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# Role of the Basic C-Terminal Half of Caldesmon in Its Regulation of F-Actin: Comparison between Caldesmon and Calponin

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**We previously reported that caldesmon (CaD), together with tropomyosin (TM), effectively protects actin filaments from gelsolin, an actin-severing protein. To elucidate the structure/function relationship of CaD, we dissected the functional domain of CaD required for the protection. The basic C-terminal half of rat nonmuscle CaD (D3) inhibits gelsolin activity to the same degree as intact CaD, although a smaller C-terminal region of D3 does not. This smaller C-terminal region contains the minimum regulatory domain responsible for the inhibition of actomyosin ATPase, and for the binding to actin, calmodulin and TM. These results suggest that the domain responsible for the inhibition of gelsolin activity lies outside the minimum regulatory domain, and that the positive charge possessed by the C-terminal half of CaD is important for its interaction with actin. Moreover, while the D3 fragment promotes the aggregation of F-actin into bundles as reported previously, this bundle formation is inhibited by the acidic N-terminal half of CaD, as well as by poly-L-glutamate. It seems likely that the acidic N-terminal half of CaD neutralizes the superfluous basic feature of the C-terminal half. A comparison between D3 and calponin, another actin-binding protein that is also basic and has similar actin-regulatory activities, is also discussed.**

**Key words: actin protection, caldesmon, calponin, electrostatic interaction, structure/function relationship.**

Abbreviations: CaD, caldesmon; CaM, calmodulin; TM, tropomyosin; CaP, calponin; NTCB, 2-nitro-5-thiocyanatobenzoic acid; DTT, dithiothreitol; pI, isoelectric point.

Caldesmon (CaD), a protein that is associated with the thin filaments in smooth muscle and in non-muscle cells, has been shown to play an important role in the regulation of actin. The cell cycle–dependent reorganization of the actin-based cytoskeleton is controlled by the cdc2-mediated serine/threonine phosphorylation of CaD (*1*, *2*). This regulatory process allows for the orderly disassembly of stress fibers and the rounding up of an anchored cell immediately prior to mitosis. Additionally, CaD inhibits actin-activated ATPase activity and the motility of phosphorylated myosin in smooth muscle cells, an inhibition that is reversed by calmodulin (CaM) in the presence of $Ca^{2+}$ (*3*–*7*). Functionally, CaD can be divided into two domains (Fig. 1): an acidic N-terminal domain that binds to the S1/S2 junction of myosin (*8*, *9*), and a basic C-terminal domain that binds to actin, tropomyosin (TM), tubulin, CaM and other $Ca^{2+}$-binding proteins, and inhibits actomyosin interaction (*10*–*15*). Low-affinity binding sites for actin and TM are also present in the N-terminal region of CaD (*16*, *17*), but the precise binding sites in this region have not been identified. The functional properties of the C-terminal domain of CaD have been well studied using fragments of CaD obtained from limited proteolysis, chemical cleavage and bacterial expression systems. The major functional motifs of CaD involved in actin-binding, CaM-binding and inhibition of actomyosin ATPase activity have been localized to its C-terminus (*13*, *15*, *16*, *18*–*20*). The actin-binding capacity and the inhibitory activity of the C-terminal half of CaD can be enhanced by smooth muscle TM and is released by the $Ca^{2+}$-binding protein CaM (for review, see Refs. *10* and *21*). Moreover, CaD can protect actin filaments from severing by gelsolin (*22*), a protective effect that is also enhanced by TM. In this study, we dissected the functional domain of CaD required for protection.

We also compared D3, the C-terminal half fragment of CaD, and calponin (CaP), another actin-binding protein with similar actin-regulatory activities. CaP is a 32-kDa smooth muscle–specific protein (*23*, *24*) that has been isolated from a variety of tissues, and has been implicated in the regulation of smooth muscle contraction (*25*–*28*). Previous studies have shown that CaP can bind to actin, TM, $Ca^{2+}$/CaM and myosin (*25*, *26*, *29*–*31*), and that CaP inhibits the actin-activated ATPase activity of myosin (*25*–*27*, *29*, *32*). Exogenous CaP attenuates the $Ca^{2+}$-induced contraction of permeabilized rabbit mesenteric arterial smooth muscle in a concentration-dependent manner (*33*). The inhibitory effect of CaP can be alleviated by phosphorylation catalyzed by protein kinase C (PKC) or $Ca^{2+}$/CaM-dependent protein kinase II (CaM kinase II) (*26*, *34*), and can be restored following dephosphorylation by type 2A (*35*) or 2B protein phosphatases (*36*). Phosphorylation-induced loss of inhibition results from a marked reduction in the affinity of phosphorylated CaP for actin (*26*). Several examples of the phosphorylation of CaP

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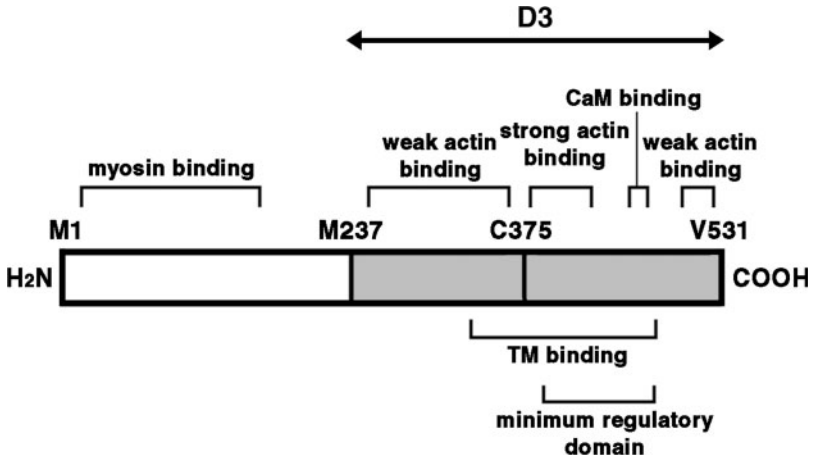


Fig. 1. **Domain structure of CaD.** The C-terminal half of CaD (D3, filled) has one strong and two weak actin-binding sites, as well as TM- and CaM-binding sites. The C-terminal side of D3 appears to include the minimum regulatory domain. The cysteine residue (C375) affected by NTCB treatment is also indicated. The isoelectric points of the C-terminal (D3) and N-terminal halves are 9.3 and 4.8, respectively.

in intact muscle in response to various contractile stimuli have been reported (*34*, *37*, *38*); however other investigators have reported that CaP is not phosphorylated in intact muscle (*39*, *40*).

Although phosphorylation of the myosin regulatory light chain has been firmly established as the primary mechanism for the regulation of smooth muscle contraction (*41*, *42*), thin filament–associated proteins such as CaD (*21*) have been suggested to play auxiliary roles that might be important for maintaining tension under low light chain phosphorylation levels. The known properties of CaP strongly suggest that it, too, might play a role in the thin filament–based regulation of smooth muscle contraction (*28*), and, recently, a number of other cellular functions that involve the actin cytoskeleton have also been suggested. The roles of CaD and CaP and their interaction in the regulation of smooth muscle contraction are controversial. The results of this study confirm the concept of independent functions for CaD and CaP, and suggest that the basic property of the C-terminal half of CaD may be important to the distinct functions of these two proteins.

## MATERIALS AND METHODS

*Proteins*—Rabbit skeletal muscle actin and chicken gizzard TM were purified as previously described (*22*, *43*). Chicken gizzard CaD (CGCaD) was purified by the method of Bretscher (*44*). The 80 kDa α-chymotryptic N-terminal fragment (NF) of CGCaD was prepared by the method of Fujii *et al*. (*45*). CGCaD was treated with 2-nitro-5-thiocyanatobenzoic acid (NTCB) as follows (*20*): CGCaD was first incubated for 1 h with 4 mM NTCB, pH 8.0, at room temperature, after which the pH of the samples was raised to pH 9.0, and the incubation was continued for 4 h at 42°C. The samples were dialyzed against buffer A [20 mM Tris-HCl, pH 8.0, 0.2 mM dithiothreitol (DTT) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)] for 30 min at 4°C, then applied to a DEAE-cellulose (DE52, Whatman) column equilibrated with the same buffer. The resulting 20 kDa C-terminal fragment (NTCB-CF) of CGCaD was eluted in the flow-through fractions. CaP was purified from chicken gizzards by the method of Abe *et al*. (*27*). Plasma gelsolin and the 40 kDa N-terminal half of gelsolin (CT40N) were obtained as previously described (*46*). The D3 fragment, the C-terminal half of rat nonmuscle CaD, was expressed and purified as described previously (*22*). The NTCB fragments of D3 were purified using a Sephadex G-25 (Pharmacia LKB, Uppsala, Sweden) column equilibrated with buffer A, after D3 was treated with NTCB as described for the preparation of the NTCB-CF of CGCaD. Protein concentrations were determined with a protein assay kit (BCA, Pierce, Rockford, IL) using bovine serum albumin as a standard. SDS–polyacrylamide gel electrophoresis was performed as described previously (*22*). Poly-L-glutamate (average molecular weight 8 kDa) was purchased from Sigma (St. Louis, MO).

*Assays for F-Actin–Binding and –Bundling*—The F-actin–binding and –bundling activities of proteins were assayed by high- and low-speed centrifugation respectively as previously described (*47*). The assay conditions for F-actin–binding are: 20 mM imidazole, pH 7.0, 1 mM $MgCl_2$, 0.5 mM ATP, 1 mM DTT, 0.1 mM $CaCl_2$, 30 or 100 mM KCl and 12 μM F-actin, and various concentrations of the examined proteins (D3 and others). The assay conditions for F-actin–bundling are: 20 mM Tris-acetate, pH 7.6, 1 mM $MgCl_2$, 1 mM ATP, 2 mM DTT, 30 or 100 mM KCl, and 12 μM F-actin, and various concentrations of the examined proteins. The formed bundles were observed by phase contrast microscopy before centrifugation to confirm that they were not the result of non-specific aggregation. Data were pooled from three experiments performed with similar results, then plotted into one graph and analyzed.

*Assays for Gelsolin Activities and Annealing of F-Actin (Assays for Gelsolin Inhibition)*—The actin-severing activity of gelsolin and the annealing process of gelsolin-severed actin fragments were monitored by flow birefringence measurement as described previously (*46*). Flow birefringence of an F-actin solution was determined with a Micro FBR MARK II (Wakenyaku Co., Japan) at a rotation speed of 500 rpm. The apparatus consists of a polarizing microscope with a small rotatable specimen stage, thus allowing the measurement of flow birefringence with as little as 150 μl of solution. By determining flow birefringence, the amount of polymerizing actin could be monitored without chemical modification of the actin molecules. The assay conditions are: 20 mM imidazole, pH 7.0, 1 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, with or without 0.2 mM $CaCl_2$, 30 or 100 mM KCl, and 12 μM F-actin, and various concentrations of the examined proteins. When actin bundle formation was

expected, the sample solution was observed by phase contrast microscopy both before and after the flow birefringence measurement. Data were pooled from two or three experiments that provided similar results, and then plotted into one graph.

## RESULTS

*Stimulation of TM Binding to F-Actin and Protection of F-Actin from Gelsolin Severing by the D3 Fragment*—The D3 fragment (Fig. 1) can stimulate the binding of TM to F-actin and can protect F-actin from gelsolin severing activity in cooperation with actin-bound TM just as intact CaD does (Fig. 2). Although TM isolated from chicken gizzards alone does not bind to F-actin in 30 mM KCl (Fig. 2A), it becomes able to bind when CaD is present (*22*, *43*). Similarly to intact CaD, D3 also causes TM to bind F-actin. The effect was saturated when the molar ratio of D3 to actin monomer approached 1/4, consistent with their binding stoichiometry molar ratio. Actin binding of TM stimulated by D3 was saturated when the molar ratio of TM to actin approached 1/5, which is also consistent with the binding stoichiometry ratio (about 1/6).

Neither D3 nor TM alone was able to repair the F-actin–severing action of the CT40N gelsolin fragment, but when both were present, the CT40N severing activity was inhibited in a concentration dependent manner. When the molar ratios of D3 and TM to actin were higher than 1/4 and 1/6, respectively, the activity of CT40N was completely inhibited (Fig. 2B). This actin-protection effect of D3 and TM was equally inducible regardless of the order of addition of the proteins and the CT40N gelsolin fragment to F-actin. Indeed, when CT40N was added first and F-actin was severed, and D3 and TM were subsequently added to the severed actin fragments, annealing and elongation of newly formed actin filaments could be observed (Fig. 2C). This annealing process was rapid and finished within about 10 min under the experimental conditions used independent of the concentrations of the added proteins. It should be noted that CT40N has the same actin-severing activity as gelsolin, except that it no longer shows calcium-sensitivity (*48*, *49*). D3 can stimulate binding between F-actin and TM and can inhibit gelsolin activities with the same efficiencies as intact CaD, which may result from the binding sites of CaD for actin, TM and other important proteins such as CaM being located in its C-terminal half region (Fig. 1) (*21*).

In contrast, neither 3 μM NTCB-CF of CGCaD nor 3 μM of the NTCB fragments of D3 stimulated binding between F-actin and TM or inhibited gelsolin activities, even though D3 at only 1 μM enabled TM to bind F-actin and to anneal actin fragments in the presence of gelsolin (Tables 1 and 2, and Fig. 3). This NTCB-CF region of CGCaD contains the minimum regulatory domain responsible for the inhibition of actomyosin ATPase and for the binding to actin, CaM and TM (*18*). These results suggest that the domain required for the inhibition of gelsolin activities lies outside the minimum regulatory domain. Moreover, the fact that cleavage by NTCB made D3 unable to stimulate binding between F-actin and TM or to inhibit the gelsolin activities suggests that a higher order structure or a closer cooperation within the D3 region is important for the functions of CaD.

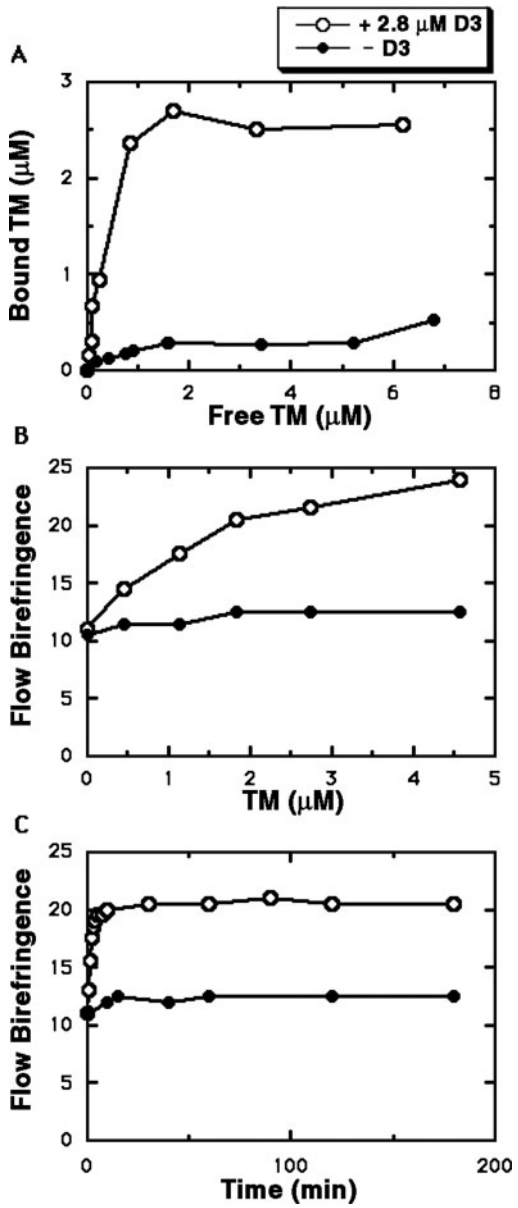


Fig. 2. **D3, the C-terminal half (E235–V531) of rat nonmuscle CaD, stimulates the actin-binding of TM, and inhibits gelsolin activities together with TM.** The concentration of D3 was 0 (as control, closed circles) or 2.8 μM (open circles). A: Stimulation of actin-binding of smooth muscle TM by D3. B: D3 together with TM inhibits the severing activity of the CT40N gelsolin fragment. The concentration of CT40N was 0.25 μM. C: D3 together with TM annealed gelsolin-severed short actin filaments into long filaments. The concentrations of CT40N and TM were 0.25 and 1.8 μM, respectively. Other conditions for the actin-binding and the gelsolin inhibition assays are described in "MATERIALS AND METHODS" (30 mM KCl).

*Bundling of F-Actin Elicited by D3*—D3 was able to force F-actin into bundles more efficiently than could CaD. For actin bundling, more than roughly 2.5 and 5 μM of D3 was required at KCl concentrations of 30 and 100 mM, respectively (Figs. 4 and 5). If the amounts of F-actin sedimented by low speed centrifugation were more than about 30%, the D3-induced actin bundles could also be observed by phase contrast microscopy.

Table 1. **Effects of NTCB-CF of CGCaD on the actin binding of TM.** No stimulation was observed. The conditions are the same as described for Fig. 2A.

| NTCB-CF of CGCaD (μM) | TM (μM) | | | |
|---|---|---|---|---|
| | + total 0.95 μM | | + total 1.9 μM | |
| | free | bound | free | bound |
| 0 | 0.80 | 0.14 | 1.4 | 0.44 |
| 2.0 | 0.79 | 0.15 | 1.7 | 0.21 |
| 3.0 | 0.81 | 0.13 | 1.7 | 0.19 |

Table 2. **Protective effect of each protein or fragment (CaP, D3, NTCB fragments of D3, or NTCB-CF of CGCaD) against the severing activity of gelsolin.** The conditions are as described in "MATERIALS AND METHODS" (30 mM KCl).

| Protein (Concentration) | Composition | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| F-actin (12 μM) | + | + | + | + | + | + | + | + | + |
| TM (1.8 μM) | – | – | + | + | + | + | + | + | + |
| D3 (1.1[a] or 3.0[b] μM) | – | – | – | +[a] | +[b] | – | – | +[b] | – |
| NTCBf (2.4 μM) | – | – | – | – | – | + | – | – | – |
| CaP (10 μM) | – | – | – | – | – | – | + | – | – |
| NTCB-CF (3.0 μM) | – | – | – | – | – | – | – | – | + |
| Gelsolin (0.3 μM) [or CT40N (0.6 μM)[c]] | – | + | + | + | + | + | + | +[c] | +[c] |
| Flow birefringence | 19.5 | 7.3 | 7.7 | 13.5 | 21.0 | 8.0 | 8.0 | 21.0 | 8.5 |

TM inhibited this D3-induced actin bundling, while TM did not affect the affinity between D3 and F-actin (Fig. 4). Actin bundles made by D3 could be inversely broken when TM was added. Poly-L-glutamate (average molecular weight 8 kDa) also could inhibit and inversely break the D3-induced actin bundling (Fig. 5). Concentrations of poly-L-glutamate above 3 μM completely prevented the actin bundle formation elicited by D3.

It is thought that actin bundle formation induced by CaD involves multiple actin-binding sites (*15*, *20*, *21*) or oligomerization of F-actin–bound CaD (*50*, *51*). D3 has one strong and two weak actin-binding sites (Fig. 1) (*15*). Poly-L-glutamate slightly decreased the ratios of D3 to F-actin, but barely altered the affinity between D3 and F-actin (Fig. 6). This suggests that poly-L-glutamate obstructs the weak actin-binding sites of D3. It has been reported that many other fragments derived from the C-terminal half of CaD, as well as D3, show stronger actin bundling activity than does intact CaD (*20*, *52*). D3, *i.e.* the C-terminal half of CaD, is basic [isoelectric points (pI) 9.3], and basic molecules can force F-actin into bundles (*53*). On the other hand, the N-terminal half of CaD is acidic (pI 4.8), and the N-terminal half fragment (NF of CGCaD), as well as poly-L-glutamate or TM (about pI 4), inhibit the actin bundle formation induced by D3 (Table 3). These results indicate that the basic nature of the C-terminal half of CaD is important for the actin bundle formation induced by CaD or D3, and that the acidic nature of the N-terminal half counteracts the effect of the C-terminal half.

*Effect of CaP on F-Actin–Binding of TM and F-Actin Protection from Gelsolin Severing Activity*—CaP increased the affinity between TM and F-actin (Fig. 7A). The actin-binding of TM stimulated by CaP saturated as the binding molar ratio approached 1/6, the binding stoichiometry ratio. In comparison with CaD or D3, much higher

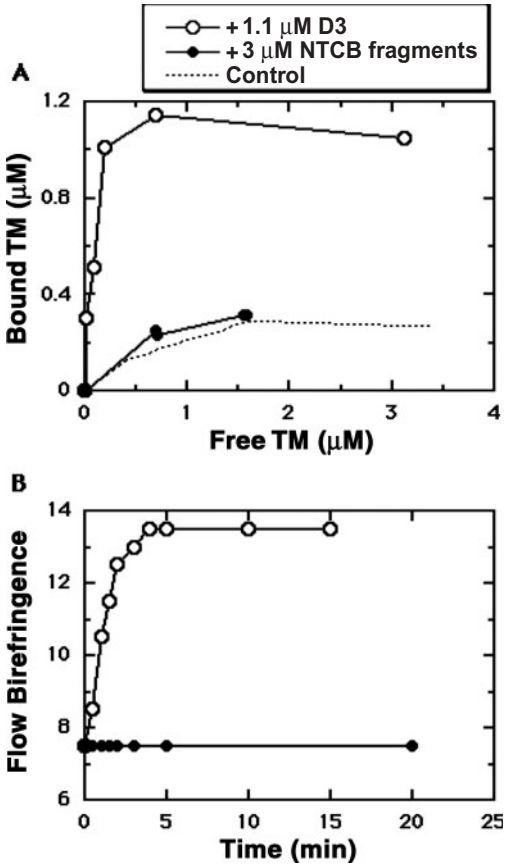


Fig. 3. **The cleavage of D3 at Cys375 by NTCB abolishes the ability of D3 to stimulate the actin-binding of TM and to anneal gelsolin-severed actin short filaments.** D3 (open circles), NTCB fragments of D3 (closed circles), or nothing (as a control, dotted line) was added to F-actin. A: Effect of cleaved D3 on the actin-binding of TM. No stimulation of actin-binding of TM was observed. B: Effect of cleaved D3 on the annealing of gelsolin-severed actin. No annealing was observed with cleaved D3. The concentrations of CT40N, TM, D3 and the NTCB fragments of D3 were 0.25, 1.8, 1.1, and 3 μM, respectively. Other conditions for the actin-binding and the gelsolin inhibition assays are described in "MATERIALS AND METHODS" (30 mM KCl).

concentrations of CaP were required to stimulate binding, probably because the binding stoichiometry of CaP to actin is 1/1 while that to CaD and D3 is about 1/6 and 1/4, respectively (*29*, *32*).

On the other hand, CaP did not inhibit gelsolin activities and did not protect F-actin from severing (Table 2 and Fig. 7B). Even under conditions where similar amounts of TM interacted with F-actin (in the presence of 3 μM D3 or 10 μM CaP), CaP did not provide any protection or annealing of actin in the presence of gelsolin, while D3 almost completely inhibited actin-severing and induced the annealing of actin fragments.

*Bundling of F-Actin Elicited by CaP*—CaP forced F-actin into bundles with a lower efficiency than D3. In the presence of 30 mM KCl, more than 6 μM CaP was required for actin bundling (Fig. 8). TM inhibited this CaP-induced actin bundling, while TM did not affect the affinity between CaP and F-actin. Actin bundles elicited by CaP could be inversely broken when TM was added.

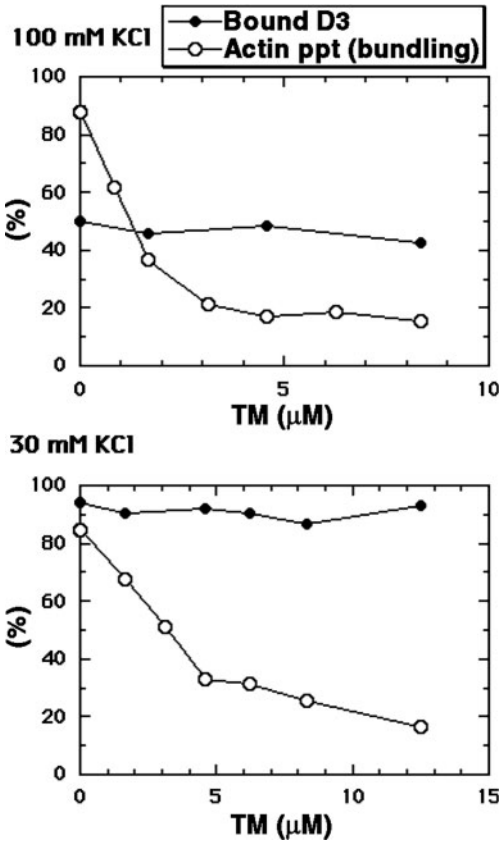


Fig. 4. **Inhibition of the actin-bundling activity of D3 by TM.** Unlike intact CaD molecules, D3 can make F-actin aggregate into bundles when the concentration of D3 is above about 2.5 μM at 30 mM KCl, or above about 5 μM at 100 mM KCl. TM inhibits the bundle formation caused by D3 at each salt concentration, and this effect is reversible. Open circles indicate amounts (%) of bundled F-actin precipitated by low speed centrifugation. On the other hand, TM does not affect the actin-binding of D3 (closed circles), in contrast with the stimulation of actin-binding of intact CaD by TM. The concentrations of D3 at 30 and 100 mM KCl were 4.3 and 5.7 μM, respectively. Other conditions for the actin-bundling and -binding assays are described in "MATERIALS AND METHODS."

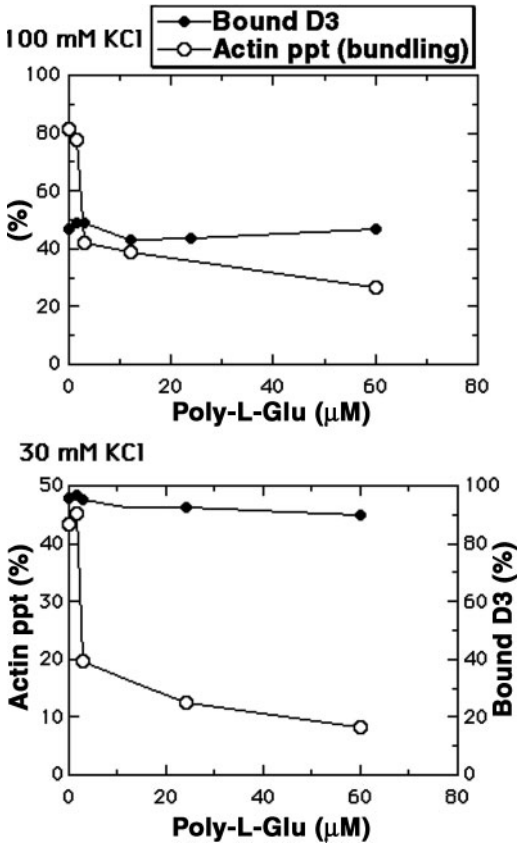


Fig. 5. **Inhibition of the actin-bundling activity of D3 by poly-L-glutamate.** The actin-bundling activity of D3 is inhibited by the addition of 3 μM poly-L-glutamate at either 30 or 100 mM KCl. Open circles indicate amounts (%) of bundled F-actin precipitated by low speed centrifugation. Closed circles indicate amounts (%) of binding D3 to F-actin precipitated by high-speed centrifugation. The concentrations of D3 at 30 and 100 mM KCl were 2.9 and 5.7 μM, respectively. Other conditions for the actin-bundling and -binding assays are described in "MATERIALS AND METHODS."

These actin-bundling activities of CaP are qualitatively similar to those of D3 (Fig. 4).

## DISCUSSION

CaD comprises an acidic N-terminal (pI 4.8) half and a basic C-terminal (D3 fragment, pI 9.3) half (Fig. 1). The C-terminal half of D3 has multiple binding sites for F-actin, TM and CaM (15, 20). The C-terminal half of D3, which is consistent with NTCB-CF of CGCaD or the C-terminal NTCB fragment of D3, also contains the minimum regulatory domain required and sufficient for the regulation of actomyosin activities in cooperation with TM and $Ca^{2+}$/CaM (*15*, *20*, *21*). In this study, we show that D3, but not its C-terminal half (*i.e.*, the minimum regulatory domain), has most of the activities for actin regulation that are possessed by the intact CaD molecule (Figs. 2 and 3, Tables 2 and 4). These results suggest that D3, the C-terminal half of CaD, is sufficient to stimulate the actin binding of TM and to protect F-actin from the activities of gelsolin, and that the domain involved in the inhibition of gelsolin activities lies outside the minimum regulatory domain, which is a much wider region than previously thought. D3 has most of the activities possessed by intact CaD, which is probably due to the fact that all binding sites for other proteins required for the activities of intact CaD are localized within this C-terminal region (*15*, *20*). Inversely, cleavage by NTCB disrupts the behavior of intact D3, suggesting that a higher order structure or a closer cooperation within the D3 region is important for the activities.

The actin bundling elicited by CaD is thought to result from CaD possessing multiple actin-binding sites (*15*, *20*, *21*) or from binding with F-actin to make an oligomer (*50*, *51*). Poly-L-glutamate decreases the affinity between D3 and F-actin only at higher D3 concentrations (Fig. 6), which suggests that poly-L-glutamate may inhibit the actin bundling activity of D3 by obstructing the weak actin-binding sites of D3. However, the above speculations do not completely explain why D3 can bundle F-actin more efficiently than intact CaD (*20*, *52*). In this study, we found that proteins or peptides that have low pI, such as the N-terminal half of CaD, TM or poly-L-glutamate, can repair

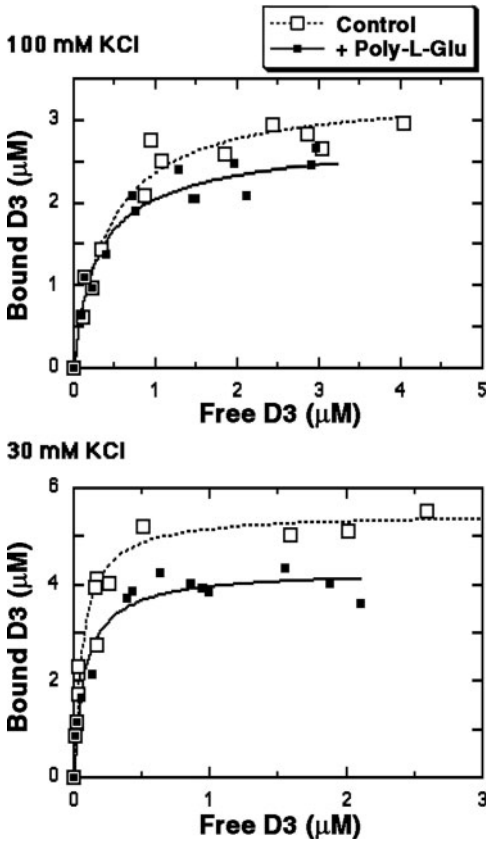


Fig. 6. **Effect of poly-L-glutamate on actin-binding of D3.** Poly-L-glutamate decreases the actin-binding ability of D3 only when higher concentrations of D3 are added with F-actin. The concentrations of added poly-L-glutamate were 60 and 6 μM at 30 and 100 mM KCl, respectively (closed squares). Open squares indicate the affinity of D3 for F-actin in the absence of poly-L-glutamate. Other conditions for the actin-binding assay are described in "MATERIALS AND METHODS." The binding stoichiometry and dissociation constant (μM) between D3 and F-actin under each condition are as the follows: 2.2 and 0.07 (without poly-L-glutamate) and 2.8 and 0.08 (with poly-L-glutamate) for 30 mM KCl, and 3.8 and 0.39 (without poly-L-glutamate) and 4.4 and 0.31 (with poly-L-glutamate) for 100 mM KCl.

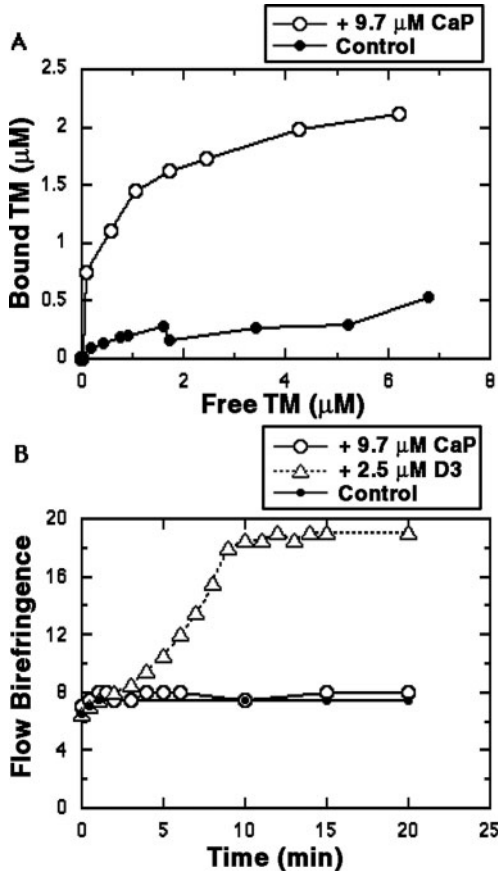


Fig. 7. **CaP isolated from chicken gizzards is also able to stimulate the actin-binding of TM (A), but not inhibit the gelsolin activity (B).** A: Stimulation of actin-binding of TM by CaP. The concentration of CaP was 0 (as a control, closed circles) or 9.7 μM (open circles). B: CaP cannot anneal gelsolin-severed short actin filaments even in the presence of TM. 9.7 μM CaP (open circles) or 2.5 μM D3 (triangles). The closed circles indicate the time course of flow birefringence in the presence of actin alone (as a control). The concentrations of gelsolin and TM were 0.3, and 1.8 μM, respectively. Other conditions for the actin-binding and the gelsolin inhibition assays are described in "MATERIALS AND METHODS" (30 mM KCl).

Table 3. **Inhibition of the actin-bundling activity of D3 by NF of CGCaD.** The N-terminus of CaD is acidic and rich in glutamic and aspartic acids, while the C-terminus of CaD is basic. The inhibition of actin-bundling by the N-terminus may explain the observation that intact CaD has little actin-bundling activity in contrast to the bundling activity of D3. The assay conditions are as described in the legend to Fig. 4. The concentration of added NF of CGCaD is 2 μM.

| Added fragment | Bundled F-actin (ppt %) | |
|---|---|---|
| | 30 mM KCl | 100 mM KCl |
| non (control) | 3.7 | 6.3 |
| D3 alone | 87 | 42 |
| D3 + NF of CGCaD | 57 | 13 |

the actin bundling caused by D3 (Table 3, Figs. 4 and 5). It already has been reported that polycationic molecules promote F-actin bundling and, inversely, that acidic amino acids disaggregate these actin bundles into single F-actin monomers (*53*). Since D3 is basic (pI 9.3), the actin bundling caused by CaD should be due to the basic nature of the C-terminal half. That explanation is supported by the fact that high concentrations of salt decrease both the actin-binding and -bundling of D3 (Figs. 4 and 5). The majority of important actin-binding proteins, other than TM, also are basic, and most important functions of CaD accumulate within D3, *i.e.* its C-terminal half region. Therefore, our conclusions are as follows: the C-terminal half of CaD developed to possess multiple functions for actin interaction and regulation during molecular evolution, which resulted in the basic nature of the region; this basic nature causes redundant actin bundling (*53*). In contrast, the acidic N-terminal half of CaD developed to neutralize this redundant actin bundling, as well the interaction with myosin. CaD physiologically shows no actin bundling activity (Table 4), probably due to the effect of the N-terminal half (*54*). The negative charges given by the N-terminal half of CaD probably inhibit the actin

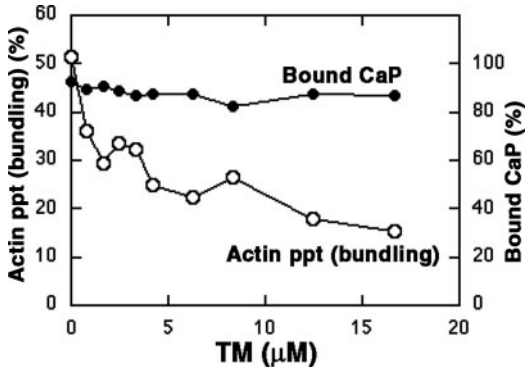


Fig. 8. **Inhibition of the actin-bundling activity of CaP by TM.** Like the D3 fragment, CaP can promote F-actin aggregation into bundles, and this actin-bundling activity is inhibited by TM (open circles). Also in this case, TM does not affect the actin-binding of CaP (closed circles), and this effect is reversible. The concentration of CaP was 9.7 μM. Other conditions for the actin-bundling and -binding assays are described in "MATERIALS AND METHODS" (30 mM KCl).

Table 4. **Actin regulatory properties of CaD and its fragments and CaP.** Summary of results obtained and comparison between CaD, D3, the NTCB fragments of D3 or the NTCB-CF of CGCaD (indicated as NTCBf), and CaP on the mode of actin interaction. Plus (+) or minus (–) indicates that the protein or fragment shows or does not show an effect on F-actin, respectively. Other results are reported in the following references as: a, Shirinsky, V.P. *et al*. (1992) *J. Biol. Chem*. 267, 15886–15892; b, Yamashiro, S. *et al*. (1995) *J. Biol. Chem*. 270, 4023–4030; c, Sobue, K. and Sellers, J.R. (1991) *J. Biol. Chem*. 266, 12115–12118; d, Ishikawa, R. *et al*. (1989) *J. Biol. Chem*. 264, 7490–7497 and 16764–16770; e, Sobue, K. *et al*. (1985) *FEBS Lett*. 182, 201–204.

| Effects | Fragment or protein | | | |
|---|---|---|---|---|
| | CaD | D3 | NTCBf | CaP |
| Inhibition of actomyosin ATPase & motility | +[a] | +[b] | +[c] | +[a] |
| Stimulation of binding between TM and F-actin | +[d] | + | – | + |
| Inhibition of gelsolin activities | +[d] | + | – | – |
| F-actin bundling | +/–[e] | + | – | + |

bundling potential of the C-terminal half of CaD without disturbing the other physiologically important activities. In fact, the activities of CaD are equal to the sum of the activities of D3 and the effect of the N-terminal half of CaD on D3. It has been reported that phosphorylation within the C-terminal half caused by cdc2-kinase during mitosis repairs the actin regulation of CaD (*2*). The negative charges arising from the phosphorylation might also be involved in the neutralization of the basic nature of the C-terminal half; but in that case, the neutralization effect is more directly and potent. On the other hand, the kinds of effects of polycationic molecules such as poly-L-lysine on the activities of D3, present a very interesting problem.

CaP isolated from chicken gizzards is, like D3, a basic protein (pI 9.9) (*55*). Both CaD and CaP are heat stable, $Ca^{2+}$/CaM-dependent F-actin–binding proteins. Both inhibit actomyosin activity in the absence of $Ca^{2+}$/CaM, and this inhibition is released by $Ca^{2+}$/CaM (*3*, *6*, *11*, *26*, *27*, *39*). Therefore, CaD and CaP were thought to play similar physiological roles. However, the presence of either CaD or CaP in excess causes the displacement of the other from the complex with F-actin (*29*). Both CaD and CaP stimulate the actin binding of TM and the formation of actin bundles; however, CaP cannot protect F-actin from gelsolin activities compared with D3 (Table 4). These results suggest that both the cooperative interaction of TM on actin binding and polycationic properties are required to protect F-actin against the activities of gelsolin. Since the stoichiometric ratios of CaD, D3 and CaP to actin are about 1/6, 1/4 and 1/1, respectively (*29*, *32*), the cross-linking of inter neighboring actin monomers comprising a filament by multiple actin-binding sites is probably important for the protection. For these reasons, if both D3 and CaP are present, it is expected that CaP will decrease the inhibitory effect of D3 on gelsolin activity. Moreover, CaD is widely distributed in many smooth muscle tissues and in non-muscle cells, while CaP is mainly detected only in smooth muscle. A differentiation-linked increase in CaP expression has been demonstrated using chicken gizzard muscle (*23*). In addition, only CaD is phosphorylated and regulated by cdc2-kinase both *in vivo* and *in vitro* (*1*, *2*). Although CaP can be phosphorylated by PKC or by $Ca^{2+}$/CaM-dependent kinase II *in vitro*, whether this phosphorylation occurs in smooth muscles remains unclear (*26*, *40*). For example, the smooth muscles of CaP knockout mice are not significantly different in their regulation compared with wild-type mice (*56*). The sum of these results suggests that CaD organizes microfilaments dynamically in many types of cells, while CaP has static effects on microfilament regulation.

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