Ca-dependent binding of actin to gelsolin

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Abstract **Ca^{2+} of 0.3–1.0 μM induces both the exposure of tryptic cleavage sites within the gelsolin molecule inaccessible in the Ca-free conformation, and binding of one actin monomer to the N-terminal half of gelsolin. On the other hand, gelsolin-induced enhancement of pyrene actin fluorescence was observed only above 50 μM Ca^{2+}, and a ternary actin/gelsolin complex preformed in 200 μM Ca^{2+} was stable only above 30 μM Ca^{2+}. These results provide direct evidence for Ca-induced transitions from closed to open conformation of the gelsolin molecule in the range of 3×10^{-7} to 10^{-6} M Ca^{2+}. They also suggest that $Ca^{2+} > 10^{-5}$ M is required to stabilize actin–actin contacts in the 2:1 actin/gelsolin complex.**

Key words: Gelsolin; Actin; Ca-dependence; Conformational change; Complex formation; Limited proteolysis

1. Introduction

Interaction of actin with gelsolin[1] is strongly Ca-dependent. At $Ca^{2+} \leq 10^{-7}$ M, no interaction has been observed. In the presence of Ca^{2+}, mammalian gelsolin forms a ternary (1:2) complex with globular actin which dissociates into a binary complex and actin upon addition of EGTA [2]. This implies that Ca^{2+}-binding changes the structure of gelsolin, rendering it more suitable for interaction with actin. Indeed, Ca-induced structural changes of both the whole gelsolin molecule and its C-terminal half were revealed by dynamic light scattering, circular dichroism, intrinsic fluorescence [3–8] and limited proteolysis [6,8–10]. It was also shown that the packing of individual segments in the gelsolin molecule is Ca-dependent, and segment shifts in response to Ca^{2+} have been proposed [11,12]. These data suggest that Ca^{2+} induces structural rearrangements of the C-terminal part of gelsolin which open up the molecule and allow actin to be bound. However, there is a discrepancy about the concentration of Ca^{2+} required for this process. Gelsolin was shown to undergo conformational changes at 10^{-7}–10^{-6} M Ca^{2+} [6,7] whereas severing of actin filaments and nucleation of actin polymerization occur at $\sim10^{-5}$ M [13–15]. The crucial point in the line of events: structural transition in gelsolin – binding of actin – severing/nucleation is the interaction of gelsolin with two actin monomers.

The aim of this work was to correlate the opening of gelsolin to the binding of one and two actin monomers, monitoring Ca-dependent conformational changes of gelsolin by limited proteolysis, and revealing the formation of ternary complexes by gelsolin-induced enhancement of pyrene actin fluorescence. Our results provide direct evidence that binding of 0.3–1.0 μM Ca^{2+} induces transition from the closed to open conformation of gelsolin with tryptic fragments of 70 and 45 kDa being generated which are characteristic of either conformation. However, a stable binding of the second actin monomer to gelsolin was observed only above 30 μM Ca^{2+}. Since gelsolin-induced enhancement of pyrene actin fluorescence occurred only above 50 μM Ca^{2+} we suggest that $Ca^{2+} > 10^{-5}$ M stabilizes the 2:1 actin/gelsolin complex by strengthening the contacts between actin monomers.

2. Materials and methods

2.1. Protein preparations

Gelsolin was purified from pig stomach smooth muscle [16] and stored as ammonium sulfate precipitate in liquid nitrogen. Before use, the precipitate was dissolved and dialyzed against PSAM buffer (10 mM imidazole, 0.5 mM EGTA, 0.2 mM DTT, 2 mM NaN_3, pH 7.0). Rabbit skeletal muscle actin was prepared according to [17] with an additional gel filtration step on Sephadex G-150. G-actin in buffer G (0.2 mM ATP, 0.1 mM $CaCl_2$, 0.4 mM β-mercaptoethanol, 1 mM NaN_3, 5 mM Tris–HCl, pH 8.2) was used within 1 week. Actin was labelled with *N*-(1-pyrenyl)iodoacetamide at Cys 374 [18]. Pyrene actin was lyophilized in the presence of 2 mM sucrose and stored at −70°C. Before use, lyophilized pyrene actin was dissolved and dialyzed over night against buffer G.

2.2. Gel chromatography

Analytical gel chromatography was performed using two columns of Superdex 200 H/R 10/30 (Pharmacia) mounted in tandem on a Pharmacia FPLC-System. To prepare the 2:1 actin/gelsolin complexes, 0.5 mg of G-actin (in buffer G) was mixed with 0.5 mg of gelsolin (in PSAM buffer) and Ca^{2+} was adjusted to 0.2 mM. 100 μl (100 μg protein) aliquots of the mixture were applied to the columns equilibrated with 0.1 M KCl, 10 mM imidazole, pH 7.0, and $CaCl_2$/EGTA at various ratios and eluted with a corresponding buffer at 0.3 ml/min.

2.3. Limited proteolysis

Gelsolin (0.5 mg/ml) either alone or as a complex with actin (0.5 mg/ml) was digested with trypsin at an enzyme/protein mass ratio of 1:5 at 22°C. At different time points, the digestion was terminated by soybean trypsin inhibitor and the samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.4. SDS–PAGE

SDS–PAGE was performed using 15% acrylamide/0.1% bisacrylamide gels in the Laemmli buffer system [19].

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[1] Gelsolin amino acid numbering used in this work is based on the horse plasma gelsolin amino acid sequence [1].

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2.5. Sequence analysis

Gelsolin fragments after limited proteolysis were separated by SDS–PAGE and transferred to PVDF-membrane by semi-dry electroblotting. The blots were transiently stained with Ponceau S and the bands of interest were cut out and subjected to automatic Edman degradation using a Knauer sequencer.

2.6. Fluorescence measurements

Fluorescence of pyrene actin was measured at 407 nm after excitation at 365 nm in a Shimadzu PC 5000 fluorometer.

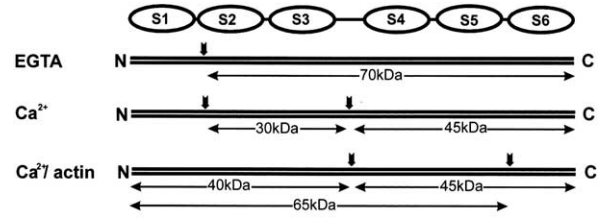
2.7. Molecular modelling and calculations of Ca^{2+}

The program Swiss Protein Database Viewer was used for displaying and labelling the molecular structure of gelsolin. [Ca^{2+}] in Ca/EGTA mixtures was calculated using the program EQCAL (Biosoft, Cambridge, UK) with regard to Mg^{2+}, ATP and pH. The respective stability constants (taken from the works of Sillen and Martell) were included in the program.

3. Results

3.1. Effect of Ca^{2+} on accessibility of gelsolin to limited proteolysis

In the absence of Ca^{2+} proteolysis of pig cytoplasmic gelsolin with trypsin was strictly limited to only one site resulting in a major ~70 kDa fragment fairly resistant to further proteolytic breakdown (Fig. 1). In 0.2 mM $CaCl_2$, cleavage at this site was accelerated and numerous new fragments appeared, indicating that several additional sites were exposed (Fig. 1). There were two major fragments of about 45 and 30 kDa. The N-terminal sequence of the 70 kDa fragment was determined as HVVPNE corresponding to a cleavage between Lys 150 and His 151. The site is located in segment 2 but close to the loop connecting segment 1 and segment 2 (Fig. 2). The N-terminal sequence of the 45 kDa fragment was determined as VPFDAA corresponding to cleavage between Arg 392 and Val 393, located within the loop connecting segments 3 and 4. The 30 kDa fragment seems to be produced when the 70 kDa fragment is cleaved at Arg 392 (Fig. 2). Thus, the results of trypsinolysis provide direct experimental evidence for a Ca^{2+}-induced structural transition of the gelsolin molecule from the closed to open state [11,12]. This transition involves exposing the sites both in segment 2 and within the loop connecting segments 3–4. The generation of either the single 70 kDa fragment or the 45 kDa fragment may serve as specific marker to characterize the EGTA- or Ca-conformation of gelsolin, respectively.



Fragment (kDa)	Conditions		N-ternimal sequence of the tryptic fragment	Cleavage site
	Ca^{2+}	Actin		
70	-/+	-	HVVPNE	Lys150-His151
45	-	-/+	VPFDAA	Arg392-Val393
40	+	+	VVEHPEFLKA	Arg392-Val393
65	+	+	VVEHPEFLKA	(Lys634-Met635)

Fig. 2. Ca-dependent tryptic cleavage of gelsolin and actin/gelsolin complex, and N-terminal sequences of generated fragments. The cleavage sites in the presence and absence of Ca^{2+} and actin, respectively, are marked by arrows. The relation of the cleavage sites to the gelsolin segments S1–S6 is shown above. N-terminal sequences of the major peptide fragments generated by tryptic cleavage are shown below together with the corresponding cleavage sites.

3.2. Ca-dependent cleavage of gelsolin in gelsolin/actin complexes

Interaction with actin changed the gelsolin proteolysis pattern (Fig. 3). Generation of the 70 kDa fragment was strongly inhibited. At the same time, in addition to the 45 kDa fragment, a major fragment of about 40 kDa was now produced.

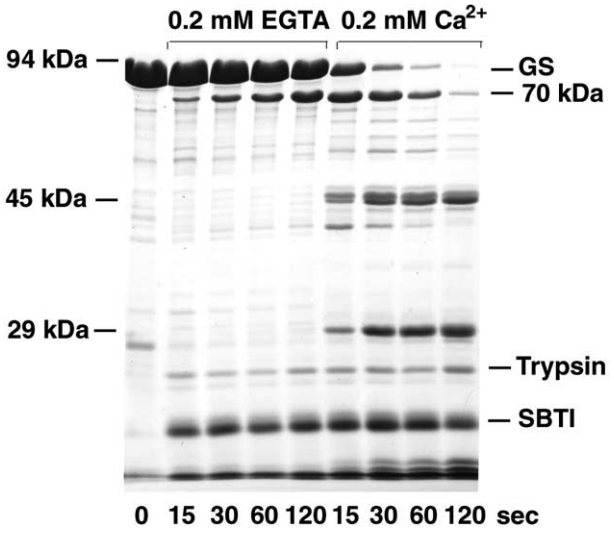


Fig. 1. Effect of Ca^{2+} on susceptibility of pig cytoplasmic gelsolin to tryptic proteolysis. 12 μM gelsolin in PSAM-buffer was cleaved with trypsin directly or in the presence of 0.2 mM $CaCl_2$ added over EGTA at an enzyme:protein mass ratio of 1:5. Proteolysis was stopped with soybean trypsin inhibitor (SBTI), and the samples were analyzed by SDS–PAGE.

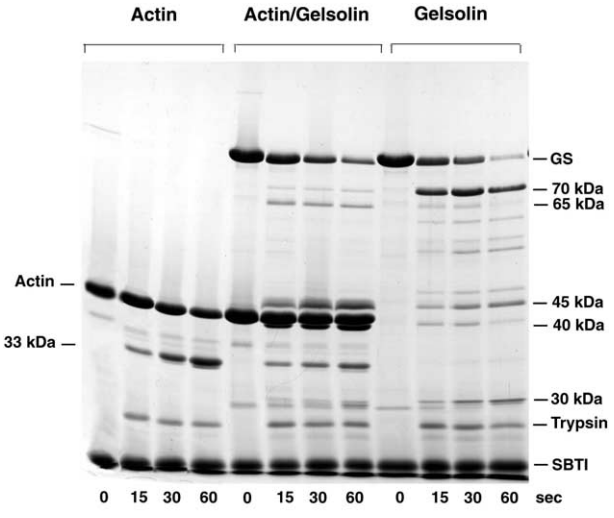


Fig. 3. Tryptic proteolysis of actin/gelsolin 1:2 complexes. 12 μM skeletal muscle G-actin (in buffer G) was mixed with 6 μM gelsolin (in PSAM buffer), and Ca^{2+} was adjusted to 0.2 mM. The actin/gelsolin mixture as well as 12 μM actin and 6 μM gelsolin alone were digested with trypsin as described in the legend to Fig. 1 and analyzed by SDS–PAGE.

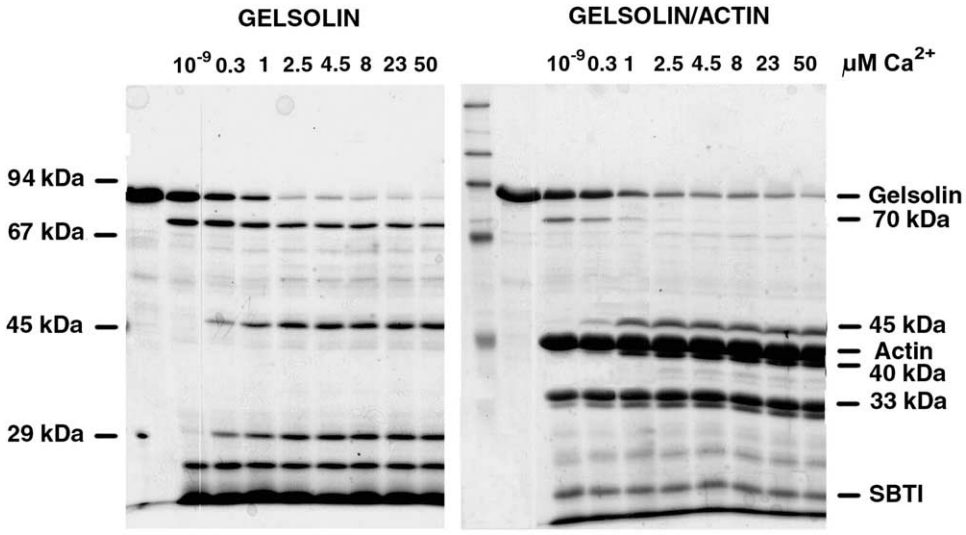


Fig. 4. Susceptibility of gelsolin and actin/gelsolin complexes to trypsinolysis at different Ca^{2+} concentrations. Gelsolin, actin and gelsolin/actin complexes were cleaved with trypsin as in Fig. 1 and analyzed by SDS–PAGE. Adjustment of Ca^{2+} in the mixtures was made by adding different concentrations of $CaCl_2$ to 0.2 mM EGTA.

The N-terminal sequence of this fragment (Fig. 2) corresponds to the N-terminal sequence of gelsolin revealing that the 40 kDa fragment is the N-terminal half of gelsolin. This fragment appears to be produced by the cleavage between Arg 392 and Val 393 within loop 3–4 while binding of actin monomer inhibits the cleavage between Lys 150 and His 151 (Fig. 2).

In the presence of actin, only one transient gelsolin fragment of about 65 kDa was formed. The N-terminal sequence of this fragment also corresponds to the N-terminal sequence of gelsolin molecule (Fig. 2). Hence, the peptide of about 15 kDa that is cleaved off is located in the C-terminal part of gelsolin. The 15 kDa fragment can be produced from the C-terminal half of gelsolin by tryptic cleavage between Lys 634 and Met 635 (unpublished data). This site is located within the loop connecting segments 5 and 6 (Fig. 2). Therefore we assume that the 65 kDa fragment of the whole gelsolin molecule observed in the presence of actin is produced by cleavage at the same site and comprises segments 1–5. Binding of actin seems to change the conformation of this part of the gelsolin molecule thereby exposing the cleavage site within loop 5–6.

3.3. Ca-sensitivity of conformational transitions in gelsolin

The Ca-dependent changes in the pattern of trypsinolysis initially appeared at 0.3 μM Ca^{2+}. They were enhanced at 1 μM Ca^{2+} and became independent of Ca^{2+} concentration above 2.5 μM (Fig. 4A). Similar proteolysis patterns were also obtained at 50–1000 μM Ca^{2+} (not shown). In the presence of actin, inhibition of proteolysis at Lys 150 was observed in the same range of Ca^{2+} concentrations (Fig. 4B), indicating that one actin monomer is bound to the N-terminal half of cytoplasmic gelsolin at 0.3–1.0 μM Ca^{2+}.

3.4. Ca-dependence of gelsolin-induced enhancement of pyrenyl actin fluorescence

Previously we have shown that binding of the second pyrene actin monomer to gelsolin under non-polymerizing conditions resulted in fluorescence enhancement. This effect was not observed if the second monomer was not labelled or upon interaction of pyrene actin with the C-terminal half of gelsolin [20]. This indicates that the enhancement of actin fluorescence in ternary actin/gelsolin complexes is mainly caused by actin–actin interaction. Therefore we used this approach to investigate how binding of the second actin within the complex depends on Ca^{2+}. Fig. 5 demonstrates that the major fluorescence increase upon addition of gelsolin to pyrene actin at a 1:2 molar ratio occurred only above 50 μM Ca^{2+}. The effect was independent of whether actin and gelsolin were mixed in the presence of EGTA or Ca^{2+}. These results do not reveal whether or not the second actin monomer is bound to gelsolin at low Ca^{2+}. However, they clearly show that specific interactions within the ternary actin/gelsolin complex occur only at high Ca-concentration.

3.5. Stability of the actin/gelsolin complexes

Ternary and binary actin/gelsolin complexes can be distinguished by their mobility in high resolution gel filtration [21].

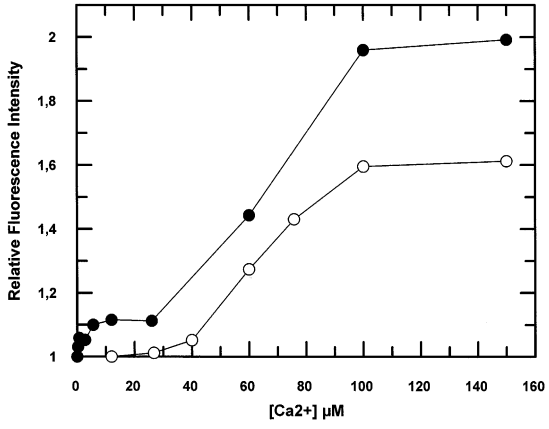


Fig. 5. Effect of Ca^{2+} on gelsolin-induced increase of pyrene actin fluorescence. 2.4 μM actin and 1.2 μM gelsolin were mixed in the presence of either 12 μM $CaCl_2$ or 200 μM EGTA. The Ca-concentration in the mixtures was increased by addition of $CaCl_2$. The data were normalized to the initial fluorescence of the pyrene actin–gelsolin mixture.

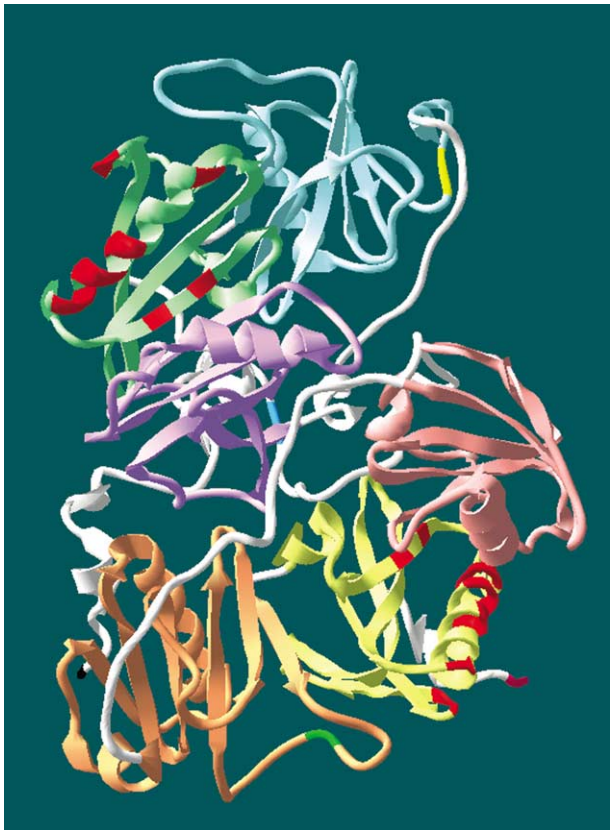


Fig. 6. Localization of the cleavage sites in the gelsolin structure. Three-dimensional model of the gelsolin structure in its Ca^{2+}-free conformation derived from [11]. Individual segments are colored in light shades of yellow (S1), orange (S2), red (S3), green (S4), blue (S5) and magenta (S6). The actin-binding sites in segments 1 and 4 are shown in bright red. The cleavage sites at Lys 150 (green), at Arg 392 (blue), and at Lys 634 (yellow) are also shown. N-terminus and C-terminus of gelsolin are marked in pink and black, respectively.

We used this method to estimate whether at low Ca^{2+} the 2:1 complexes remain stable. Actin and gelsolin were mixed in the presence of 200 μM Ca^{2+} at a 2:1 molar ratio to preform 2:1 complexes. Aliquots of the mixture were applied to gel filtration on Superdex 200. The columns were equilibrated with buffers containing different Ca^{2+} concentrations as given in Table 1. The availability coefficients K_{av} of the complexes in the presence of 200 μM Ca^{2+} and 200 μM EGTA ($Ca^{2+} < 0.01$ μM) were 0.324 and 0.361, respectively. These values were characteristic of the 2:1 and 1:1 actin/gelsolin complexes. At intermediate Ca^{2+}-concentrations (Table 1) distinct peaks were eluted as for the 2:1 and 1:1 complexes but at intermediate elution volumes, leading to the K_{av} values given in the table. Apparently, at $Ca^{2+} < 30$ μM the preformed 2:1 complex was no longer stable during gel filtration and gradually dissociated into a 1:1 complex and free actin. This caused retardation of the eluting peak as compared to that of the 2:1 complex but – since the dissociation occurred gradually during the run – the peaks eluted earlier than the 1:1 complex in EGTA.

Table 1
Gel filtration of preformed 2:1 actin–gelsolin complexes at various [Ca^{2+}] on Superdex 200HR

Protein	Ca^{2+} (μM)	K_{av}
Gelsolin	< 0.01	0.450
Actin:gelsolin, 1:1	< 0.01	0.361
Actin:gelsolin, 2:1	200	0.324
Actin:gelsolin preformed as 2:1 complex	1.3	0.361
	2.6	0.363
	4.3	0.357
	8.0	0.340
	23	0.343
	37	0.325
	51	0.325

4. Discussion

Ca^{2+}-activation of gelsolin appears to include unlatching the gelsolin distal tail helix (AS 744–754) from the N-terminal half of the molecule [8,11] and conformational changes reflected in a multistep response of tryptophan fluorescence [8]. It is generally accepted that these changes provide the conditions for gelsolin–actin interaction. However, it is not yet clear whether both actin monomers are bound at low Ca^{2+} when the observed conformational changes in gelsolin take place. Our experiments attempt to address this question.

Localization of the trypsin cleavage sites in the gelsolin structure [11] highlights several parts of the molecule where Ca^{2+}- and actin-induced conformational changes can take place (Fig. 6). In Ca^{2+}-free gelsolin the site between Lys 150 and His 151 is located in a surface-exposed loop in segment 2 and is apparently the only site susceptible to tryptic digestion. In the presence of Ca^{2+}, the cleavage at this site is accelerated whereas binding of actin inhibits it. Ca-induced acceleration of proteolytic cleavage has previously been shown for chymotrypsin [9] and plasmin [8,10]. Since the loop involving residues 150–151 is exposed already in Ca-free gelsolin (Fig. 6), this acceleration implies that binding of 0.3–1.0 μM Ca^{2+} must affect the conformation of this loop. The effect is apparently abolished by actin binding – possibly by steric hindrance – since the actin monomer is bound relatively close [12,22].

In agreement with the data obtained previously by optical methods [6,7] our proteolysis experiments show that gelsolin undergoes conformational changes from the closed EGTA-conformation to the more open Ca-conformation at 0.3–1.0 μM Ca^{2+}. In Ca-activated gelsolin, several sites become accessible to tryptic cleavage. Along with the site at His 151, the site between Arg 392 and Val 393 within the long loop connecting segments 3 and 4 is efficiently cleaved. In Ca-free gelsolin [11] loop 3–4 adopts a compact surface conformation (Fig. 6) in which the trypsin cleavage site is not exposed. Exposure of this site as well as the sites susceptible to chymotrypsin [9] and thermolysin [23] implies that in Ca-activated gelsolin the loop is extended. This structural transition is consistent with a model [12] in which loop 3–4 is long enough to span the distance between two adjacent actin molecules in the opposite strands of the actin filament. Thus, our data provide direct experimental evidence for Ca-induced shifts in the gelsolin molecule at low Ca^{2+}. Actin-induced inhibition of the cleavage at His 151 indicates that this structural transition is sufficient for one actin monomer to be bound to the N-terminal half of gelsolin.

Evidence for the calcium concentration required for binding of the second actin to gelsolin – which is necessary to efficiently nucleate actin polymerization – is more questionable. The lack of trypsin-sensitive sites in the C-terminal half of

gelsolin did not allow us to reveal Ca-dependent binding of the second actin to gelsolin. The 2:1 actin/gelsolin complex can be detected by gel filtration [21] or by gelsolin-induced enhancement in fluorescence of labelled actin [2,20]. Both approaches showed that at 200 μM Ca^{2+} the 2:1 actin/gelsolin complex was formed and stable. However, a preformed complex became unstable and dissociated into the 1:1 actin/gelsolin complex and actin during gel filtration at $Ca^{2+} < 30$ μM. Similarly, the half-maximal increase of NBD-fluorescence was found to occur at 25 μM Ca^{2+} [15], and a ~2-fold enhancement of pyrenyl-fluorescence was observed above 50 μM Ca^{2+}. These values lie in a range of Ca^{2+} required for gelsolin nucleating [13,15], capping [24] and severing [25] activities.

Thus, none of these approaches reveals whether or not the second actin is bound to gelsolin at low Ca^{2+}. However, they clearly show that the stable binding of the second actin monomer requires higher Ca^{2+} and is accompanied by enhancement of actin fluorescence. This suggests that if the second actin is bound to gelsolin already at low Ca^{2+}, this binding is weak and does not affect the fluorescence of the complex. High Ca^{2+} may change the conformation of the C-terminal half of gelsolin, bringing the two actin monomers into a contact similar to that they have in F-actin. This would stabilize the ternary actin–gelsolin complex and result in efficient nucleation of actin polymerization.

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