Domain 2 of gelsolin binds directly to tropomyosin

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Abstract Gelsolin is an actin filament severing protein composed of six similar structured domains that differ with respect to actin, calcium and polyphospho-inositide binding. Previous work has established that gelsolin binds tropomyosin [Koepf, E.K. and Burtnick, L.D. (1992) FEBS Lett. 309, 56–58]. We have produced various specific gelsolin domains in *Escherichia coli* in order to establish which of the six domains binds tropomyosin. Gelsolin domains 1–3 (G1–3), G1–2 and G2 all bind tropomyosin in a pH and calcium insensitive manner whereas binding of G4–6 to tropomyosin was barely detectable under the conditions tested. We conclude that gelsolin binds tropomyosin via domain 2 (G2).

Key words: Gelsolin; Tropomyosin; Cytoskeleton

1. Introduction

The actin cytoskeleton is principally responsible for the structural integrity and motility of cells. The tendency of monomeric actin (G-actin) to polymerise and the structure of the resulting microfilament array is modulated by as many as 60 distinct classes of actin binding proteins [1].

Gelsolin is a widely expressed actin binding protein [2–4] that severs filaments and caps the barbed end of the filament [5]. The protein is composed of six repeating sequence segments [6] that encode six similar structural domains (G1–6) consisting of about 120 amino acids each [7]. It is presumed that the protein arose from a series of gene duplications, first to produce a G1–3 containing protein (such as severin and fragmin) [5] that was then itself duplicated to form G1–6. This notion is supported by the observation that domains in the N-terminus map best to the equivalent domain, in rank order, in the C-terminus (G3–6), (G1·G4, G2·G5, G3·G6), in both structure [6,7] and function [8].

Gelsolin requires calcium to sever filaments at neutral pH, but there is no such requirement for calcium at pH 6.0 [9]. The calcium regulation of gelsolin seems to be brought about by movements in G4–6 [10] since G1–3 alone severs filaments without calcium. Gelsolin has two binding sites for G-actin, in G1 and G4, and one filamentous actin (F-actin) binding site in G2. G1 binds G-actin tightly ($K_d = 5$ pM) in a calcium insensitive manner while G4 has a moderate affinity ($K_d = 1.8$ μM) [8] actin binding activity that requires calcium. The F-actin binding site in G2 has a lower affinity ($K_d = 5$–7 μM) and binds to the same actin monomer as G1 within the filament [11]. The regulation of gelsolin activity in vivo is less certain [12].

Tropomyosins are found in almost all eukaryotic cells where they bind laterally to microfilaments [13]. They are elongate, α-helical proteins that dimerise to form a parallel α-helical coiled-coil interacting with other tropomyosin dimers through their N- and C-termini. In muscle cells, tropomyosins work together with troponins to form a calcium-regulated switch for actomyosin contraction. Their function in non-muscle cells is less clear but they appear to selectively stabilise groups of microfilaments and to modulate binding of other actin binding proteins [14]. Many isoforms of tropomyosin are produced in vertebrate cells although little correlation between the various isoforms and their cellular function has yet been made [15].

In addition to actin and troponins, tropomyosin binds many proteins including caldesmon [16], calponin [17,18], S100 proteins [19,20], the LIM protein enigma [21], Rad GTPase [22] and it has been reported to bind gelsolin [23,24]. In this study, we confirm that tropomyosin binds gelsolin, that this binding takes place mainly through G2 and that the gelsolin:tropomyosin interaction has many similarities to the interaction between actin microfilaments and gelsolin.

2. Materials and methods

2.1. Protein purification

Tropomyosin was prepared from rabbit back and leg muscle and turkey breast muscle by the method of Bailey [25]. Recombinant human cytoplasmic gelsolin was produced in *Escherichia coli* carrying a full length cDNA encoding gelsolin in the pMW172 plasmid as previously described [26] and purified by a modification of a previously described method [27]. Briefly, a 1 l culture was harvested by centrifugation and the cell pellet taken up in lysis buffer (50 mM Tris pH 8.0, 1 mM EGTA, 25% sucrose) and freeze-thawed with sonification three times. The supernatant was collected after a 10 min centrifugation at $13\,000 \times g$, and dialysed against 10 mM Tris pH 8.0, 1 mM NaN_3, 1 mM EGTA, 1 mM benzamidine. A DE52 (Whatman) ion exchange column was equilibrated with the same buffer and the protein added after clarification (10 min at $13\,000 \times g$). After a thorough wash, gelsolin was eluted off the column by replacing 2 mM $CaCl_2$ for the EGTA in the buffer. Gelsolin produced by this method was pure as judged by SDS–PAGE. The gelsolin fragments G1, G2, G1–2, G1–3, G4–6 were produced and purified as previously described [26,28,29]. Recombinant human non-muscle cofilin was produced in BL21 (DE3) transfected with a T7-based vector (pMW172) carrying the human cofilin cDNA fragment. Cofilin was purified using the same method previously used for human ADF [30], with an additional CM52 (Whatman) column step. Protein concentrations were determined spectrophotometrically using the following extinction coefficients

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Abbreviations: G-actin, monomeric actin; F-actin, filamentous actin; BSA, bovine serum albumin; G1–G6, the six repeated domains of gelsolin in order

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(A_{280} = 1.0 cm^{-1}) and were: tropomyosin = 80 μM, gelsolin = 8.93 μM, cofilin = 74.0 μM, G1 = 49.7 μM, G1+10 = 49.7 μM, G2 = 79 μM, G1–2 = 30.3 μM, G1–3 = 21.0 μM, G4–6 = 15.5 μM.

2.2. Affinity chromatography

Rabbit and turkey skeletal tropomyosin were linked to CNBr-activated Sepharose beads according to the manufacturer's recommendations (Pharmacia) and subsequently washed three times with 0.1 M Tris–HCl pH 7.5 to block any remaining active sites. 5 ml of tropomyosin-Sepharose beads was packed into 'EconoColumns' (Bio-Rad) and equilibrated with various running buffers (see the individual figure legends). 1 ml of test protein was added in the relevant buffer to columns and 1 ml fractions collected under gravity feed. Protein elution was monitored by the optical density at 280 nm and, where required, SDS–PAGE. Each column could be used repeatedly without loss of binding capacity.

2.3. Light scattering measurements

Light scattering (90°) was measured at 400 nm in a temperature-controlled Perkin-Elmer LS-3B fluorescence spectrometer at 21°C. Buffers and protein solutions (see the individual figure legends for details) were filtered using 0.2 μm filters and de-gassed before each experiment.

2.4. Sedimentation assays

Sedimentation of various fragments of gelsolin with tropomyosin was performed in a Beckman bench top ultracentrifuge (TL-100). 15 μM turkey breast tropomyosin was incubated with 5 μM each of the various gelsolin domains in 10 mM Tris pH 7.0, 2 mM $CaCl_2$ for 1 h at room temperature. The centrifuge tubes were then sedimented at 100 k for 15 min at 4°C. The supernatants were removed and the pellets taken up in sample buffer and analysed by SDS–PAGE.

3. Results

We have confirmed that tropomyosin binds directly to cytoplasmic gelsolin (Fig. 1). Experiments with whole gelsolin were conducted with tropomyosins from turkey or rabbit muscle but no difference was seen (data not shown). Furthermore, we have discovered that the tropomyosin interaction with gelsolin is both pH and calcium dependent. At pH 8.0, calcium is required for maximal binding, whereas at pH 6.5, calcium is not. Affinity chromatography (data not shown) and turbimetric assays (Fig. 4) show that magnesium cannot be substituted for calcium. G4–6 does not bind appreciably to tropomyosin; conversely, G1–3 does bind. However, the pH/calcium dependency seen with the whole gelsolin molecule is not observed.

Table 1
The binding of various proteins to skeletal muscle tropomyosin affinity columns

Protein	EGTA		Calcium	
	pH 6.5	pH 8.0	pH 6.5	pH 8.0
Gelsolin	0.43	0.29	0.50	0.81
G1	0.0	0.0	0.08	0.0
G1(+10)	nd	0.03	0.0	nd
G2	0.41	0.39	0.48	nd
G1–2	nd	0.65	0.85	nd
G1–3	nd	1.18	1.06	nd
G4–6	nd	0.0	0.17	0.11
Tropomyosin	nd	2.0	1.8	nd
Cofilin	0.0	0.0	0.0	0.0
BSA	0.0	0.0	0.0	0.0

Data are expressed as the ratio of protein that flowed through the column and the protein that was eluted off the column by 0.5 M KCl (i.e. integration of fractions 0–20 over fractions 25–30, in Fig. 1). (nd = not done).

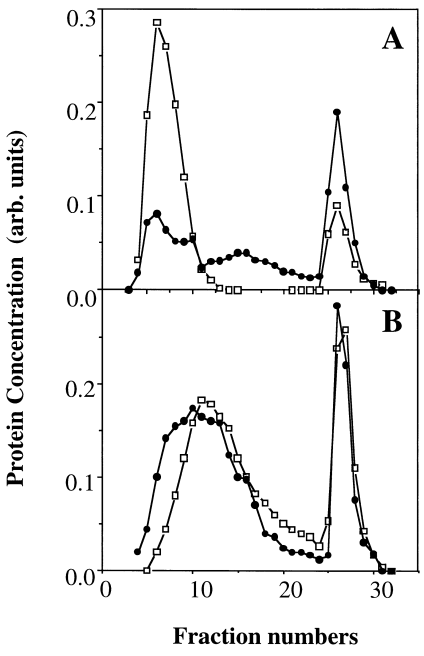

Fig. 1. Elution of human recombinant cytoplasmic gelsolin from a turkey skeletal muscle tropomyosin affinity column. (A) Excess gelsolin (1 ml at 16.5 μM) was added to the column so that some came straight through (flow through fractions 4–20), the remaining specifically bound gelsolin was eluted off the column with 0.5 M KCl (fractions 25–30). Conditions: (●) 25 mM Tris pH 8.0, 1 mM NaN_3, 1 mM $CaCl_2$. (□) 25 mM Tris pH 8.0, 1 mM NaN_3, 1 mM EGTA. (B) (●) 25 mM imidazole pH 6.5, 1 mM NaN_3, 1 mM $CaCl_2$. (□) 25 mM imidazole pH 6.5, 1 mM NaN_3, 1 mM EGTA.

Results of several experiments such as those shown in Fig. 1 are presented in Table 1. Tropomyosin itself binds to the tropomyosin affinity column (but not Sepharose alone, data not shown). Bovine serum albumin (BSA) did not bind tropomyosin. Human recombinant ADF, human recombinant cofilin, actophorin (an ADF/cofilin member from *Acanthamoeba*) were also found not to bind the tropomyosin column (data not shown). The smallest gelsolin fragment that binds

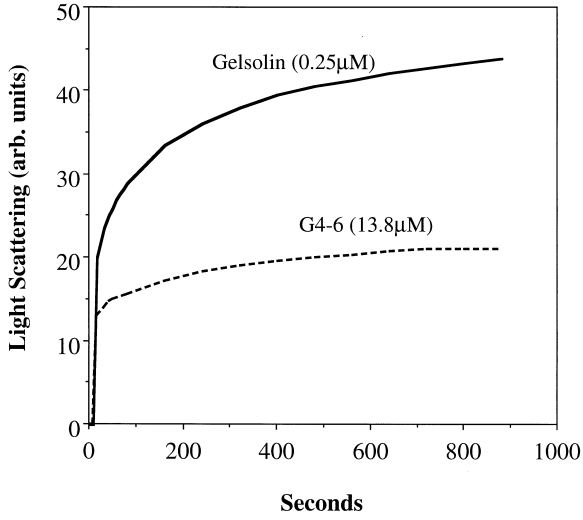

Fig. 2. Tropomyosin at 2.5 μM in 10 mM Tris pH 6.8, 1 mM $CaCl_2$, 1 mM NaN_3. Gelsolin or G4–6 added at time zero. Light scattering measured at 400 nm.

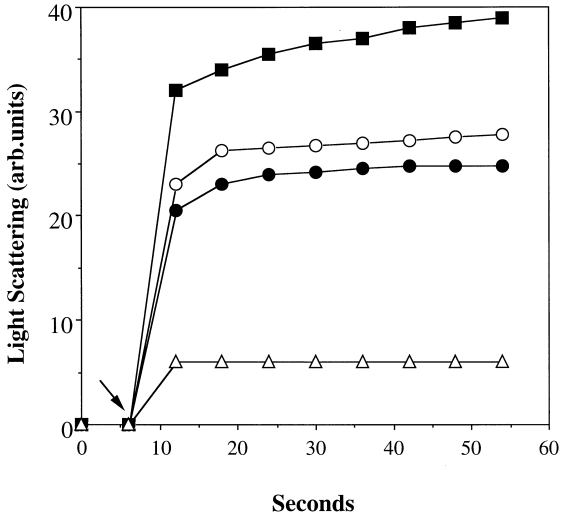

Fig. 3. The effect of G2 and G1–2 on the light scattering of tropomyosin. G2 inhibits the ability of G1–2 to increase the light scattering of tropomyosin solutions. 5 μM tropomyosin and 2.5 μM G1–2 added at 6 s (■). 5 μM tropomyosin with 2.5 μM G2 and 2.5 μM G1–2 added at 6 s (○). 5 μM tropomyosin with 5 μM G2 and 2.5 μM G1–2 added at 6 s (●). 5 μM tropomyosin with 2.5 μM G2 added at 6 s (△). Conditions: 10 mM Tris pH 7.0, 2 mM $CaCl_2$, 1 mM NaN_3.

tropomyosin is G2 although the affinity of the G2 domain seems to be less than G1–2. Binding of G2 to tropomyosin is not calcium sensitive.

Aggregation of tropomyosin caused by gelsolin and gelsolin fragments was monitored by light scattering (Figs. 2 and 3). Whole gelsolin caused a rapid increase in light scattering, but only a very modest increase was observed in the presence of large concentrations of G4–6 (Fig. 2). Although G2 did not cause an increase in the light scattering of tropomyosin (Fig. 3), it inhibited the increase in light scattering produced by

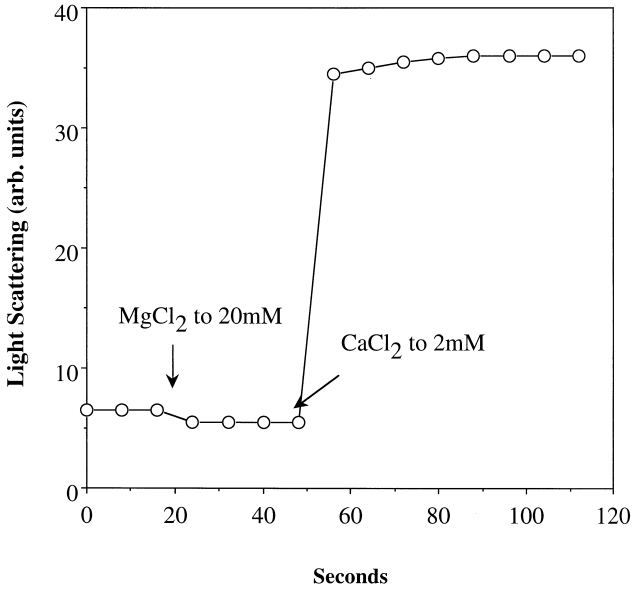

Fig. 4. Cation specificity of the gelsolin-induced aggregation of tropomyosin. 2.5 μM tropomyosin was incubated with G1–3 in 10 mM Tris pH 7.0, 1 mM NaN_3 and $MgCl_2$ added to 20 mM at 20 s. At 50 s, $CaCl_2$ was added to a final concentration of 2 mM. Similar results were obtained with intact gelsolin (data not shown).

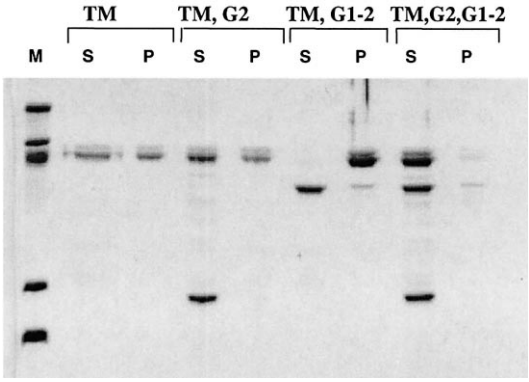

Fig. 5. Co-sedimentation of tropomyosin in the presence of G2 and/or G1–2. Supernatant (S) and pellets (P) for each experiment are shown. Conditions: 10 mM Tris pH 7.0, 1 mM $CaCl_2$.

G1–2. Note that G2 itself produced a small amount of light scattering but that this amount was equivalent to the arithmetic sum of that produced by tropomyosin and the G2 proteins alone. Whereas the light scattering produced by G1–2 and tropomyosin was in huge excess of the arithmetic sum of the two individual proteins. The inhibitory effect of G2 on light scattering by G1–2 was enhanced by pre-incubation of G2 with tropomyosin (data not shown).

Mixing gelsolin, domains of gelsolin and tropomyosin under certain conditions was found to cause rapid precipitation. We used this observation to investigate the gelsolin:tropomyosin interface by measuring the turbidity increase caused by the gelsolin:tropomyosin precipitation (Table 2). The gelsolin:tropomyosin precipitates were collected by co-sedimentation and analysed by SDS–PAGE. Co-sedimentation experiments (Fig. 5) were in good agreement with the turbidity data (Table 2). Under the conditions used, tropomyosin itself pelleted to a limited extent, this was (if anything) slightly inhibited by G2. G2 did not sediment either with tropomyosin or alone (data not shown). In some experiments of this type, there was no visible effect of G2 on pelleting of tropomyosin (data not shown). G1–2 was markedly different, causing a significant amount of tropomyosin to pellet. This was strongly inhibited by G2.

4. Discussion

Gelsolin is a well characterised, calcium and pH sensitive

Table 2
Tropomyosin co-precipitation with gelsolin and various gelsolin domains in the presence of calcium

Protein	Precipitation
Gelsolin	+++
G1–3	+++
G1–2	++
G1–2 and G1–3	++
G1	−
G2	−
G2 and G1–3	+
G2 and G1–2	+
G4–6	+/−− (weak)

Turkey breast tropomyosin (15 μM) was incubated with 5 μM each of the various gelsolin domains in 10 mM Tris pH 7.0, 2 mM $CaCl_2$.

actin binding protein [2,3,5]. Gelsolin binds both actin and tropomyosin in the presence of calcium at pH 8.0, but both interactions are calcium independent at pH 6.5. Calcium sensitivity of both interactions at pH 8.0 is lost if G4–6 is removed. It is known that gelsolin changes shape [31,32] upon binding calcium. Recent data [7] suggest that this involves the release of connections between the N- and C-termini. This study has revealed that the manner in which gelsolin binds tropomyosin is remarkably similar to the actin binding conditions. We believe that it is likely that the tropomyosin binding site on G2 (and the actin binding sites) become exposed in the presence of calcium when the G4–6 moves away and that this movement can also occur at low pH. We have found evidence for a weak interaction of tropomyosin for G4–6 in addition to the stronger interaction with G2. It is possible that whole gelsolin contains two tropomyosin binding sites, indeed the work of Koepf and Burtnick [23] suggests that this is the case. The second binding site may reside within G4–6 as the gelsolin molecule has been duplicated (G1•G4, G2•G5 and G3•G6) [6,7]. Although the second tropomyosin binding site in G4–6 seems to be a low affinity site, it is of course expected that the net affinity for whole gelsolin and tropomyosin would be greater than the sum of the two individual sites.

The finding that tropomyosin binds gelsolin, possibly in a manner that would prevent gelsolin binding F-actin, allows us to speculate that this may be a regulatory mechanism operating in the cell. Microfilaments seem refractory to gelsolin-induced disassembly in some cell types [33–35]. Also, gelsolin does not produce overt actin rearrangement upon micro-injection into fibroblasts [12], despite the very efficient actin filament severing activity observed in vitro. This is probably not as a result of inadequate cellular calcium, since calcium was also micro-injected into these cell with gelsolin. When G1–3 was introduced to the cell, however, the expected microfilament collapse was seen. There are of course a number of possible explanations for this [12]. It is possible that the calcium introduced to the cell was very rapidly removed, and that this leads to inactivation of the gelsolin, whereas G1–3 being calcium insensitive remodelled the actin cytoskeleton to produce the results observed [12]. Another possibility is that tropomyosin switched the whole gelsolin off as far as actin binding is concerned, by binding to it, but that due to the absence of the second site (within G4–6), the lower affinity of G1–3 for the inhibitory tropomyosin was low enough for G1–3 to sever filaments. The discovery that tropomyosin affords a degree of protection to actin filaments against severing of gelsolin in vitro [36–38] is compatible with this view. It is not likely that the inhibition of gelsolin is severing results from competition of the two actin binding proteins for actin since their affinity for actin is so disparate.

Surprisingly, the structure of ADF/cofilin [39] is similar to G1 despite [40] having no recognisable sequence homology. Like gelsolin, the severing activity of ADF/cofilin is inhibited by tropomyosin and this fact together with the structural similarity encouraged us to investigate the binding of the cofilins to tropomyosin. However, we found no conditions under which any of the ADF/cofilin family members tested would bind to the tropomyosin affinity columns. The lack of ADF/cofilin binding together with the fact that gelsolin domains other than G2 do not bind indicate that the interaction is specific and has little to do with the overall structural fold.

It is becoming clear that gelsolin may have a part to play in a number of signal transduction pathways. Gelsolin binds and regulates the activity of phospholipase D [41], phospholipase C, either by phospholipid binding [42,43] or by a direct interaction [44], and gelsolin has been implicated in the process of apoptosis [45]. A β-actin 'zipcode' binding protein has been characterised [46] that is suggested to localise the β-actin mRNA to the leading edge of fibroblasts. Interestingly, gelsolin and tropomyosin both immuno-precipitated with the β-actin mRNA binding protein, indicating that these three proteins may form a trimeric complex. There is evidence that in at least some cases, gelsolin is localised to the leading edge of cells with tropomyosin, the later reportedly not complexed with actin but with S100A2 proteins [20]. This interaction, like that between gelsolin and tropomyosin, is calcium dependent. It remains to be seen if tropomyosin can bind S100A2 simultaneously with gelsolin.

In addition to a possible role for gelsolin in signalling pathways, gelsolin [47], and tropomyosin [48], gene expression alterations have been correlated with oncogenic change. It is tempting to speculate that the interaction between tropomyosin and gelsolin (through G2) may be involved in the regulation of these signalling pathways.

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