# Calponin Interaction with α-Actinin-Actin: Evidence for a Structural Role for Calponin

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ABSTRACT The purpose of this study was to address the paradox of calponin localization with α-actinin and filamin, two proteins with tandem calponin homology (CH) domains, by determining the effect of these proteins on the binding of calponin to actin. The results show that actin can accommodate near-saturating concentrations of either calponin and α-actinin or calponin and filamin with little change or no change in ligand affinity. Little direct interaction occurred between α-actinin and calponin in the absence of actin, so this effect is not likely to explain the co-distribution of these proteins. Calponin, like α-actinin, induced elastic gel formation when added to actin. When α-actinin was added to newly formed calponin/actin gels, no change was seen in the mechanical properties of the gel compared to calponin and actin alone. However, when calponin was added to newly formed α-actinin/actin gels, the resulting gel was much stronger than the gels formed by either ligand alone. Furthermore, gels formed by the addition of calponin to α-actinin/actin exhibited a phenomenon known as strain hardening, a characteristic of mechanically resilient gels. These results add weight to the concept that one of the functions of calponin is to stabilize the actin cytoskeleton.

## INTRODUCTION

Calponin is a 32–36-kDa actin-binding protein found first in smooth muscle (Takahashi et al., 1986) and select nonmuscle tissues (reviewed in Chalovich and Pfitzer, 1997). Calponin has properties that indicate it may regulate muscle contraction, including the ability to inhibit actomyosin ATPase activity (Winder and Walsh, 1990), force production in muscle fiber preparations (Itoh et al., 1994), and movement in the in vitro motility assay (Shirinsky et al., 1992; Haeberle, 1994). However, other observations suggest that calponin is a structural protein. For example, the binding of calponin to actin causes bundling of the actin (Tang et al., 1997). Also, calponin is primarily localized with other structural proteins such as α-actinin (North et al., 1994; Mabuchi et al., 1996) and desmin (Mabuchi et al., 1996, 1997) on the surface of smooth muscle dense bodies. Calponin is also found in the cardiac myocyte Z-line (Masuda et al., 1996). The picture is complicated by evidence that the interaction of calponin with actin is dynamic. Parker et al. (1994, 1998) reported that in relaxed muscle calponin is localized with α-smooth muscle actin and myosin, but in stimulated muscle is present in the cell cortex, a region rich in β-nonmuscle actin. Understanding the complex relationships of calponin with actin and other actin-binding proteins is a key to understanding the function of calponin.

Why calponin is preferentially associated with the cytoskeletal fraction of actin is not known. Calponin does not appear to bind tighter to β-actin than to α-actin as measured by co-sedimentation with actin, although some selectivity is suggested by a greater degree of light scattering with β-actin (Parker et al., 1998). It may be that other cytoskeletal actin-binding proteins increase the affinity of calponin for β-actin. However, α-actinin, which co-localizes with calponin, binds to very similar, if not the same, regions of actin as calponin does (Hodgkinson et al., 1997; McGough et al., 1994). Both proteins (filamin, spectrin, dystrophin, and fimbrin) contain a region known as the calponin homology domain or CH domain (Carugo et al., 1997). Therefore, rather than synergism, one might predict antagonism toward actin binding among these proteins.

The present report examines the binding of calponin to actin in the presence of α-actinin and filamin. A negative interaction was observed between filamin and α-actinin, which resulted in a partial displacement of one protein by the other. Calponin binding to actin was largely unaffected by the presence of either α-actinin or filamin. The simultaneous binding of calponin and α-actinin to actin altered the storage modulus of the actin solution. Interestingly, the magnitude of the change in storage modulus was dependent on the order of addition of ligand proteins. The addition of calponin to a mixture of actin and α-actinin did produce a large increase in the storage modulus of actin, and the resulting gel exhibited strain hardening. These properties were not observed when the order of addition of α-actinin and calponin were reversed.

*Received for publication 29 April 1999 and in final form 27 August 1999.*

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0006-3495/99/12/3208/10 $2.00

## METHODS

### Preparation of proteins

Skeletal muscle actin was isolated from rabbit muscle according to the method of Spudich and Watt (1971) as modified by Eisenberg and Kielley

(1972). Actin used for rheology studies was further purified by gel filtration chromatography. Filamin and α-actinin were isolated from turkey gizzards using the method of Feramisco and Burridge (1980). Smooth muscle tropomyosin was prepared from turkey gizzards according to the method of Bretscher (1984). Calponin was isolated from turkey gizzards using the following modification of the method of Abe et al. (1990): 400 g of cleaned fresh turkey gizzards were ground and dispersed with a Polytron homogenizer in five volumes of buffer containing 50 mM imidazole, pH 7.0, 300 mM KCl, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.01 mg/ml leupeptin, and 0.05 mg/ml soybean trypsin inhibitor. Aliquots of ~200 ml were placed in a boiling water bath for ~10 min with frequent agitation and allowed to cool on ice for at least 1 h. The mixture was centrifuged at low speed to remove insoluble material. The supernatant was clarified by an additional centrifugation at 100,000 × *g* for 20 min. The clarified supernatant was brought to 30% saturation with ammonium sulfate and the calponin precipitate was collected by centrifugation for 30 min at 100,000 × *g*. The pellet was dissolved in a minimal volume of 1 M NaCl, 20 mM sodium acetate, pH 5.6, 6 M urea, 0.1 mM EGTA, 0.01% sodium azide, and 0.5 mM dithiothreitol. The resulting solution was dialyzed twice against 20 volumes of the same buffer without the NaCl. The dialysate was centrifuged for 20 min at 100,000 × *g* and loaded onto an Integrated Separation Systems (ISS, Hyde Park, MA) CM-500 Spherilose column equilibrated with the dialysis buffer. Calponin was eluted with a 400 ml gradient in which the KCl concentration was increased to 300 mM. Calponin was normally present in fractions eluting between 0.2 and 0.25 M KCl. Fractions containing pure calponin, judged by SDS polyacrylamide electrophoresis, were pooled. With the CM-500 Spherilose column there was usually no need for subsequent gel filtration chromatography. The inset to Fig. 1 shows an SDS-polyacrylamide gel of the proteins.

All proteins were stored on ice in a buffer composed of 140 mM potassium propionate, pH 7.0, 10 mM imidazole, pH 7.0, 2 mM magnesium chloride, and 1 mM dithiothreitol. Concentrations of proteins were measured by absorbance at 280 nm and corrected for light scattering at a nonabsorbing wavelength using the following absorption coefficients and molecular weights: actin ($\epsilon = 1.15$, $M_r = 42{,}000$), filamin ($\epsilon = 0.74$, $M_r = 250{,}000$), and α-actinin ($\epsilon = 1.23$, $M_r = 100{,}000$) and calponin ($\epsilon = 0.74$, $M_r = 32{,}300$). The extinction coefficients for filamin and calponin were from Shizuta et al. (1976) and Stafford et al. (1995), respectively. The extinction coefficient for α-actinin was determined for this study by the method described earlier (Stafford et al., 1995). The monomer molecular weights were used for calculating the concentrations of α-actinin and filamin.

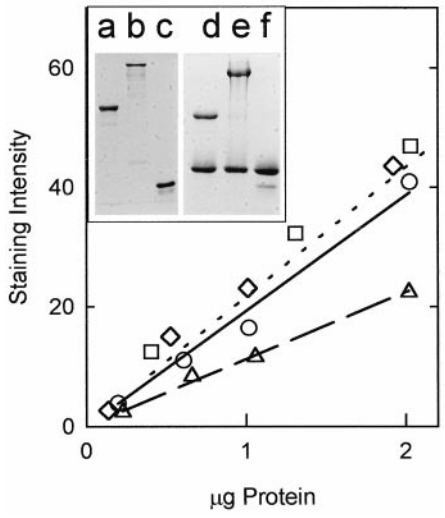


FIGURE 1 SDS-polyacrylamide gels of ligand proteins and the relationship between staining intensity and the amount of protein. Standard curves are shown for actin (□), α-actinin (◇), calponin (○) and filamin (△); the actin curve was linear to twice the protein concentration shown here. The density of each protein band was determined by the average of 3 digital images of a single gel. *Inset:* 6% polyacrylamide gels showing α-actinin (*a*), filamin (*b*), calponin (*c*), as well as pellets from the co-sedimentation with actin of α-actinin (*d*), filamin (*e*) and calponin (*f*).

## Sedimentation assay

In order to measure the binding of several proteins to actin without the use of multiple labeling we used a gel quantification method. All actin-binding proteins were clarified by centrifugation at 100,000 × *g*, 4°C for 20–30 min immediately before use. The proteins were mixed by gentle pipetting in polyallomer ultracentrifuge tubes. Typically, 50 μl of 20 μM actin was added to 150 μl of a solution containing 140 mM potassium propionate, pH 7.0, 10 mM imidazole, pH 7.0, 2 mM $MgCl_2$ and 1 mM dithiothreitol, and the actin-binding protein of interest. Samples were centrifuged in a Beckman 42.2 rotor at 100,000 × *g* (30,000 rpm) for 30 min at 20°C. Supernatants and pellets were run on 10% polyacrylamide gels in denaturing conditions. Images of gels were digitized using a ScanJet IIxc/T scanner and the DeskScan II software package (Hewlett-Packard, Inc., Wilmington, DE). The density of each band was measured in triplicate using ImageQuant (Molecular Dynamics Inc., Sunnyvale, CA) and the intensity of the ligand protein was normalized to the intensity of actin which was constant in any given experiment. Approximately 95% of the actin sedimented during the centrifugation. Fig. 1 shows a calibration curve for standard proteins determined from polyacrylamide gel electrophoresis. The protein levels measured in actual experiments were within the range of those used for construction of these standard curves. Standards were run on every gel used for analysis.

To correct for the fraction of ligand that sedimented in the absence of actin, two high concentrations of ligand were used and the average of these values was used for the correction. Typically, <5% of calponin, α-actinin, and filamin sedimented in the absence of actin. The fraction of ligand that did not bind to actin was measured using an excess of actin. Virtually all of the ligand was found to bind under these conditions. The amount of actin that sedimented was measured by densitometry of each actin pellet.

## Analysis of data

The McGhee-von Hippel equation (McGhee and von Hippel, 1974) was fit to values of $\upsilon$ ($\text{ligand}_{\text{bound}}/\text{actin}_{\text{total}}$) as a function of free ligand, $L$:

$$\frac{\upsilon}{L} = K \cdot (1 - n\upsilon) \cdot \left\{\frac{(2\omega - 1)(1 - n\upsilon) + \upsilon - R}{2(\omega - 1)(1 - n\upsilon)}\right\}^{n-1} \cdot \left\{\frac{1 - (n + 1)\upsilon + R}{2(1 - n\upsilon)}\right\}^{2}$$

where

$$R = \{[1 - (n + 1)\upsilon]^2 + 4\omega\upsilon(1 - n\upsilon)\}^{1/2}$$

Where $K$ is the affinity of a single ligand for an isolated binding site, $n$ is the number of actin monomers a ligand occupies, and $\omega$ is a measure of cooperativity between ligands. $K\omega$ is the contiguous site affinity or the affinity of a ligand for an actin site adjacent to one occupied site. The program MLAB (Civilized Software Inc., Bethesda, MD) was used to perform the nonlinear regression.

## Native gel electrophoresis

Bidirectional native gel electrophoresis was performed with 3% acrylamide/0.5% agarose gels in a 20 × 20 cm horizontal electrophoresis unit. The gel buffer was either 150 or 50 mM Tris propionate, pH 7.0. The chamber buffer was 50 mM Tris propionate. Samples loaded in the wells

were in the same 140 mM potassium propionate buffer used for the sedimentation assays. Temperature was held within the range of 25–30°C by means of a circulating water bath and pH changes were kept within 1 pH unit with buffer exchange ports. Dilution of the samples was prevented by pouring the buffer only to the top of the gel, hence the use of a horizontal electrophoresis unit. Protein migration in the anode direction was monitored by use of bromophenol blue.

## Rheology

The rheological measurements were performed using a Rheometrics RFS II rheometer (Rheometrics Scientific, Piscataway, NJ). The same instrument has been used to characterize actin gels as previously described (Janmey et al., 1994). A flat bottom plate was driven by a mechanical motor to oscillate with a tiny amplitude at a fixed frequency. A time-dependent shear was applied to the test protein solution sandwiched between a plate and a cone having a 0.02 radian tilt angle (much like a gyrus, but with the tip cut and ~50× compressed vertically), and the resulting torque acting on the top cone was detected electronically. In our experiments, a cone-and-plate pair of 5 cm diameter was selected, which required an ~700 $\mu$l sample for each measurement. The electronically detected torque is in general sinusoidal, but has a phase lag from the driving oscillations and can be decomposed to an in-phase component and an off-phase component. The in-phase component tells how elastic a sample is and the corresponding component of stress divided by the strain is defined as the storage modulus, $G'$, with a unit of pascal (Pa, 1 Pa = 10 dyn/cm$^2$). The component that is 90° off phase is defined as the loss modulus, $G''$. The values of $G'$ and $G''$ and their ratio indicate how elastic, or gel-like, a protein mixture is. If $G'$ is large and $G''/G' \ll 1$, then the sample is very elastic and little energy is dissipated. On the contrary, if $G''$ is large and $G''/G' \gg 1$, the sample is viscous and dissipative. A detailed discussion of the viscoelastic properties of polymers can be found elsewhere (Ferry, 1980).

Actin filaments were maintained at an average length of 2 $\mu$m by adding gelsolin to a molar ratio of gelsolin/actin of 1:760. G-actin at a concentration of 24 $\mu$M was mixed with either 5 $\mu$M $\alpha$-actinin or 5 $\mu$M calponin followed by the addition of salt to start actin polymerization. In the case of three-protein mixtures, the third protein was added within ~1 min of the initiation of polymerization after a brief vortex mixing.

## RESULTS

Before examining the competition of calponin, $\alpha$-actinin, and filamin for actin binding, the binding profile of each ligand was determined individually. Fig. 2 shows the titration of 5 $\mu$M actin with $\alpha$-actinin, filamin, and calponin. Two protein preparations were used for the calponin and $\alpha$-actinin curves. In the case of calponin, the data could be described with a single actin monomer comprising a calponin site (i.e., $n = 1$) with negative cooperativity, $\omega = 0.28$ and contiguous site constant $K\omega = 1.9 \times 10^5$ M$^{-1}$. The addition of bovine serum albumin to the binding assays to minimize nonspecific binding did not significantly alter these parameters.

The McGhee-von Hippel equation was fit to the data for the binding of $\alpha$-actinin and filamin for actin. Reasonable fits for $\alpha$-actinin were obtained when the $\alpha$-actinin site on actin was assumed to consist of either three or four actin monomers ($n = 3$ or 4, or in terms of dimeric $\alpha$-actinin, $n =$ 6–8 actin monomers). For the case $n = 3$, it was necessary to introduce negative cooperativity into the fit ($\omega = 0.4$, $K = 7.2 \times 10^5$ M$^{-1}$), whereas for $n = 4$, the binding was slightly positively cooperative ($\omega = 3.1$, $K = 4.6 \times 10^5$ M$^{-1}$). For filamin, reasonable fits were obtained for any value of $n$ from 3 to 5 (or 6 to 10 for filamin dimers) as long as $\omega$ was allowed to alter from negative to positive cooperativity, i.e., 0.22 to 6.3 ($K = 3.2 \times 10^5$ M$^{-1}$ to $K = 1.8 \times 10^5$ M$^{-1}$). As in the case with $\alpha$-actinin, negative cooperativity can compensate for a decrease in the value of $n$. Fig. 2, *A* and *B* also show the binding of $\alpha$-actinin and filamin to actin in the presence of smooth muscle tropomyosin (*solid symbols*). Tropomyosin reduces the affinity of both proteins to actin. Because our earlier results show that

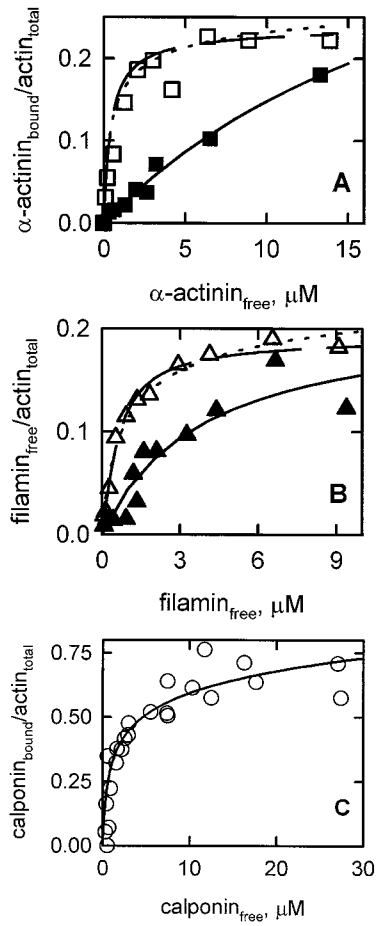


FIGURE 2 Sedimentation assay of binding of $\alpha$-actinin, filamin, and calponin to actin and actin-tropomyosin. *Conditions:* 5 $\mu$M actin and varied ligand concentrations at 20°C in a buffer composed of 140 mM potassium propionate, pH 7.0, 10 mM imidazole, pH 7.0, 2 mM $MgCl_2$, and 1 mM dithiothreitol. The equation of McGhee-von Hippel was used to generate the curves. (*A*) Binding of $\alpha$-actinin. In the absence of tropomyosin (□) the values of $n$, $K$, $\omega$, are 3, $7.2 \times 10^5$ M$^{-1}$, 0.4 (*dotted line*) or 4, $4.6 \times 10^5$ M$^{-1}$, 3.1 (*dashed line*), respectively. The presence of 3 $\mu$M tropomyosin (■) causes a reduction in $K$ to $1.7 \times 10^4$ M$^{-1}$ with positive cooperativity of 6.2 when $n = 4$. (*B*) Binding of filamin. In the absence of tropomyosin (△) the binding could be adequately described with values of $n$, $K$, and $\omega$ of 3, $3.2 \times 10^5$ M$^{-1}$, 0.22 (*dotted line*), 4, $2.8 \times 10^5$ M$^{-1}$, 1.7 (not shown), or 5, $1.8 \times 10^5$ M$^{-1}$, 6.3 (*dashed line*). In the presence of tropomyosin (▲) when $n = 5$, the values of $K$ and $\omega$ were $4.1 \times 10^4$ M$^{-1}$ and 6.8, respectively. (*C*) Binding of calponin. In the absence of tropomyosin $n = 1$, $\omega = 0.28$, $K = 6.8 \times 10^5$ M$^{-1}$ and $K\omega = 1.9 \times 10^5$ M$^{-1}$. The addition of 10 $\mu$M bovine serum albumin to reduce any nonspecific interactions had no effect on the data.

tropomyosin does not inhibit calponin binding to actin (Lu et al., 1995) calponin data were collected only in the absence of tropomyosin.

Filamin and α-actinin both utilize CH domains to bind to actin, but it was unclear whether these proteins compete with each other for actin binding. The effect of α-actinin on the binding of filamin to actin was examined and the results are shown in Fig. 3. While α-actinin did bind to actin in the presence of 2.5 μM filamin, there was a depression in binding. Thus, at a free α-actinin concentration of 1 μM, the value of θ was 0.037 in the presence of filamin but 0.11 in the absence of filamin (compare the *solid* and *dashed curves* in Fig. 3). The cause of this depression in binding could not be uniquely defined. The difference between the solid and dashed curves could be simulated equally well by assuming that filamin decreased the affinity or increased the number of actin monomers interacting with a single α-actinin monomer. Fig. 3 also shows that with increasing levels of bound α-actinin, the amount of filamin bound to actin decreased. However, the binding of α-actinin did not totally displace the filamin. Over the range of 2.5–6 μM free α-actinin, the value of θ for both ligands was ~0.14.

Unlike α-actinin and filamin, calponin has only one CH domain. The effect of α-actinin and filamin on the binding of calponin to actin was examined. Fig. 4 *A* shows that little displacement of calponin occurred over the range of free α-actinin concentrations giving maximum binding of α-actinin to actin. Approximately 20% of the bound calponin (*circles*) was displaced at higher concentrations of α-actinin. Therefore, the possibility of a small reduction in affinity or number of binding sites cannot be excluded. The binding of α-actinin to actin (*squares*) was unimpeded by the presence of bound calponin as shown by the close correlation of the data with the dashed curve showing the binding of α-actinin to actin in the absence of other ligands (taken from Fig,. 2). Similarly, Fig. 4 *B* shows that the binding of calponin to actin (*circles*) did not appreciably reduce the amount of bound α-actinin to actin (*squares*); the fitted line through the α-actinin data has a slope of ≈0. The binding of calponin to actin (*circles*) was not greatly affected by the presence of bound α-actinin, as seen by the close correlation of the data to the dashed curve generated for the binding of calponin to actin in the absence of other ligands (from Fig. 2). It is not possible to state definitively from these data that α-actinin had no effect on the stoichiometry of binding of calponin to actin. However, it is possible to obtain a complex in which actin has 80% of the expected amount of bound calponin and 100% of the expected bound α-actinin (>80% may be possible at higher calponin concentrations). Binding of nearly stoichiometric amounts of both proteins could occur if there were no overlap between calponin and α-actinin. Similar results could occur if both proteins had partially overlapping sites (see Fig. 7).

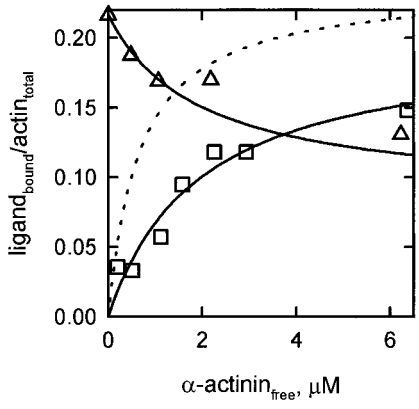


FIGURE 3 Influence of filamin on the binding of α-actinin to actin. *Conditions:* 2.5 μM filamin, 5 μM actin, titrated with α-actinin in the buffer described in Fig. 2. Filamin (△) was initially saturating and decreased with increasing α-actinin concentration. The binding of α-actinin to actin in the presence of filamin (□ and *solid line*) was depressed somewhat compared with the case in the absence of filamin (*dashed line*; taken from Fig. 2 *A*).

Fig. 4 also shows that there is little competition between calponin and filamin. When calponin was held constant and the actin was titrated with filamin there was little displacement of bound calponin (*circles*) until the filamin concentration was saturating (Fig. 4 *C*). The binding of filamin to actin was virtually the same in the presence of saturating calponin (*triangles*) as in the absence of other ligands (*dashed curve*). When filamin was held constant and actin was titrated with calponin there was virtually no displacement of filamin (*triangles*, Fig. 4 *D*); the fitted line through the squares had a slope of approximately zero. Similarly, the binding of calponin to actin (*circles*) was unchanged by the presence of saturating filamin.

A possible complication of the experiments involving calponin and either α-actinin or filamin is the possibility of a direct interaction between calponin and these other ligands. Such an interaction between two ligands could mask a competition for binding to actin. The binding of α-actinin and calponin was qualitatively assessed by native gel electrophoresis as described in Materials and Methods. Fig. 5 *A* shows that a change in migration (or band broadening) of α-actinin was detected, but only at high calponin concentrations (above 25 μM calponin). At 50 μM calponin there was substantially more peak broadening than in the presence of 25 μM calponin. These results were obtained with a 50 mM Tris-propionate buffer system. Similar results were obtained with 150 mM Tris-propionate, though the migration was much diminished. Fig. 5 *B* is a positive control of the assay in which 5 μM calponin was titrated with $Ca^{2+}$-calmodulin. The formation of a complex at the anode position occurred in this case even though the concentrations of proteins were much lower than in Fig. 5 *A*. Calponin was shown to be present in this complex via Western analysis (data not shown). The binding of calponin to calmodulin was further verified by a visible decrease in calponin mass in lane 2 compared to lane 1. For a negative control a mixture of 5 μM bovine serum albumin and 50

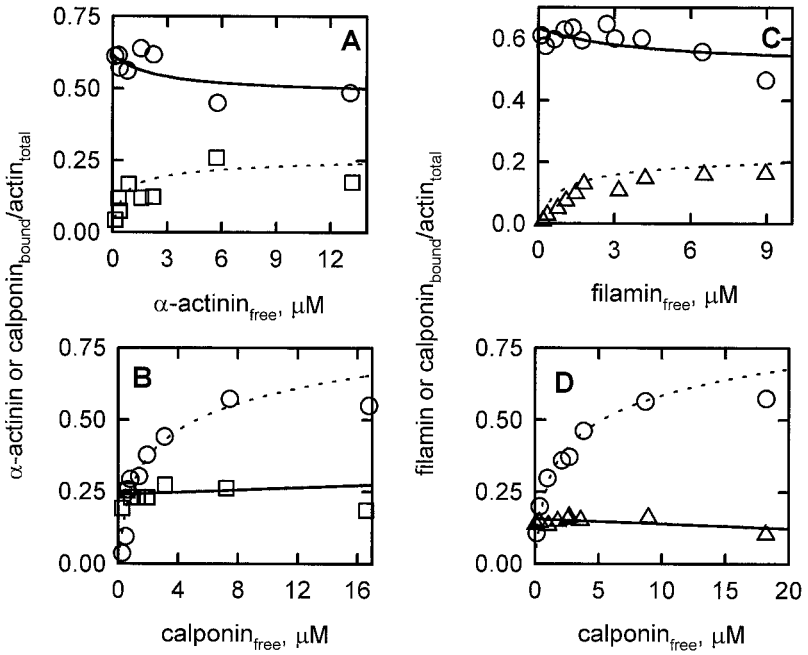


FIGURE 4 Influence of $\alpha$-actinin and filamin on the binding of calponin to actin. *Symbols:* ○, calponin; □, $\alpha$-actinin; △, filamin. Binding was measured at 20°C using 5 $\mu$M actin in the buffer described in Fig. 2. (*A*) Titration with $\alpha$-actinin in the presence of 10 $\mu$M calponin. (*B*) Titration with calponin in the presence of 7 $\mu$M $\alpha$-actinin. (*C*) Titration with filamin in the presence of 10 $\mu$M calponin. (*D*) Titration with calponin in the presence of 5 $\mu$M filamin. The dashed lines were taken from Fig. 2 (binding of a single ligand) to show the lack of effect of the presence of the second ligand.

$\mu$M calponin was examined (data not shown). No band broadening or other indication of interaction was observed between bovine serum albumin and calponin. Similar experiments were more difficult in the presence of filamin due to poor migration of filamin in the native gel. However, no band broadening of calponin was observed in the presence of filamin, so there were no obvious indications of a calponin-filamin interaction. It did not appear that interactions between calponin and either $\alpha$-actinin or filamin were substantial enough to create the false appearance of binding to actin in the presence of a competing ligand, as observed in Fig. 4. Thus, calponin can bind directly to actin in the presence of either filamin or $\alpha$-actinin.

The simultaneous binding of both $\alpha$-actinin and calponin to actin altered the properties of actin. For example, the complex of actin with both ligands could be collected by centrifugation at low speed, indicating that a large network of actin had formed. Because of this observation, the colocalization of these proteins in cells, and the reported ability of $\alpha$-actinin to increase the elasticity of actin gels, the rheological properties of the $\alpha$-actinin–actin-calponin complex was examined. Fig. 6 shows that under the conditions employed by our experiments the storage modulus approximately doubled when 5 $\mu$M $\alpha$-actinin was added to 24 $\mu$M actin during polymerization. The same amount of calponin increased the storage modulus fourfold. When both calponin and $\alpha$-actinin were mixed with actin the storage modulus depended on the order of addition. When $\alpha$-actinin was added into rapidly polymerizing actin in the presence of 5 $\mu$M calponin, the mixture formed a gel, having a storage modulus comparable to that caused by 5 $\mu$M calponin alone.

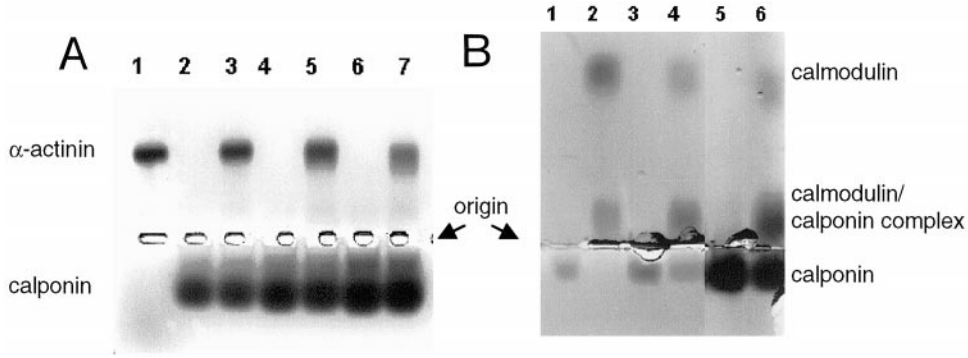


FIGURE 5 Interactions of calponin with $\alpha$-actinin and calmodulin detected with horizontal native gel electrophoresis. Calmodulin and $\alpha$-actinin migrate toward the anode (top of gel) while calponin migrates toward the cathode. (*A*) $\alpha$-Actinin binding. *Lane 1:* 2 $\mu$M $\alpha$-actinin. *Lanes 2 and 3:* 25 $\mu$M calponin without and with 2 $\mu$M $\alpha$-actinin. *Lanes 4 and 5:* 35 $\mu$M calponin without and with 2 $\mu$M $\alpha$-actinin. *Lanes 6 and 7:* 50 $\mu$M calponin without and with 2 $\mu$M $\alpha$-actinin. (*B*) Calmodulin binding. *Lanes 1 and 2:* 1 $\mu$M calponin without and with 5 $\mu$M calmodulin. *Lanes 3 and 4:* 5 $\mu$M calponin without and with 5 $\mu$M calmodulin. *Lanes 5 and 6:* 50 $\mu$M calponin without and with 5 $\mu$M calmodulin.

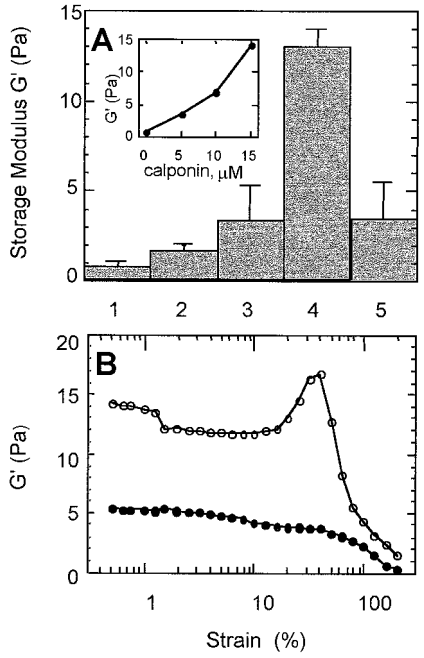


FIGURE 6 Effect of order of addition of calponin and α-actinin on the storage modulus ($G'$) of F-actin. (*A*) The storage modulus, $G'$ is shown for 24 μM actin in the presence of 5 μM α-actinin and 5 μM calponin added either during polymerization of actin or after polymerization was completed. *Lane 1:* actin alone. *Lane 2:* actin + α-actinin. *Lane 3:* actin + calponin. *Lane 4:* actin + α-actinin, then calponin. *Lane 5:* actin + calponin, then α-actinin. *Inset:* Calponin (in absence of α-actinin) concentration dependence of the storage modulus when added during actin polymerization. (*B*) The storage modulus, $G'$, as a function of shear strain for α-actinin-actin-calponin complexes. *Filled circles:* α-actinin was added to a mixture of co-polymerized actin and calponin. *Open circles:* calponin was added to co-polymerized actin and α-actinin. The gel to which calponin was added last experienced a sharp increase in the storage modulus (strain hardening) at ~40% strain.

In contrast, when calponin was added into a rapidly polymerizing actin and α-actinin mixture, the resulting mixture formed a much stronger gel than either calponin or α-actinin did alone (Fig. 6 *A, bar 4*). The storage modulus of the complex formed in this manner was 16 times that of actin alone.

The ternary complex formed by adding calponin last also was resistant to higher strains than in the complex in which α-actinin was added last (Fig. 6 *B*). When the dynamic storage modulus $G'$ was measured with incrementally increased strains, the complex formed with calponin added last had a two to threefold higher value of $G'$ at low strain and underwent a sharp increase in $G'$ at ~40% strain. This increase in $G'$ is a phenomenon known as strain hardening, which is characteristic of a resilient elastic gel. In contrast, when α-actinin was added last, the same combination of proteins produced a weaker and more fragile gel, which did not show any noticeable strain hardening before the gel collapse. Therefore, we conclude that calponin can reinforce a preexisting actin gel cross-linked by α-actinin, but not vice versa, i.e., when the order of addition of the actin-binding proteins was reversed.

## DISCUSSION

Smooth muscle actin is present in two compartments. The nonmuscle or β isoform of actin is present in the cell cortex, while the muscle or α and γ isoforms of actin are present in contractile complexes along with myosin. Some actin-binding proteins are segregated to a single compartment (North et al., 1994; Parker et al., 1994, 1998; Mabuchi et al., 1996) but the factors contributing to this segregation are largely unknown. The problem becomes more intriguing when one considers that some of the actin-binding proteins (i.e., calponin; Parker et al., 1994, 1998) move between the two compartments. Several factors may contribute to the differential distribution of actin-binding proteins such as different affinities for β and γ actin and attractive or competitive interactions among actin-binding proteins. Differences in affinity of calponin, α-actinin, and filamin for one actin isoform versus another have only a minor effect on selectivity. Unpublished data from one of our laboratories (J.X.T.) indicate less than a twofold higher affinity of α-actinin for β-actin compared with skeletal actin. Similarly, we observed the affinities of calponin for α-skeletal actin and γ-smooth muscle-actin to be the same (Lu et al., 1995). High-speed sedimentation could not discern differences in the affinity of calponin for skeletal actin versus β-actin, but a difference was seen when light scattering was monitored (Parker et al., 1998). Therefore, we focused not on the actin type but on the effect of various actin-binding proteins. We chose to use skeletal muscle actin because rabbit skeletal muscle actin can be highly purified in the large quantities required for co-sedimentation and rheology studies.

The present results show that smooth muscle tropomyosin does inhibit the binding of both α-actinin and filamin to actin. This observation confirms earlier reports (Maruyama and Ohashi, 1978; Zeece et al., 1979; Nomura et al., 1987). The apparent absence of tropomyosin from α-actinin-rich regions of actin such as in dense bodies (Small et al., 1986) and Z-lines (Szczesna and Lehrer, 1992) may result, in part, from this mutually inhibitory interaction. This competition of binding together with the strong cooperativity for tropomyosin binding (Wegner, 1979) would make it energetically unfavorable for filamin and tropomyosin to be randomly distributed along actin filaments. It is interesting that Pruliere et al. (1986) observed that tropomyosin caused clustering of filamin at the ends of actin filaments. Calponin did not dramatically affect α-actinin binding to actin in the presence of tropomyosin. Calponin did, however, seem to increase inhibition of filamin binding to actin in the presence of tropomyosin (data not shown). This may be relevant to the exclusion of tropomyosin from the dense bodies as already discussed.

Because tropomyosin is excluded from regions of actin containing calponin, α-actinin, and filamin, it was equally important to study the interactions of these proteins in the absence of tropomyosin. Neither filamin nor α-actinin stimulated the binding of calponin to actin. Rather, there was a limited competition between these proteins that nevertheless

permitted near-stoichiometric binding of calponin and α-actinin or calponin and filamin, or to a somewhat lesser extent α-actinin and filamin to actin. Thus, the binding of one of these ligands to actin does not appear to stimulate or inhibit the binding of the others to actin. The packing of these proteins on actin is an interesting structural problem particularly when one considers that these proteins utilize similar domains for binding to actin. An added component to the complexity of the structure formed is that the properties of the actin filament appear to be dependent on subtle differences in the arrangement of the ligand proteins.

Both α-actinin and filamin contain tandem CH domains that are thought to be involved with actin binding (Lebart et al., 1993, 1994). Cryoelectron micrographs of both calponin-actin (Hodgkinson et al., 1997) and α-actinin-actin (McGough et al., 1994) implicate the same region of actin in the binding of both ligands. Despite these common areas of interaction, Zeece et al. (1979) did not observe competition between filamin and α-actinin for binding to actin. However, that study was limited to low ligand concentrations. We reexamined the competition between these proteins under conditions where both ligand concentrations were high enough to be saturating in the absence of an inhibitor. Under these conditions a unit of seven actin monomers contained, on average, a single bound filamin monomer and a single bound α-actinin monomer. In the absence of a competing ligand, each filamin monomer bound to three to five actin monomers, while each α-actinin monomer bound to three to four actin monomers. The interpretation of this change in stoichiometry in structural terms is ambiguous because α-actinin and filamin are dimers and each cross-links actin filaments differently, forming a unique structure (Critchley, 1993; Gorlin and Hartwig, 1993). The binding studies shown here do not distinguish between binding of either *dimer* to one or two actin filaments. To the degree that binding occurs to only a single filament, the value of $n$ observed will be an underestimate of the true value. For pure monodendate binding, the ratio of actin monomers to ligand will be underestimated by a factor of 2 because two monomers of ligand are involved in binding to $n$ monomers on a single actin filament. In competition experiments involving both α-actinin and filamin, it is unlikely that both ligands will be able to bind to two actin filaments. This means that the values of $n$ for both ligands will be underestimated to some degree.

The seemingly incomplete competition of competition between α-actinin and filamin may be due to either 1) a change from bidendate to monodendate binding of one or both of the ligands so that additional actin monomers become available for binding, or 2) the ability of both ligands to bind to the same unit of $n$ actin monomers. If both ligands bind to the same unit of $n$ actin monomers, then filamin and α-actinin must be able to bind in a staggered manner in which the two ligands are overlapped along the actin filament. The x-ray crystal structures of actin-binding domains containing tandem CH domains of fimbrin (Goldsmith et al., 1997) and spectrin (Carugo et al., 1997), as well as cryoelectron micrographs of these regions in α-actinin (McGough et al., 1994) and fimbrin (Hanein et al., 1998) indicate that the actin-binding region is an ellipsoid mass that connects subdomain 1 and subdomain 2 of adjacent monomers. If only two actin monomers make tight contact with α-actinin and filamin it becomes possible to arrange both proteins within a group of seven actin monomers.

Cryoelectron microscopy studies indicate that calponin binds to two actin monomers (Hodgkinson et al., 1997) in much the same way that α-actinin (McGough et al., 1994) binds. However, calponin, with its single CH domain, exhibited little if any competition with either α-actinin or filamin for actin binding. In the absence of α-actinin or filamin, a block of 10 actin monomers could contain five molecules of calponin. This same block of 10 actin monomers could also accommodate two monomers of either α-actinin or filamin without displacing the calponin. This absence of competition confirms the observation by others that the CH domain of calponin does not contribute significantly to calponin binding to actin. Gimona and Mital (1998) showed by deletion mutagenesis that the CH domain of mammalian calponin is not sufficient for actin binding. Similarly, Mezgueldi et al. (1992) observed that a fragment of gizzard (basic) calponin containing only the CH domain did not bind actin, while a slightly larger fragment did bind.

The lack of competition between calponin and either α-actinin or filamin might mean that no common sites on actin are involved in binding. It is also possible that there is overlap of both filamin and α-actinin with a site that has only a small effect on the stability of calponin for actin. For example, calponin appears to bind optimally to two actin monomers although the interaction with a single monomer is sufficient. The unstable interaction between calponin and the second actin monomer may be displaced with 1) a large amount of free calponin, hence the gradual approach to a stoichiometry of 1:1 when the concentration of free calponin is high; and 2) α-actinin and filamin, which use tandem CH domains to bind actin. Case 1 may occur if a large ligand has different types of interactions with actin along its length. In the presence of a competing ligand, those interactions of lowest affinity may be displaced, allowing the ligand to "peel away" from actin so that the protein is held by those interactions that are not competitive or have high affinity (Fig. 7). A model describing competition when one ligand has a mosaic of binding sites has been presented earlier (Chen and Chalovich, 1992).

The interaction of ligands such as α-actinin, calponin, and filamin to actin present several complications that might affect the observed binding constants. The formalism of McGhee and von Hippel, which was used in the present analysis, accounts for the parking problem that occurs when a ligand binds to multiple sites on a lattice. That formalism also accounts for cooperativity among adjacent ligands. Grazi et al. (1993) noted that the apparent affinity of α-actinin for actin changes with the concentration of actin. They suggested that as the actin concentration increases, the diffusion rate of actin decreases, thus making cross-linking less

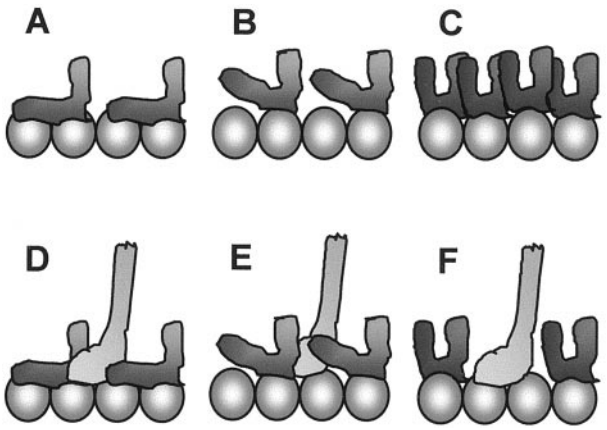


FIGURE 7 Possible modes of binding of calponin and α-actinin to actin. At ionic strengths exceeding 110 mM the binding of calponin to actin can be described as a 1:2 complex with positive cooperativity between adjacent calponin molecules (*A*) or as a 1:1 complex with negative cooperativity (*B*). In the latter case alternate actin monomers are blocked by steric or other repulsive forces. Calponin can bind in a 1:1 complex to actin under conditions of high calponin concentration and at ionic strengths <110 mM (*C*). In the presence of α-actinin calponin binds to actin with a stoichiometry of 1:2. Three possible cases are shown in *D–F* where α-actinin is depicted as a truncated monomer. In cases *D* and *E*, α-actinin and calponin bind to different sites on the same actin monomer. In case *E* the unfavorable free energy required to bend calponin as shown in (*B*) is partially compensated for by a weak calponin/α-actinin interaction shown in Fig. 5. In case *F*, α-actinin and calponin bind to different actin monomers.

likely. In addition, there is an increase in filament entanglement that tends to favor the formation of cross-links. These effects combine to produce a complex pattern of binding. The dissociation constant at infinite actin dilution (when there is no filament entanglement and filament diffusion is unhampered) was calculated to be $4.6 \times 10^5\ M^{-1}$, which is in good agreement with $6.2 \times 10^5\ M^{-1}$ reported for the recombinant actin-binding domain of gizzard α-actinin (Xu et al., 1998) and similar to the values of *K* obtained in the present study ($4.6 \times 10^5\ M^{-1}$ to $7.2 \times 10^5\ M^{-1}$).

In an earlier report of the binding of $^{14}C$-labeled calponin to actin we observed that the value of *n* changed from 1 to 2 as the ionic strength was increased above 110 mM (Lu et al., 1995). By using a different method measure binding under slightly different assay conditions and at higher calponin concentrations, we presently observed a value of *n* of 1.5 actin monomers (Fig. 2 *C*) where we previously observed a value of 2. Interestingly, the present data could also be fit well with $n = 1$ as long as negative cooperativity ($\omega < 1$) was included between adjacent calponin molecules bound to actin. This raises the possibility that the salt-dependent change in apparent stoichiometry (Lu et al., 1995) and other effects (Kolakowski et al., 1995) reported earlier could be due to a change in the degree of cooperativity rather than to a change in stoichiometry. Changes in cooperativity and stoichiometry are related and sometimes difficult to distinguish (Schwarz and Watanabe, 1983). That is to say that in Fig. 7, the transition from a 1:2 complex to a 1:1 complex of calponin to actin can be equally well-described as a loss of affinity of a secondary weak binding site (Fig. 7 *A*) or, if only a single binding site exists, as the loss of a steric or repulsive blocking of an adjacent actin monomer (Fig. 7 *B*). Other possibilities were described earlier (Lu et al., 1995).

As shown in Fig. 6 *B*, the complex of actin, calponin, and α-actinin formed by adding calponin last was resistant to higher strains than that in which α-actinin was added last. Such behavior could have several causes. As already mentioned, these proteins do not compete with each other but they might nevertheless influence the manner in which each is bound to actin. For example, actin that is involved in a 1:1 complex with calponin might behave differently from actin in which two actin monomers interact with a single calponin molecule. It is also possible that calponin and α-actin produce different types of actin networks and that the final structure is dependent on the order of addition of the components. Another possibility is that the order of addition affects the degree of actin polymerization of the actin or the length of the polymers formed. The actin was studied for hours after addition of both proteins so it is unlikely that the final extent of polymerization would depend on the order of addition. We have not examined the length distribution of the actin or the structure of the various complexes.

The order of addition effect observed here is significant only if there is some cellular control over the exposure of actin to various actin-binding proteins. Such temporal control could occur in many ways. An interesting possibility is that the inherent rate constants of binding might dictate the order of binding. We have noted previously that the rate constant of binding of calponin to actin is very slow ($<10^6\ M^{-1}\ s^{-1}$) compared with that of caldesmon binding to actin (Lu et al., 1995) at 15°C. Goldmann and Isenberg (1993) reported that the apparent rate constant for binding of smooth muscle α-actinin to a fluorescent actin derivative (NBD-actin) is $1 \times 10^6\ M^{-1}\ s^{-1}$ at 20°C. The apparent constant for binding of the actin-binding domain of α-actinin at 15°C is $1.4 \times 10^6\ M^{-1}\ s^{-1}$ to $1.8 \times 10^6\ M^{-1}\ s^{-1}$ (Kuhlman et al., 1994, Xu et al., 1998). The possibility exists that the slow rate of calponin binding may favor formation of the more strain-resistant ternary complex. Better estimates of the kinetics of binding of these proteins to actin are necessary before conclusions can be drawn as to the role of kinetics in establishing the type of actin complex formed in vivo.

Although biochemical and cryoelectron microscopy studies have shown that α-actinin and calponin binding sites on actin are very close to each other, the present studies indicate that the binding sites on actin are different. This raises the possibility that calponin could strengthen the α-actinin/actin gel (Fig. 6) either by 1) interacting with both actin and α-actinin (Fig. 7, *D* and *E*), or 2) causing a conformational change in actin that makes the α-actinin/actin bond more resistant to strain (Fig. 7, *D–F*).

A potentially important factor in the interaction of proteins with actin is the level of phosphorylation of the actin-binding proteins. The phosphorylation of both calponin

(Winder et al., 1993) and filamin (Ohta and Hartwig, 1995) have been reported to inhibit actin binding. Our calponin preparation was not significantly phosphorylated and essentially 100% of the calponin was competent in actin-binding assays. Preparations of calponin from other sources have also been reported to be largely unphosphorylated (Winder et al., 1993; Rokolya et al., 1996; Mino et al., 1995). Using anti-phosphoserine antibodies we did not detect phosphoserine in our filamin preparations, although some phosphoserine was present in our $\alpha$-actinin preparations (data not shown). It would be interesting in future studies to see how the level of phosphorylation affects the affinity of these proteins to actin and the subsequent changes in actin rheology.

In summary, calponin can bind to actin filaments that contain either bound filamin or $\alpha$-actinin. There are no obvious synergistic interactions between calponin and $\alpha$-actinin either in the absence or presence of actin that would explain the intracellular co-localization of these proteins. Although $\alpha$-actinin and filamin have similar actin-binding domains, they can simultaneously bind to actin in nearly stoichiometric amounts. Similarly, despite similarities in the actin-binding domains, there is little displacement of calponin by either filamin or $\alpha$-actinin. The organization of these proteins on actin remains an interesting question. The properties of the $\alpha$-actinin-actin-calponin complex depend on the order of addition of the ligands; adding calponin last creates a structure that is more resistant to strain. This raises the possibility that the mechanical properties of actin are dictated not only by the composition of actin-binding proteins but also by the order in which these proteins are added to actin. The order of addition of these proteins to actin might be controlled by regulation of synthesis, transport, or by the intrinsic rate constant of binding of the various ligands. The localization of calponin at the surface (Mabuchi et al., 1997) of $\alpha$-actinin containing dense bodies together with the changes in rheological properties caused by calponin support the possibility that calponin reinforces the $\alpha$-actinin-actin cross-links so that they can resist the strains imposed during muscle contraction.

Chicken gizzards were a gift from Swift Inc., Wallace, North Carolina.

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