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Bundle Formation of Smooth Muscle Desmin Intermediate Filaments by Calponin and Its Binding Site on the Desmin Molecule[1]

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Smooth muscle basic calponin, a major actin-, tropomyosin-, and calmodulin-binding protein, has been examined for its ability to interact with desmin intermediate filaments from smooth muscle cells using sedimentation analysis, turbidity changes, chemical cross-linking, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI TOF/MS), and electron microscopic observations. Calponin interacted with desmin intermediate filaments in a concentration-dependent manner *in vitro*. The binding of calponin to desmin produced dense aggregates at 30°C. The dense aggregates were observed by electron microscopy to be composed of large anisotropic bundles of desmin filaments, indicating that calponin forms bundles of desmin filaments. The addition of calmodulin or S100 to the mixture of calponin and desmin caused the removal of calponin from the desmin filaments and inhibited bundle formation in the presence of Ca^{2+}, but not in the presence of EGTA. Calponin-related proteins including G-actin, tropomyosin, and SM22, had little effect on the binding of calponin to desmin filaments, whereas tubulin weakly inhibited the binding. Desmin had little influence on the calponin-actin and calponin-tubulin interactions using the zero-length cross-linker, EDC. Domain mapping with chymotryptic digestion showed that the binding site of calponin resides within the central α-helical rod domain of the desmin molecule. The chemical cross-linked products of calponin and synthetic peptides (TQ27, TNEKVELQELNDRFANYIEKVRFLEQQ; EE24, EEELRELRRQVDALTGQRARVEVE) derived from the rod domain were detected by MALDI TOF/MS. Furthermore, the calponin-desmin interaction was significantly inhibited by the addition of EE24, but only slightly by TQ27. These results suggest that calponin may act as a cross-linking protein between desmin filaments as well as among intermediate filaments, microfilaments and microtubules in smooth muscle cells.

Key words: bundling, calponin, cytoskeleton, desmin intermediate filament, rod domain.

Intermediate filaments are a major cytoskeleton component with a diameter of about 10 nm. A variety of intermediate filament species have been observed in different eukaryotic cell types (*1*, *2*). When intermediate filaments are disorganized and released from the membrane cytoskeleton, the cells become fragile and susceptible to rupture by mechanical stress (*3*). Recently, proteins that induce the cross-bridging of intermediate filaments to one another and cross-linking between intermediate filaments and other cytoskeletal components and anchors on the cytoplasmic surface have been found; these proteins seem to act as mechanical integrators of the cytoplasm and function to resist mechanical force (*2*, *3*).

Basic calponin is an actin-, tropomyosin-, and calmodulin-binding protein specifically expressed in smooth muscle cells (*4–6*). The binding of calponin to F-actin produces an inhibitory effect on the actin-activated Mg^{2+}-ATPase activity of myosin without affecting phosphorylation of the myosin light chain (*7–12*). Both binding and ATPase inhibition are reversibly abolished by the addition of calmodulin or S100 in a calcium-dependent manner (*7*, *10–12*). Calponin has also been reported to interact with myosin (*13*), microtubules (*14*, *15*), caldesmon (*16*), and phospholipids (*17*), indicating that this protein also exhibits functions other than the regulation of the actomyosin system. Immunocytochemical techniques have shown that calponin is distributed not only in the contractile but also in the cytoskeletal zones of smooth muscle (*18*, *19*). In fact, calponin has recently been reported to interact with desmin, a major intermediate filament in smooth muscle cells (*20*, *21*). However, little is known about the properties of the interaction.

In this study, we have found that the binding of calponin to desmin induces the formation of desmin filament bundles *in vitro*. The calponin-binding site is contained within

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Abbreviations: DIFP, diisopropyl fluorophosphate; DTT, dithiothreitol; EDC, 1-ethyl-3[3-(dimethylamino)propyl]carbodiimide; MES, 2-(*N*-morpholino)ethanesulfonic acid; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

the acidic central rod domain of the desmin molecule. Some characteristics of the binding and bundling and the effect of desmin on calponin binding to F-actin and microtubules are described.

MATERIALS AND METHODS

Preparation of Proteins—Calponin, tropomyosin, and SM22 were purified from chicken gizzard smooth muscle according to Fujii (*22*), Fujii *et al.* (*23*), and Lees-Miller *et al.* (*24*), respectively. The following proteins were isolated by means of the cited methods: rabbit skeletal muscle actin (*25*), porcine brain calmodulin (*26*), porcine brain S100 (*26*), and porcine brain tubulin (*27*). Desmin was isolated from chicken gizzard by the method of Lazarides and Granger (*28*) with some modifications. The desmin-rich fraction obtained from dry muscle was subjected to chromatography on CM-cellulose and DEAE-Sephacel in the presence of 6 M urea. The desmin fractions were collected, concentrated by ultrafiltration, and dialyzed against 10 mM Tris/acetate (pH 8.3). After centrifugation at 50,000 ×*g* for 60 min at 4°C, the purified desmin was stored for 1–2 weeks on ice until use.

Cosedimentation Assays—Desmin binding and bundling assays were performed by cosedimentation with desmin in bundling buffer (25 mM MES-NaOH, 5 mM Tris-acetate, 0.5 mM $MgCl_2$, 0.05 mM DTT, 20 mM NaCl, pH 7.1) containing 5 μM desmin and various concentrations of calponin and other proteins. After incubation for 30 min at 30°C, aliquots (100 μl) of each mixture were sedimented at high-speed (100,000 ×*g*, for 30 min at 25°C) to assess desmin filament assembly and at low-speed (14,000 ×*g*, for 30 min at 25°C) to assess desmin filament bundling. The obtained supernatants and pellets were analyzed by 10% SDS-PAGE using the discontinuous buffer system of Laemmli (*29*). The amount of protein present in each of the Coomassie Blue-stained bands was quantified by densitometry.

Electron Microscopy—The formation of desmin filaments and bundles was assessed by electron microscopy. Samples (25 μl) from the cosedimentation experiments were applied to carbon-coated 400-mesh grids and allowed to adsorb for 3 min. The grids were stained with 2% aqueous uranyl acetate. The uranyl acetate was withdrawn with filter paper and the grids were air-dried. Samples were visualized in a JEOL JEM-2010 operating at 90 keV.

Turbidity Change—Turbidity was monitored by recording the change in absorbance at 350 nm using a Hitachi spectrophotometer equipped with an automatic recorder at 30°C in a thermostatically regulated sample chamber. The reaction mixture was the same as used to assay the binding and bundling of calponin to desmin.

Cross-Linking—A mixture of calponin (4.4 μM) and desmin (12 μM) in 20 mM Tris-HCl (pH 7.2), and either actin (3.5 μM) or tubulin (3.3 μM) was incubated with freshly prepared 3 mM 1-ethyl-3[3-(dimethylamino)propyl]carbodiimide (EDC). After incubation for 30 min at 25°C, the reaction was stopped by the addition of one-fifth volume of 5 × electrophoresis sample buffer (155 mM Tris-HCl, pH 6.8, 5% SDS, 25% glycerol, 0.75% β-mercaptoethanol, and 0.0025% pyronin Y), then heated to 95°C for 2 min. The samples were then subjected to electrophoresis.

MALDI-TOF/MS Analysis—Calponin, with or without peptides treated with EDC, was subjected to DE MALDI-TOF mass spectrometric analysis. Sample solutions (1 μl) containing (15–25 pmol) and 2 μl of matrix solution (10 mg of sinapinic acid in 1 ml of 3:7 mixture of acetonitrile/water containing 0.1% trifluoroacetic acid) were mixed vigorously and centrifuged in a microcentrifuge for 1 min. One microliter of the supernatant was loaded onto a sample plate with 100 sample positions. The plate was loaded into a Voyager™ Elite DE Biospectrometry™ Workstation (PerSeptive Biosystems) with an N_2 laser (337 nm) in the linear mode. The resolution of the ion peak was determined by the resolution calculator using the GRAMS/386 software supplied with the instrument.

Amino Acid Sequence Analysis—Following separation by SDS-PAGE, the polypeptides were electroblotted onto a PVDF membrane and detected by Coomassie-Blue staining. The amino acid sequences of the polypeptides were determined using a gas-phase protein sequencer (Shimadzu PPSQ-21).

Other Procedures—Desmin was digested with chymotrypsin (1:400 w/w) in 10 mM Tris-acetate (pH 8.5). After incubation for 5 min at 25°C, the reaction was terminated by the addition of diisopropyl fluorophosphate (DIFP) to a final concentration of 2 mM. Calponin was coupled to CNBr-activated Sepharose 4B as described previously (*14*). The protein concentration was measured using the Bradford (*30*) Coomassie Blue dye binding method using bovine serum albumin as the standard. Alternatively, proteins were spectrophotometrically determined using appropriate extinction coefficients: A_{280} of 5.5 for desmin, A_{290} of 0.65 for actin, A_{275} of 1.8 for calmodulin, A_{280} of 3.44 for S100, and A_{278} of 2.9 for tropomyosin.

RESULTS

Binding of Calponin to Desmin Filaments—The properties of smooth muscle basic calponin binding to desmin filaments were determined by sedimentation assay (Fig. 1A). In the absence of calponin, high-speed centrifugation (at 100,000 ×*g* for 30 min) sedimented about 70% of the desmin, while low-speed centrifugation (at 14,000 ×*g* for 30 min) sedimented about 20% of the desmin under the established conditions (Fig. 1B). On the other hand, in the presence of calponin, the amount of desmin sedimented under both centrifuge conditions increased with increasing added calponin. When the concentration of calponin was over 10 μM in the mixture solution, most of the desmin was recovered in the pellet and the molar ratio of calponin bound to desmin reached a plateau of about 1.1 under both conditions. The apparent K_d value of calponin for desmin using high-speed centrifugation condition was approximately 5.3 μM. The binding ratio of calponin to desmin was only slightly affected when calponin was mixed with preformed desmin filaments under either set of centrifuge conditions (data not shown).

We examined the effect of calponin on the turbidity of desmin (Fig. 2). Mixing calponin with desmin solution increased the turbidity of the values obtained by the summation of the turbidity of each protein solution. The extraordinary increase in turbidity was observed in proportion to the amount of calponin added (3–6 μM) when the desmin concentration was fixed at 3 μM. A precipitate developed at 30°C, but did not occur in an ice bath for at least 30 min. These results suggest that calponin may accelerate

the rate of assembly of desmin filaments and induce the bundling or aggregation of desmin filaments through direct interaction.

Visualization of Desmin Bundles by Electron Microscopy—The effect of increasing calponin on desmin filament formation was assessed by electron microscopy. Single filaments of desmin were observed in the absence of calponin (Fig. 3A). When calponin was added to the solution, anisotropic bundles of desmin intermediate filaments were observed (Fig. 3B). These observations indicate that the lateral association of desmin filaments is mediated by calponin.

Calponin Binding and Bundling Are Regulated by Ca^{2+}/Calmodulin and Ca^{2+}/S100—It is well known that intermediate filaments containing desmin filaments are relatively stable compared with microtubules and microfilaments. Recently, S100 has been reported to induce the disassembly of desmin filaments in the presence of Ca^{2+} (*31*). In addition, both calmodulin and S100 interact with calponin and these interactions can modulate the binding of calponin to actin filaments and microtubules depending on the calcium concentration (*7–12*, *37*). Thus, we examined the effects of these proteins on the reconstitution of desmin filaments and calponin-desmin filament interaction using the sedimentation assay. After high-speed centrifugation, most of the desmin filaments were recovered in the pellet fraction in both the absence and presence of calponin as described in Fig. 1. When calmodulin or S100 was added to the mixture, the amount of desmin in the pellet in the presence of 0.5 mM $CaCl_2$ was reduced to 30 and 20% of the total desmin added, respectively (Fig. 4A). In the presence of calponin, the binding of calponin to desmin filaments was weakened in the presence and absence of Ca^{2+}. The inhibitory effect in the presence of Ca^{2+} was stronger than that in its absence. In the presence of Ca^{2+}/S100, neither desmin nor calponin was little recovered in the pellet. At low-speed centrifugation, the amount of desmin in the pellet was only slightly affected by calmodulin or S100 irrespective of the Ca^{2+} concentration (Fig. 4B). However, the bundling of calponin disappeared in the presence of Ca^{2+}/calmodulin or Ca^{2+}/S100. Interestingly, S100 had a more potent inhibitory effect than calmodulin. These results suggest that the addition of calmodulin or S100 in the presence of Ca^{2+} inhibits not only desmin assembly but also the calponin-induced bundle formation of desmin.

Effects of Actin, Tubulin, Tropomyosin, and SM22 on Calponin Binding to Desmin Filaments—In order to elucidate the nature of calponin binding to desmin, we examined the effects of other calponin-binding proteins including actin,

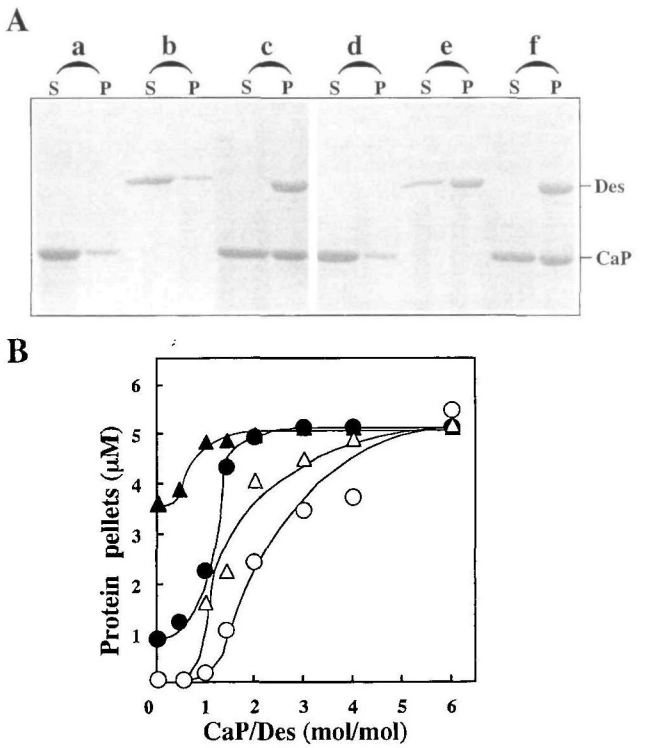

Fig. 1. **Sedimentation analysis of the interaction between calponin and desmin filaments.** (A) Desmin was incubated with various concentrations of calponin. The binding of calponin to desmin filaments was examined by the low-speed (pairs a–c) and high-speed (pairs d–f) sedimentation methods, as described in "MATERIALS AND METHODS", in which the protein compositions of the supernatants (S) and pellets (P) were analyzed by 10% SDS-PAGE. The concentrations of calponin and desmin were 8 and 5.2 μM, respectively. Pairs a and d, calponin; pairs b and e, desmin; pairs c and f, calponin + desmin. (B) Desmin was incubated with various concentrations of calponin. The amounts of calponin (○, △) and desmin (●, ▲) in the pellets obtained by low-speed (○, ●) and high-speed (△, ▲) sedimentation were determined by densitometry. CaP, calponin; Des, desmin.

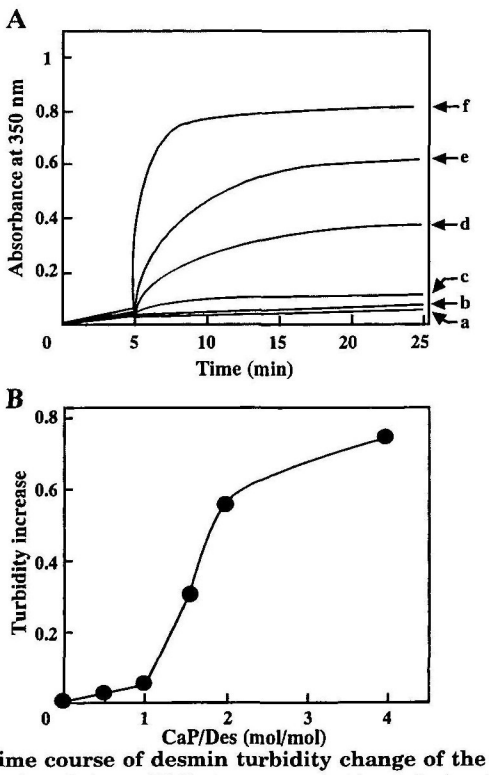

Fig. 2. **Time course of desmin turbidity change of the calponin–desmin mixture.** (A) Various concentrations of calponin were added after preincubation of desmin (3 μM) for 5 min at 30°C. The change in turbidity was monitored as the increase in absorbance at 350 nm in bundling buffer. The concentrations of calponin were 0 (a), 1.5 (b), 3 (c), 4.5 (d), 6 (e), and 12 μM (f). (B) The increase in turbidity increase is expressed relative to the optical density at 350 nm for 20 min.

tropomyosin, and tubulin, and SM22 on the binding of calponin to desmin filaments (Fig. 5). These proteins did not sediment by themselves by either the low- and high-speed centrifugations. To avoid the polymerization of G-actin to F-actin, we prepared non-polymerized actin using mycalolide B isolated from a marine sponge as previously reported (*14*, *33*). The addition of actin treated with mycalolide B had only a slight inhibitory effect on calponin binding to desmin filaments at an actin to calponin molar ratio of 2. On the other hand, a slight decrease in calponin bound to desmin filaments was found when tubulin was present at the same molar ratio. Tropomyosin had little effect on binding. SM22, a basic smooth muscle protein whose amino acid sequence shows a high degree of homology with calponin (approximately 41%), (*24*, *34*) did not interact directly with desmin filaments with significant affinity. In addition, this protein had little influence the bundling of calponin to desmin filaments. Similar results were also obtained in binding assays using high-speed centrifugation.

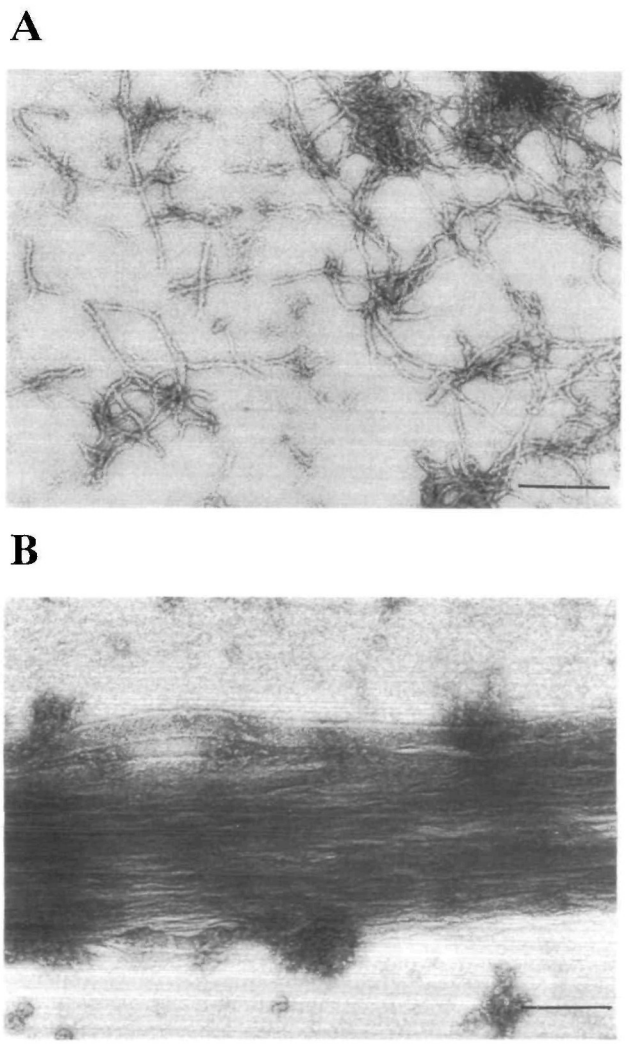

Fig. 3. **Electron microscopic visualization of a calponin–desmin filament mixture.** Desmin (4 μM) was incubated in bundling buffer for 30 min at 30°C in the absence (A) and presence (B) of calponin (4 μM). Samples were applied to carbon-coated grids and stained with 2% uranyl acetate. Bar, 200 nm.

Effects of Desmin on Calponin Binding to Actin and Tubulin—When mixtures of calponin-actin and calponin-tubulin were incubated with the zero-length cross-linker EDC, cross-linking products with an apparent molecular mass of 76 kDa (calponin-actin) and 86 kDa (calponin-tubulin) were generated and detected on SDS-PAGE (*14*, *35*). When calponin and desmin were mixed with EDC under the same conditions, a cross-linking product with an apparent molecular mass of 90 kDa was generated (Fig. 6). This 90 kDa band was immunostained with anti-calponin antibodies (data not shown). Furthermore, the addition of desmin had little effect on the formation of the calponin-actin and calponin-tubulin complexes, and there was no evidence of ternary complex formation. These results suggest that the desmin binding site(s) on calponin and the driving force of the interaction may be different from the calponin-actin and calponin-tubulin interactions.

Identification of the Calponin-Binding Domain in the Desmin Molecule—Desmin, like other intermediate filament constituents, possesses a tripartite substructure consisting of head, rod, and tail domains (*2*, *36*). When desmin is digested with chymotrypsin (1:500, w/w) for 5 or 20 min at 25°C, the 54 kDa polypeptide (native desmin) nearly disappears and the primary 38 kDa fragment is generated. Sedimentation assays have shown that calponin cosedi-

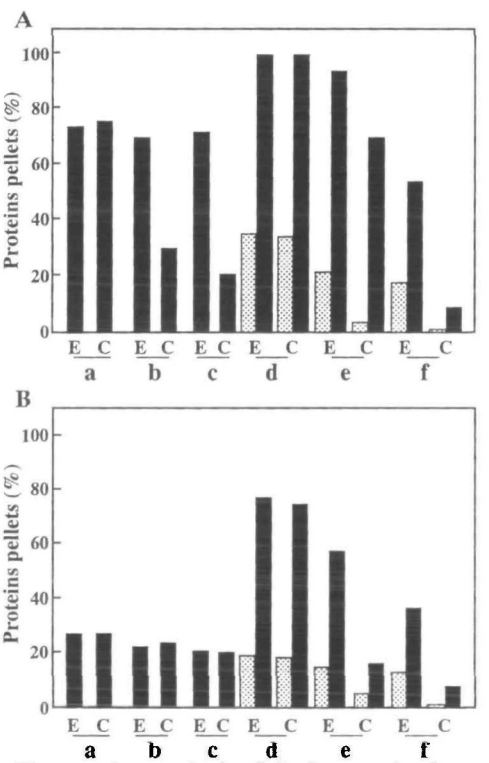

Fig. 4. **Sedimentation analysis of the interaction between calponin and desmin in the presence of EGTA, $CaCl_2$, calmodulin, and S100.** The assay conditions were the same as in Fig. 1A in the presence of 0.2 mM EGTA (E) or 0.5 mM $CaCl_2$ (C). Aliquots were sedimented at high-speed to assess desmin filament binding. The concentrations of calponin, desmin, calmodulin and S100 were 8, 5, 24, and 24 μM, respectively. CaM, calmodulin. a, Desmin; b, desmin + calmodulin; c, desmin + S100; d, desmin + calponin; e, desmin + calponin + calmodulin; f, desmin + calponin + S100. The amounts of calponin (dotted columns) and desmin (closed columns) were determined from densitometric scans of the SDS-gels.

ments with the 38 kDa fragment like native desmin during low-speed centrifugation (Fig. 7A). Amino acid sequence analysis shows that the 38 kDa fragment begins with Gln-Gly-Ala-Gly-Glu - - -, which corresponds to the central rod domain fragment from Gln74 as previously described (*36, 37*).

Further digestion was performed to determine the calponin-binding domain in more detail. Desmin was digested with chymotrypsin (1:100, w/w) for 30 min at 25°C and the reaction was stopped by the addition of DIFP at a final concentration of 2 mM. The major products appeared as 26, 18, and 12 kDa fragments on SDS-PAGE (Fig. 7B). The sample was applied to a calponin-Sepharose 4B column. The bound fraction eluted by 150–200 mM NaCl contained 26 and 18 kDa fragments. The amino acid sequences showed that the 26, 18, and 12 kDa fragments begin at Gln74, Arg139, and Thr323, respectively. These polypeptides are contained in the rod domain of the desmin molecule (Fig. 8).

Interaction of Calponin with Peptides Derived from the Rod Domain of Desmin and Their Effects on the Calponin-Desmin Interaction—Recently, we reported that calponin

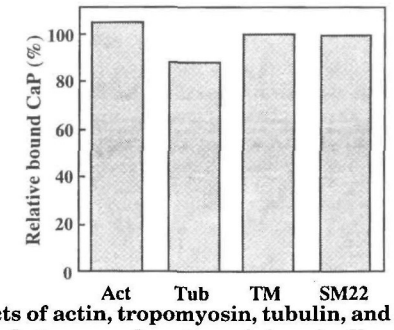

Fig. 5. **Effects of actin, tropomyosin, tubulin, and SM22 on the interaction between calponin and desmin filaments.** The assay conditions were the same as in Fig. 1 for low-speed centrifugation. The concentrations of calponin, desmin, actin, tubulin, tropomyosin, and SM22 were 8, 5, 16, 16, 4, and 16 μM, respectively. The relative amount of bound calponin is expressed as a percentage of the amount of calponin bound to desmin in the presence of added protein as opposed to its absence. Act, actin; TM, Tub, tubulin; TM, tropomyosin.

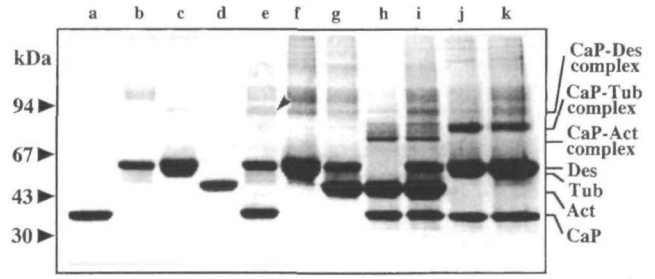

Fig. 6. **Effect of desmin on the cross-linking of calponin-actin and calponin-tubulin complexes.** The cross-linking reaction was performed with 3 mM EDC for 30 min at 30°C in the standard assay mixture containing 4.4 μM calponin, 12 μM desmin, 3.4 μM actin, and 3.4 μM tubulin as described in "MATERIALS AND METHODS." a, calponin; b, desmin; c, tubulin; d, actin; e, calponin + desmin; f, desmin + tubulin; g, desmin + actin; h, calponin + actin; i, calponin + actin + desmin; j, calponin + tubulin; k, calponin + tubulin + desmin. The cross-linked materials were separated by 5–15% SDS-PAGE. The arrowhead indicates the 90 kDa (calponin-desmin) cross-linked product.

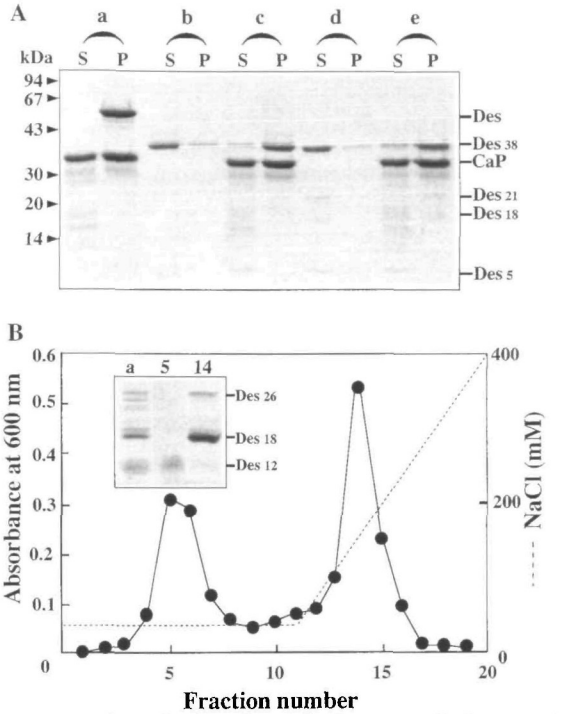

Fig. 7. **Interaction of calponin with chymotrptic fragments of desmin.** (A) Desmin was digested with 1/400 chymotrypsin (w/w) for 5 and 20 min at 25°C. The 38 kDa enriched fraction (0.54 mg/ml) was mixed with calponin (10 μM) and the procedure for the cosedimentation assay at low-speed centrifugation was the same as for Fig. 1. a, desmin + calponin; b, digested desmin (5 min); c, digested desmin (5 min) + calponin; d, digested desmin (20 min) ; e, digested desmin (20 min) + calponin. (B) Desmin was digested with 1/100 chymotrypsin (w/w) for 30 min at 25°C. The digest (4 mg) was loaded onto a calponin-Sepharose column equilibrated with 10 mM Tris-HCl (pH7.1), 0.5 mM $MgCl_2$, 0.2 mM DTT, 25 mM NaCl, 0.1 mM DIFP, 1 μg/ml pepstatin. The column was washed with the above solution and the bound protein was eluted with a linear gradient of 25–400 mM NaCl in the same buffer (-----). The insert shows SDS-PAGE profiles for the applied mixture (a) and fractions 5 and 14.

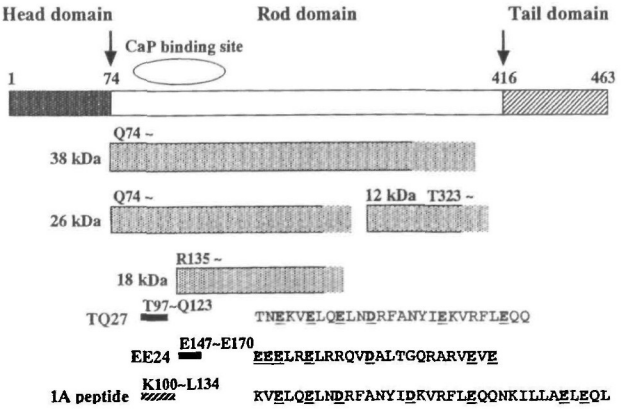

Fig. 8. **Localization of chymotryptic fragments and synthesized peptides in the rod domain of desmin.** The diagram shows the head, rod, and tail domains of the desmin molecule. The positions of the chymotryptic fragments and peptides TQ27, EE24, and peptide 1A are indicated. The sequence numbering follows that of chicken desmin (*56*).

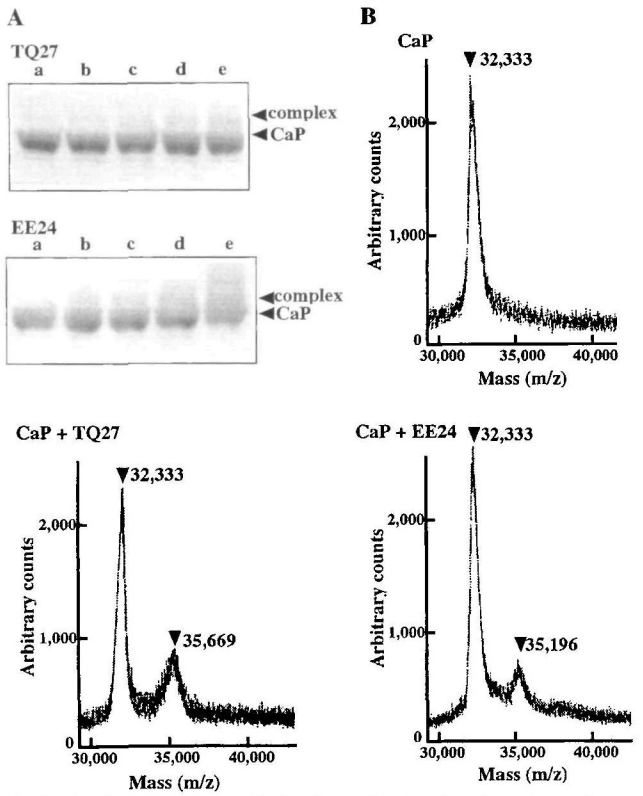

Fig. 9. **Analysis of cross-linked products of calponin and synthesized peptides by SDS-PAGE and MALDI-TOF/MS.** (A) Calponin (10 μM) was cross-linked with EDC to the synthetic peptides TQ27 (TNEKVELQELNDRFANYIEKVRFLEQQ) and EE24 (EEELRELRRQVDALTGQRARVEVE) as shown in Fig. 6. The peptides to calponin molar ratios were 0 (a), 10 (b), 25 (c), 50 (d), and 100 (e). The samples were analyzed by 5–15% SDS-PAGE. (B) The samples (molar ratio = 25) were analyzed by MALDI TOF/MS. Accelerating voltage: 21,000, grid voltage: 85% of the accelerating voltage, grid wire voltage: 0.2% of the accelerating voltage, delay: 100 ns, laser step: 3,000, scan average: 100. Myoglobin (molecular mass = 16,950.7), bovine insulin (molecular mass = 5,733.53), and angiotensin I (molecular mass = 1,296.7) were used as external standards to calibrate the spectra.

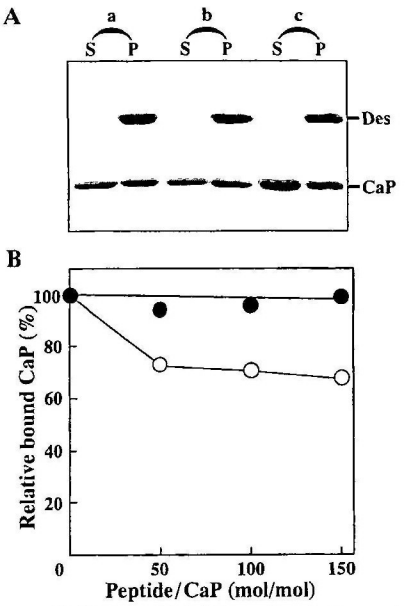

Fig. 10. **Effects of TQ27 and EE24 on the interaction between calponin and desmin filaments.** (A) The assay procedures were as described for Fig. 1 under the low-speed centrifugation. The concentrations of calponin, desmin, TQ27, and EE24 were 5, 5, 750, and 750 μM, respectively. Pair a, calponin + desmin; pair b, calponin + desmin + TQ27; pair c, calponin + desmin + EE24. (B) Various concentrations of TQ27 (●) or EE24 (○) were mixed with the calponin and desmin solution. The amounts of calponin bound to desmin filaments were determined from densitometric scans of SDS-gels.

interacts with synthetic peptides derived from the acidic regions of α and β tubulins (*15*). Acidic polyamino acids, including poly-L-aspartic acid and poly-L-glutamic acid, potentially inhibit the binding of calponin to desmin filaments in sedimentation assays (data not shown). The acidic region in the rod domain of desmin is thought to be essential for the binding to calponin. We prepared two synthetic peptides corresponding to T97–Q123 (TQ27; TNEKVELQELNDRFANYIEKVRFLEQQ; molecular mass = 3,355) and E147–E170 (EE24; EEELRELRRQVDALTGQRARVEVE; molecular mass = 2,882) derived from the rod domain (see Fig. 8). When calponin and the peptides were mixed with EDC as shown in Fig. 6, cross-linking products with apparent molecular masses of 35–36 kDa on 5–15% SDS-PAGE were generated in a concentration-dependent manner (Fig. 9A). The complexes were clearly confirmed by MALDI-TOF/MS. The molecular mass observed for the major species was 32,333, which is in perfect agreement with the value calculated from the amino acid sequence of chicken gizzard calponin (*38*). When calponin and either TQ27 or EE24 were incubated with EDC, new peaks with molecular masses of 35,669 (calponin-TQ27) and 35,196 (calponin-EE24) were observed (Fig. 9B). These sesults show the formation of 1:1 (mol:mol) calponin-peptide complexes.

We examined the effects of the two synthetic peptides on calponin binding to desmin filaments using the cosedimentation assay. As shown in Fig. 1, calponin cosediments with desmin filaments. A progressive decrease in the amount of calponin bound to desmin filaments was observed with increasing concentrations of EE24 under low-speed centrifugation conditions (Fig. 10). On the other hand, TQ27 had little effect on binding. These results suggest that calponin specifically recognizes the N-terminus acidic region in the rod domain of the desmin molecule.

DISCUSSION

The present data demonstrate that calponin interacts with desmin filaments and that this interaction can promote the bundling of desmin filaments *in vitro*. Wang and Gusev (*20*) and Mabuchi *et al.* (*21*) have reported that calponin cosediments with desmin filaments during high-speed centrifugation (at 100,000 ×*g* for 30 min). The apparent K_d value of calponin to desmin was calculated to be 5.3 μM with 1.1 mol of calponin maximally bound per 1 mol of desmin (Fig. 1). This K_d value is in fair agreement with a previous report (3–15 μM) (*22*). The apparent K_d values of calponin to F-actin and tubulin have been reported to be 0.19–6 μM (*22*, *39*, *40*) and 5.2 μM (*14*), respectively. Furthermore, the content of calponin in chicken gizzard smooth muscle is relatively high (about 80 μM) compared with calmodulin (24–

30 μM), caldesmon (about 10 μM), and myosin light-chain kinase (about 5 μM) (*4*, *41*, *42*). These data suggest that calponin is an adequate desmin-binding protein. The maximum amount of calponin bound to desmin is relatively high compared with the reported values (0.05–0.25 mol/mol) (*20*, *21*), indicating that bundle formation occurs at a high molar ratio of calponin to desmin filaments. In fact, no turbidity increase was observed at molar ratios of calponin to desmin below 0.5 (Fig. 2).

Synemin (molecular mass 230 kDa), paranemin (molecular mass 280 kDa), and IFAPa-400 (molecular mass 400 kDa) have been identified as desmin filament-associated proteins in smooth and skeletal muscles (*2*, *43*). However, the biochemical properties and physiological functions of these proteins on desmin filaments are still unknown. Sequence analyses have revealed that both synemin and paranemin may be intermediate filament proteins rather than intermediate filament-associated proteins, because they contain about 300 amino acids similar to the rod domain of intermediate filament proteins (*44*, *45*). Furthermore, no extensive similarities were found with calponin (*38*, *44*, *45*). A number of actin-binding proteins, including actin-bundling proteins, have been examined (*46*, *47*). However, little is known about the desmin-bundling protein in smooth muscle. Like other intermediate filaments, desmin seems to be a mechanical integrator of the cytoplasm that functions to resist mechanical stress. Compared with desmin alone, the presence of calponin produces distinguishable filamentous networks that aggregate into filamentous desmin bundles (Fig. 3). The mechanical integrity may be reinforced by the bundling of desmin filaments by calponin.

Intermediate filaments are widespread from the cell periphery to the nucleus. Georgatos *et al.* (*48*) have suggested ankyrin and lamin B as candidates for the association of desmin with the plasma membrane and nuclear envelope. The binding sites of ankyrin and lamin B on the desmin molecule are present in the head and tail domains, respectively. Desomoplakin, an intercellular junction component, interacts through its C-terminal region with keratin as well as vimentin and desmin at their head domains (*49*). Plectin (molecular mass 300 kDa), which is thought to mediate the association of intermediate filaments with contact structures at the plasma membrane, also interacts with microtubules, microfilaments, intermediate filaments, and membrane adhesion sites as well as with itself (*50*, *51*). Foisner *et al.* (*52*) have shown in solid-phase binding experiments that plectin binds to the helical rod domain of vimentin digested by limited chymotryptic digestion. As shown in Figs. 7 and 9, calponin also binds to the central rod domain of desmin. It is generally believed that calponin primarily regulates the interaction between actin and myosin filaments by binding to actin filaments. The binding of desmin to calponin has little influence on calponin-actin filaments and calponin-microtubules interactions (Fig. 6). When calponin is digested by chymotrypsin, fragments of 22 and 13 kDa are primarily generated (*5*, *53*). The binding sites of calmodulin, S100, actin, tropomyosin, and phospholipids are located in the 22 kDa fragment from Asn7 to Tyr182 in the amino acid sequence (*17*, *32*, *53*, *54*). As previously reported (*20*), we also confirmed that the binding domain to desmin lies in the 22 kDa fragment (data not shown). Further studies are needed to determine the order of the interaction and the binding sites within the 22 kDa fragment at the molecular level.

The isoelectric points of calponin and desmin are around 10 and 5.6, respectively (*53*, *28*). The calponin-desmin interaction is sensitive to the ionic strength (*20*). It seems that electrostatic interactions play an important role in the interaction. We think that the binding of calponin to desmin is specific because the binding is modulated by calmodulin and S100 in a Ca^{2+}-dependent manner. Previously, we and other groups have reported that Ca^{2+}-binding proteins, including calmodulin and S100, bind to calponin and modulate calponin–F-actin and calponin–microtubule interactions in a Ca^{2+}-dependent manner (*7–12*, *14*). Sedimentation analyses of the desmin in the presence of Ca^{2+}/calmodulin or Ca^{2+}/S100 show that only 10–50% of the desmin filaments are recovered in the pellet fractions after high-speed centrifugation (Fig. 4). Similar interactions have been reported by Garbuglia *et al.* (*31*), who found that the direct interaction of S100 with desmin inhibits the assembly of desmin and, furthermore, induces the disassembly of desmin filaments in a Ca^{2+}-dependent manner. Both Ca^{2+}/calmodulin and Ca^{2+}/S100 inhibit the binding of calponin to desmin and the formation of desmin filament bundles by calponin.

Calponin has been reported to inhibit the actin-activated Mg^{2+}-ATPase activity of myosin by binding to F-actin, and both calmodulin and S100 remove the inhibitory effect of calponin on actomyosin ATPase activity in the presence of Ca^{2+} (*7*, *9–11*, *32*). Unlike calmodulin and S100, desmin does not relieve the calponin-induced inhibition of actomyosin Mg^{2+}-ATPase (data not shown).

This is the first report of the determination of the binding site of calponin on the desmin molecule. Limited digestion of desmin by chymotrypsin mainly generates a 38 kDa rod domain fragment as described previously (*36*, *37*). This fragment cosediments with calponin, like native desmin (Fig. 6). Desmin fragments digested further with chymotrypsin do not sediment by themselves under the conditions employed. The location of the calponin-binding site is restricted using calponin-Sepharose chromatography (Fig. 7). N-terminal sequence determination shows that the fragments bound to calponin contain the N-terminal region of the rod domain. We designated two synthetic peptides (TQ27 and EE24) derived from the rod domain as shown in Fig. 8. Binding assays show that these peptides interact with calponin (Figs. 9 and 10), indicating multiple contributions of calponin binding. TQ27 regularly contains four XXE(D) sequences in the N-terminus region that overlap the 1A and 1B helical regions and L1 linker region (*2*), indicating the surface exposure of the acidic region in the higher order structure. On the other hand, EE24 contains acidic clusters in the N-terminal region located in the 1B helical region. These results suggest that the clustering of acidic residues is essential for the binding to calponin.

Goldman *et al.* (*55*) have reported that the synthetic peptide K103–L137 (peptide 1A, KVELQELNDRFANYIDKVRFLEQQNKILLAELEQL) derived from vimentin rod domain can disassemble to form vimentin filaments at a 1:1 molar ratio *in vitro*. The amino acid sequence shows high homology to that of TQ27 (identity, 23/27 residues) and K100–L134 of desmin (identity, 27/35 residues) as shown in Fig. 8. However, no significant disassembly of desmin filaments by TQ27 was observed by sedimentation assay. This discrepancy may depend on the different kinds of interme-

diate filaments and the length of the peptides. Interestingly, peptide 1A induces not only the disassembly of intermediate filaments but also the depolymerization of microtubules and microfilaments when 1A is microinjected into cultured fibroblasts (*55*). We examined the effects of TQ27 and EE24 on calponin-microtubules and calponin–F-actin interactions. We observed no significant influence on binding until the molar ratio of the peptides to calponin reached 150 (data not shown).

Calponin has been shown to interact with various cytoskeletal components including F-actin, tropomyosin, microtubule proteins, myosin, calmodulin, S100, caldesmon and phospholipids, including phosphatidylserine and phosphatidylinositol. Taken together, these results suggest that calponin is capable not only of modulating the actin-myosin interaction but also of promoting the bundling of desmin intermediate filaments and the linking of intermediate filaments to other cytoskeletal structures and the membrane cytoskeleton.

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