity was measured as described in the legend to Figure 1. Results are means $\pm$ S.D. (B) The net effect of tropomyosin on caldesmon-induced inhibition of actomyosin ATPase activity in the absence (□) and presence (▨) of gelsolin, expressed as a percentage of the increase in actomyosin ATPase activity (actomyosin + tropomyosin/actomyosin; actomyosin + tropomyosin + gelsolin/tropomyosin + gelsolin).

Table 2 Inhibition of gelsolin-severing activity by tropomyosin and caldesmon

Actin (10 μ M final concentration, containing 10% pyrene-actin) was polymerized in the presence of 0.033 μ M gelsolin (for precapping) in buffer containing 1 mM ATP, 2 mM $MgCl_2$, 0.1 M KCl, 0.3 mM $CaCl_2$ and 10 mM imidazole, pH 7.2, for 2 h at 25 °C. To the respective samples tropomyosin and caldesmon were added at molar ratios to actin of 1:5 and 1:3, and the mixture was incubated for 15 min at 25 °C. Gelsolin was then added at various molar ratios to actin, and after 2 min to allow for severing, the mixture was diluted into 0.1 M KCl/2 mM $MgCl_2$ /0.3 mM $CaCl_2$ /1 mM ATP/10 mM imidazole, pH 7.2, to a final concentration of 200 nM actin. The initial depolymerization rate after dilution was calculated from the decrease in fluorescence. The increase in depolymerization rate after addition of gelsolin was used to measure severing and to determine the severing efficiency. The relative inhibition of severing was calculated from the decrease in depolymerization rates as caused by tropomyosin, caldesmon or both. To compensate for the variation in depolymerization rates of the unsevered (control) actin filaments depending on the actin-binding protein added, the inhibition had to be normalized. Therefore the term 'inhibition of severing' expresses the ratio of depolymerization rates between actin alone and the complexes after normalization against the samples without additional gelsolin.

Gelsolin/actin (mol/mol)	Actin depolymerization rate (nM/min)	Actin + caldesmon		Actin + tropomyosin		Actin + caldesmon + tropomyosin	
		Depolymerization rate (nM/min)	Inhibition of severing (%)	Depolymerization rate (nM/min)	Inhibition of severing (%)	Depolymerization rate (nM/min)	Inhibition of severing (%)
—	5.25	5.25	—	5.5	—	2.75	—
1:200	8	6	24.5	6.5	21	3.25	28
1:100	12.5	7.25	41	8.5	33	4.75	30
1:50	14.75	9.25	42	10	34	5.5	34
1:20	24.75	13.75	44	15.25	39	6.25	54
1:10	36	18.75	48	22	40	6.5	67

actin alone, it was only about half for the mixture of the two proteins.

Caldesmon and tropomyosin at molar ratios to actin above nominal saturation decreased the depolymerization rates significantly at all gelsolin to actin ratios investigated, indicating inhibitory effects of both proteins. When the relative inhibitions were calculated from the depolymerization rates, the effect of caldesmon was slightly higher than that of tropomyosin alone but the effect was significantly higher with the caldesmon/

tropomyosin combination (Table 2). On the other hand, under none of the experimental conditions used was complete inhibition of severing observed.

The relative inhibition increased with the concentration of gelsolin, i.e. it rose from 28 to 67% in the presence of both tropomyosin and caldesmon (Table 2). This indicates that, in contrast with actin alone, only a limited number of potential severing sites was available on the composite filaments. Therefore at higher concentrations of gelsolin, the relative inhibition was

Figure 3 Electron microscopy of actin filaments in the presence of tropomyosin and caldesmon before and after addition of gelsolin

Tropomyosin and caldesmon were added to F-actin (0.1 mg/ml) at molar ratios of 1:6 and 1:5 respectively. After 30 min of incubation, gelsolin was added at a molar ratio of 1:25 relative to actin. Samples were then negatively stained with uranyl acetate. (A), (C), (E) Controls, no gelsolin; (B), (D), (F), (G) samples containing gelsolin. (A), (B) Actin + tropomyosin; (C), (D) actin + caldesmon; (E), (F) actin + tropomyosin + caldesmon; (G) actin + gelsolin.

progressive. This may be explained by the fact that, even at high molar ratios of tropomyosin and caldesmon well above saturating concentrations, some regions on the actin filament may be either temporarily or permanently free and therefore accessible to gelsolin. Alternatively, the binding of caldesmon and tropomyosin may not always fully cover the binding sites for gelsolin. It is conceivable that not all regions of the elongated molecules are in permanent contact with the actin filament and may therefore leave occasional gaps for the access of gelsolin.

These results were in part corroborated by electron-microscopic investigations under conditions comparable with those used for the fluorimetric experiment (Figure 3). A significant shortening of filaments by gelsolin was observed even in the presence of tropomyosin and caldesmon, although the filaments with caldesmon were found to be shorter than with tropomyosin and almost as short as in the control sample (Figure 3G). Measurements of filament length on electron micrographs gave average values of 0.12 μm for the actin–gelsolin control, 0.23 μm for actin–caldesmon–gelsolin, 0.31 μm for actin–tropomyosin–caldesmon and 0.68 μm for actin–tropomyosin–caldesmon–gelsolin (between 110 and 180 filaments were measured for each sample). In the presence of caldesmon, actin tended to form bundles [10,20] (Figure 3C). These bundles largely disappeared after addition of gelsolin, only small pieces of bundle-like aggregates being observed (Figure 3D). Hence the bundling

Figure 4 Effect of gelsolin on sedimentation of actin in the presence of tropomyosin and caldesmon

Gelsolin (molar ratio to actin 1:25) was added to either F-actin (left), which had been preincubated with tropomyosin and caldesmon, or G-actin (right), which was then polymerized, and thereafter incubated with tropomyosin and caldesmon. The molar ratios of tropomyosin and caldesmon to actin were 1:6 and 1:5 respectively, and actin concentration was 1 mg/ml. After centrifugation in an airfuge and SDS/PAGE of the pellets, the relative amounts of actin pelleted (as a percentage of total actin) were determined by densitometric quantification. $\square$, Actin alone; ▨ , actin + tropomyosin; ▩ , actin + caldesmon; ▧ , actin + tropomyosin + caldesmon. The data represent mean values from two experiments.

alone did not inhibit the severing activity of gelsolin. With a combination of tropomyosin and caldesmon the situation was significantly different, since many bundles were still found after the addition of gelsolin. However, some severing activity was observed in these samples also since outside the bundles shorter filaments were also visible (Figure 3F).

When the samples used for electron microscopy (before 10 times dilution) were subjected to high-speed centrifugation, the amounts of actin found in the pellets differed significantly (Figure 4). Under the conditions applied, only 5% of F-actin was pelleted in the presence of gelsolin, whereas without gelsolin 90% of actin was found in the pellet (not shown). Again, tropomyosin and caldesmon individually increased the amount of actin in the pellet, but much more so when in combination. The situation was somewhat different when gelsolin was added to actin before polymerization, and both caldesmon and tropomyosin were added subsequently. Under these conditions the relative amount of actin in the pellets of the sample with caldesmon was higher than before and that of the sample with tropomyosin was lower than before. However, in the sample containing both caldesmon and tropomyosin, about the same amount of actin was pelleted.

DISCUSSION

Our experiments show that the three actin-binding proteins tropomyosin, caldesmon and gelsolin mutually affect each other in their effects on the actin activation of myosin Mg^{2+} -ATPase activity.

The Ca^{2+} -dependent influence of gelsolin on the ATPase activity may be exclusively attributed to its modulation of the actin filament length. Shorter actin filaments apparently provide more favourable sterical conditions for the stimulation of ATPase activity by gelsolin under low-ionic-strength conditions, when

myosin filaments tend to aggregate and precipitates of actomyosin are formed [8]. In this respect it should be noted that the stimulatory effect of gelsolin is observed only under conditions of actomyosin ATPase assay, i.e. in an insoluble system. Acto-subfragment 1 ATPase is not stimulated by gelsolin but, at high ratios of gelsolin to actin, is inhibited both in the presence and absence of tropomyosin and caldesmon (results not shown) (see also ref. [8]). This gives additional evidence that the sterical arrangement of filaments under precipitating conditions as they occur during actomyosin ATPase measurements are modified by shorter actin filament length.

Gelsolin-induced stimulation of actomyosin ATPase activity was significantly reduced in the presence of high caldesmon concentration. However, direct measurements of the gelsolin severing activity with the actin–caldesmon complex showed that the relative protection by caldesmon against severing was only 25–48% (depending on the gelsolin concentration) under these conditions when compared with the gelsolin-treated F-actin control. Furthermore electron micrographs revealed no dramatic changes in actin filament length under these conditions relative to gelsolin-treated F-actin control. This discrepancy may be explained by the effect of caldesmon on the binding of gelsolin to actin. According to the atomic model of actin complexed with gelsolin segment 1, which plays a central role in the severing and capping activities of gelsolin, the cleft between subdomain 3 and subdomain 1 of actin serves as a docking region for gelsolin [31]. Therefore the association of caldesmon with the C-terminal region of actin [32,33], which is located near the cleft, may induce conformational changes in actin and affect its interaction with gelsolin.

Similarly, the C-terminus of the actin molecule contains the binding site for tropomyosin [34]. Therefore the additive enhancement of gelsolin-induced actomyosin ATPase activity by tropomyosin can be attributed to conformational changes in the actin filament by tropomyosin that are independent of the filament length. On the other hand, decreasing gelsolin-induced severing of actin filaments (Table 2) and increasing the length of the resulting fragments by tropomyosin (Figure 3B) can result from the direct binding between gelsolin and tropomyosin [35] as well as from reannealing of the resulting fragments [36].

When both caldesmon and tropomyosin are bound to actin, their effect on actin filament stability is significantly increased. In this case the mutual interaction between these two proteins also strengthens their bonds with the actin filament [11] and probably prevents gelsolin from finding its target-binding site on actin. Reversal of the severing action of gelsolin by tropomyosin coupled with caldesmon can result from dissociation of gelsolin from the capped ends of short filaments which enable their reannealing [36]. Simultaneously caldesmon, which has the capacity to nucleate actin polymerization [37,38], may induce formation of long actin filaments *de novo* from depolymerized actin.

However, even under saturating levels of both caldesmon and tropomyosin we found a residual severing activity of about 30–35%. It is possible that the caldesmon–tropomyosin–actin complex is not entirely stable, and that, owing to an occasional ‘opening up’ of the assembly, the binding sites on the actin filaments for gelsolin become available and this finally leads to their fragmentation. In this respect our results are at variance with the data of Ishikawa et al. [13] who observed complete inhibition of gelsolin activity in the presence of smooth-muscle caldesmon and tropomyosin. This difference could be due to either different binding affinities of the tropomyosin and caldesmon used or the different methods of determining the severing activity of gelsolin. The method we have used gives a

more direct result than the flow birefringence studies performed by Ishikawa et al. [13]. It is worth mentioning that recently published models of thin filaments take into account the possibility that part of the caldesmon molecule (N-terminal domain) can be released and tethered to the main body of the actin filament [39]. This region of the filament may be the area that is susceptible to severing.

It should be noted that actin filaments formed in the presence of gelsolin, and therefore shorter than gelsolin-severed filaments, interacted only weakly with caldesmon and tropomyosin. This is suggested by the lack of stimulatory effect of tropomyosin and the weak inhibitory effect of caldesmon on the ATPase activity of actomyosin reconstituted from these very short filaments (Table 1). However, caldesmon seems to be more effective than tropomyosin in increasing their length, since the amount of actin filaments pelleted in the presence of caldesmon is higher than that pelleted in the presence of tropomyosin (Figure 4).

The decrease in caldesmon-induced inhibition of actin-activated Mg^{2+} -ATPase activity of myosin by gelsolin may be relevant to the actin-linked regulation of smooth-muscle contraction. It has been suggested that, by reducing caldesmon inhibition of the ATPase, gelsolin present in native thin filaments (at a molar ratio to actin of about 1:100) increases the potency of Ca^{2+} /calmodulin in reversing this inhibition [40].

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