Association of Calponin with Desmin Intermediate Filaments*

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Our previous immunoelectron microscopy studies of chicken gizzard smooth muscle cells showed that in certain areas the distribution of anti-calponin exhibits a high degree of overlap with β-actin, filamin, and in particular, desmin, suggesting that in situ a fraction of calponin may be associated with intermediate filaments of the cytoskeleton. In this work we further explore this idea by studying the interaction between calponin and desmin. We found that at physiological salt concentrations, calponin bound only weakly to synthetic desmin intermediate filaments. On the other hand, calponin bound strongly to nonfilamentous desmin tetramers and was incorporated into intermediate filaments when the two proteins were mixed in a buffer containing 6 M urea and dialyzed into a buffer containing 0.15 M NaCl. Anti-calponin was found to label a portion of intermediate filaments and dense bodies isolated from gizzard tissues. Our findings suggest that in chicken gizzard smooth muscle cells, calponin may be an integral component of desmin intermediate filaments in the vicinity of dense bodies. Since calponin is also known to bind actin, we hypothesize that one of the functions of calponin might be to bridge intermediate filaments with actin in dense bodies.

Calponin (CP)1 is a ~33-kDa basic protein that is specifically expressed in smooth muscle cells (SMC) in adult animals. The properties of CP have been reviewed by Gimona and Small (1) and Winder and Walsh (2). Soon after its discovery, several striking biochemical properties of CP were rapidly established. (a) It is an actin-binding protein (3, 4). (b) It is capable of inhibiting actomyosin ATPase activity (4, 5). (c) It can bind calmodulin in the presence of Ca2+ with low affinity, and the complex does not bind to F-actin nor inhibit the ATPase activity (3, 6, 7). (d) It can be phosphorylated (4, 8), and the phosphorylated protein is no longer capable of binding actin nor of inhibiting actomyosin ATPase activity (4). It is based on these in vitro findings that CP is widely regarded as a thin filament-based regulator of actin-myosin interaction. However, its true physiological role remains unknown.

Aside from regions that contain myosin and contractile actin, SMCs have an extensive cytoskeletal network made of desmin and/or vimentin intermediate filaments (IFs), β-actin filaments, α-actinin, and filamin (reviewed by Small (9), Small et al. (10), and Bagby (11)). They also have dense bodies, often thought to be equivalent to the Z-disks of skeletal muscle, but unlike Z-disks, dense bodies contain noncontractile β-actin (12). It has been proposed that in gizzard SMC, the desmin IFs connect the dense bodies, but the molecular mechanism of how they are connected is not known (9). As is well known, IFs are polymerized at ~0.15 M NaCl from desmin tetramers, which are stable at low ionic strengths and in urea up to ~5 M (13).

Studies by Lehman showing that the majority of CP did not immunoprecipitate with anti-Ca2+ (14) but did with anti-filamin (15) suggested a cytoskeletal association for CP. More recently, North et al. (16) reported immunoelectron microscopy (IEM) results showing that CP is localized in both the cytoskeletal and the contractile regions of chicken gizzard SMCs. Using a novel fixation method that clearly distinguished cytoskeletal regions from myosin-containing contractile regions, we found that the density of CP in regions rich in myosin filaments is much lower than that in and around the cytoskeleton (17). This indicates that it is not likely for CP to directly regulate actin-myosin interaction in these cells. The distribution of CP is such that in certain regions of the cell it overlapped precisely with the cytoskeletal proteins β-actin, filamin, and in particular, desmin. This suggests that a fraction of CP may be associated with desmin IFs in situ and may function to link IFs with various elements of the cytoskeleton.

In this work we seek further evidence for the association of CP with IFs and investigate the nature of this association. Our results indicate that CP can be incorporated into synthetic IFs as a co-polymer with desmin in vitro and that some CP remains associated with native IFs and dense bodies even after extraction with solutions containing 0.4 M NaCl.

EXPERIMENTAL PROCEDURES

Materials—Common reagents were from Sigma, urea was from U. S. Biochemical Corp., materials for gel electrophoresis were from Bio-Rad, and materials for recombinant DNA procedures were from Difco.

Protein Preparation—Mouse desmin cDNA previously characterized by Li et al. (18) was inserted into the protein expression vector pET11d (Novagen, Madison, WI) and transformed into Escherichia coli BL21/DE3. Bacteria from a 2-liter culture were pelleted and kept at ~20 °C until use. The pellets were suspended in 150 ml of wash solution containing 0.1 M NaCl, 2 mM EDTA, 1 mM dithiothreitol (DTT), 1% Triton X-100, and 20 mM Tris-HCl, pH 7.5. The suspension was centrifuged at 120,000 × g for 25 min, and the resulting residue was homogenized in 100 ml of extraction buffer containing 6 M urea, 1 mM DTT, 2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 25 µg/ml trypsin inhibitor, and 20 mM Tris-HCl, pH 8.0. After removal of the insoluble materials by centrifugation, the extracts were applied onto a DE52 (Whatman) fast flow column pre-equilibrated with the same buffer. The column was washed with the extraction buffer that contained 50 mM NaCl but without the trypsin inhibitor. The bound proteins were then eluted with a 50–200 mM NaCl gradient. A desmin peak at ~110 mM

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Calponin-Desmin Association

Nylon was pooled and dialyzed against a solution containing 0.15 mM NaCl and 20 mM Tris-HCl, pH 7.5, to polymerize the desmin. Desmin filaments were collected by centrifugation (30 min at 70,000 × g), dissolved with 6 M urea in 20 mM Tris-HCl, pH 8.5, and stored at −20 °C. We obtained about 100 mg of desmin from a 2-liter culture. Desmin concentration was determined by the method of Bradford (2). A convenient means of indicating desmin concentration as that of the monomeric single-peptide chain throughout.

Expression and purification of recombinant chicken gizzard CP were described earlier (19). The purified CP was kept as freeze-dried powder at −20 °C. When needed, it was dissolved in a solution containing 6 M urea and 10 mM DTT then dialyzed against the same buffer. Since some fraction of CP is always aggregated upon storage, we routinely centrifuge CP solution at 220,000 × g for 20 min before each application.

**Dense Body Preparation** — The method of Tsukita et al. (20) was modified as follows. Chicken gizzard homogenates were first thoroughly washed as described for the preparation of washed myofibrils (21). We extracted actomyosin with 5 mM ATP, 10 mM MgCl₂, 1 mM EDTA, 0.1 mM NaCl, and 20 mM Tris-HCl, pH 7.5 (ATP/EGTA solution). Actomyosin was removed by three cycles of homogenizations of the myofibrils or residues in the ATP/EGTAMgCl₂ solution and centrifugations at 5,000 × g for 10 min. The residue after the third extraction, termed the dense body/IF-enriched fraction, was further suspended in the ATP/EGTA solution containing 0.15–0.4 M NaCl and then centrifuged at 150,000 × g for 10 min to compare the extractability of CP with that of CaD or Tm.

**Formation of Intermediate Filaments** — Desmin (4 μM) and CP (0, 2, and 4 μM) were dissolved in a solution containing 6 M urea, 10 mM Tris-HCl, pH 8.5, and the urea was replaced with IF buffer consisting of 0.15 M NaCl and 10 mM imidazole, pH 7.0, by successive dialyses versus solutions of 10 mM Tris-HCl, pH 8.5, containing 4, 2, and 0 M urea, and finally versus the IF buffer. After each dialysis, small amounts of solutions were put aside for electron microscope examination.

**Co-sedimentation and Co-polymerization Experiments** — For co-sedimentation experiments, 4 μM CP with or without 8 μM desmin IFs in 100 μl of solution containing 0.1 M NaCl, 0.5 mM DTT, and 20 mM Hepes, pH 7.5 (0.1 M NaCl buffer) was centrifuged at 100,000 × g for 10 min using the Beckman TL-100 ultracentrifuge and a TLA 100.2 rotor. For co-polymerization experiments, 5 μM each of proteins were mixed in 6 μl urea, the urea was replaced with the IF buffer as described above, and 200 μl was used for analysis. After centrifugation, 40 μl each of the supernatants and all the resolubilized pellets were subjected to SDS-PAGE.

**Electron Microscopy** — For rotary shadowing, proteins were adsorbed onto mica by placing a sheet of freshly cleaved mica on a drop of protein solution prepared as follows: 1 μg/ml desmin in 30% glycerol, 20 mM Tris-HCl, pH 8.5, with or without 4 μM urea, or 200-fold diluted dialyzed samples (see "Formation of Intermediate Filaments" below). The mica was then transferred to the above solution containing neither urea nor protein to remove adsorbed protein and urea. Adsorbed protein was then treated by treatment with uranyl acetate. After rinsing away the uranyl acetate, the mica was processed for rotary shadowing as described earlier (22, 23).

For imaging, the antibody-labeled CP-desmin complex, a monoclonal anti-CP (ascites, Sigma) was purified on a CP affinity column as described for the preparation of washed myofibrils (21). CP and anti-CP were then mixed at 5-fold molar excess, and a monoclonal anti-mouse antibody (BioCell, Cardiff, UK) was added to the mixture at 5-fold dilution and further incubated overnight at 4 °C.

**Desmin Affinity Chromatography** — Desmin (~15 mg) was dissolved in 6 M urea, 20 mM Tris-HCl, pH 8.5, and dialyzed versus 4 μM urea, 100 mM sodium potassium phosphate buffer, pH 8.0. About 0.5 g of CNBr-Sepharose 4B (Sigma) was quickly washed with the same buffer and mixed with the desmin solution. The mixture was left at 4 °C over a weekend with gentle constant agitation and then packed into a 1 × 2-cm column. After repeated washes with 0.1 mM acetic acid and 0.1 mM Tris solutions, the column was equilibrated with a solution containing 75 mM NaCl, 1 mM DTT, 1 mM EDTA, and 20 mM Tris-HCl, pH 7.5. 0.5 ml of a 0.8 mg/ml CP solution in 75 mM NaCl and 20 mM Tris-HCl, pH 7.5, was applied onto the desmin column containing ~2 ml of desmin-conjugated resin. The column was washed with ~10 ml of the buffer above then eluted with a 0.075–1 M NaCl gradient.

**SDS-PAGE and Densitometric Quantitation of Proteins in Gel Slabs** — Gel electrophoresis as described by Laemmli (25) was carried out using SE 250 vertical slab units ( Hoefer Scientific Instruments, San Francisco) according to manufacturer protocols. Images of gel slabs were captured by Data Transfer Frame Grabber (Maribor, MA) using a model CCD-72 (Dage-MTI, Inc., Michigan City, IN) camera equipped with a Sony TV zoom lens. Amounts of proteins associated with gel bands were estimated from the digitized images using the program NIH Image 1.54.

**RESULTS**

Co-sedimentation and Co-polymerization Experiments — To assess whether CP binds directly to IFs, CP (4 μM) and preformed desmin IFs (8 μM) were mixed in a solution containing 0.1 M NaCl and subjected to centrifugation. SDS-PAGE of the supernatant and pellet fractions (Fig. 1, lanes 3 and 4, respectively) show that whereas all the IFs were sedimented, only a small amount of CP sedimented with the IFs. Densitometric protein quantitation showed that 19% CP was found in the pellet fraction. Since ~10% CP sedimented by itself under the same conditions (Fig. 1, lanes 1 and 2), the fraction of sedimented CP attributable to binding to the synthetic IFs is no more than 9%. We conclude that the interaction between CP and preformed synthetic IFs is weak at best.

To examine whether CP can form a co-polymer with desmin, CP (5 μM) was mixed with desmin (5 μM of monomer) in 6 M urea as described under “Experimental Procedures.” The resultant filaments were centrifuged, and the supernatant and pellet fractions were subjected to SDS-PAGE (Fig. 1, lanes 5 and 6). It can be seen that a considerable amount of CP (52%) sedimented along with desmin. When CP without desmin was dialyzed versus the no-salt buffer, almost all the CP appeared to have stuck to dialysis membranes, and further dialysis versus the IF buffer solubilized only one-half of the CP. In contrast, with desmin, −44% of the original 5 μM was recovered in the IF fraction, whereas very little desmin was lost. Using this final CP concentration of ~2.2 μM rather than the initial 5 μM and assuming that ~10% of the 2.2 μM was self-sedimented, 42% of the CP co-polymerized with desmin IFs, clearly larger than the 9% in the co-sedimentation experiment (see above).

To ascertain whether the sedimented CP is indeed associated with the filaments, the sediments from an initial mixture of 2 μM CP and 4 μM desmin were resuspended in the IF buffer and examined by gold IEM using the monoclonal anti-CP. It can be
seen that the gold particles overlap well with the filaments (Fig. 2C). In control experiments using filaments formed with desmin alone, almost all the particles were found in the background (data not shown). It appears that the overall shape of the filaments is dependent on the relative content of CP; whereas filaments formed from an initial mixture of 2 μM CP and 4 μM desmin (Fig. 2B) were similar in shape compared with desmin IFs (Fig. 2A), those formed from 4 μM each of protein were wider, less dense, and had a flared appearance (Fig. 2D). These observations indicate that CP and desmin can form a co-polymer and imply that CP may interact with tetrameric desmin.

Binding of CP to Nonfilamentous Form of Desmin—It has been reported that tetrameric desmin is stable at 4 M urea (13). We attempted to image the rotary-shadowed images of desmin in 4 M urea (Fig. 3A) is indeed similar to those in the absence of salt (Fig. 3B). We further found that in the presence of 4 M urea, desmin does not polymerize in 100 mM phosphate buffer at pH 8.5 (data not shown), a property that we exploited to construct a desmin affinity column (see “Experimental Procedures”). Fig. 4 shows the elution profile of CP from the desmin column (panel A) and the corresponding SDS-gel electrophoretogram of selected fractions (panel B). No CP was found in the flow-through, indicating that all the CP was bound. Upon elution with a 0.075–1 M NaCl gradient, CP was eluted at ~0.3 M NaCl (fractions 22–26). In another experiment, the bound CP was eluted with stepwise increases in the concentration of NaCl; the majority of the CP also eluted at 0.3 M NaCl. This result shows that CP interacts with the unpolymerized form of desmin and that this interaction is likely to be quite strong since 0.3 M NaCl is required to dissociate CP from the desmin column.

The binding of CP to tetrameric desmin was also examined using the so-called “dot-blot assay,” in which tetrameric desmin (0–0.48 μM) was first spotted onto nitrocellulose then incubated with CP (0–0.9 μM). The bound CP was visualized using anti-CP and quantitated by densitometric imaging. The results (not shown) show that at 0.2 M NaCl, CP binds to desmin tetramers in a concentration-dependent and saturable manner, further indicating that this interaction can occur.

Imaging of the CP-Desmin Complex—We attempted to image the complex between CP and tetrameric desmin by mixing CP and desmin in the presence of 6 M urea followed by dialysis into salt-free buffer. Rotary shadowing electron microscopy of this preparation revealed large globular structures with many projections. Since it is well known that under this condition desmin is in the tetrameric form (see also Fig. 3B) whereas CP forms aggregates, we surmised that the projections are desmin tetramers, and the globules are CP aggregates. Such structures can form if CP binds to or near the ends of the desmin tetramers. We obtained clearer pictures from a preparation obtained by mixing 1 μg/ml desmin with 0.5 μg/ml CP in the presence of ~1 mM salt, conditions in which desmin molecules stay as tetramers and most of the CP molecules stay monomeric because of the very low protein concentration. We observed images of triangularly shaped anti-CP IgG molecules (26) localized primarily at the very ends of individual or aggregated desmin tetramers (Fig. 3D, marked by arrowheads) and, much
less frequently, at about one-third from the ends (marked by arrows).

Dense Body/IF Fractions—SDS-gel electrophoretograms of the residue left after extensive ATP/EGTA extraction (the dense body/IF-enriched fraction; see “Experimental Procedures” for details) showed that it contained primarily α-actinin, desmin, actin, and CP together with small amounts of filamin, myosin, CaD, and Tm (Fig. 5, lane 3). Further extraction of the dense body/IF-enriched fraction with the ATP/EGTA solution containing higher concentrations of NaCl removed CaD at 0.15 M and Tm almost completely at 0.3 M (lane 9). In contrast, a significant amount of CP remained with desmin even after extraction with 0.4 M NaCl (lane 11). We found that anti-β-actin (Sigma) reacted much stronger to actin in the residue after 0.4 M NaCl extraction than to actin in total gizzard homogenates (data not shown), indicating that β-actin is also enriched. It should be noted that several unidentified proteins were also present in the 0.4 M NaCl-washed fraction. These results show that desmin and CP are co-purified, but whether they associated with each other is not determined yet.

Electron microscopic examination of the dense body/IF-enriched fraction showed the presence of dense body-like dark objects, some of them connected by IFs (Fig. 6A). Upon reaction with anti-CP and gold-conjugated anti-IgG, many gold particles were seen to be associated with the dense body-like objects (Fig. 6B). However, details were difficult to discern, probably because filaments bearing CP became aggregated by the IgG molecules. When IEM examination was carried out on the residue after the 0.3 M NaCl extraction, association of gold particles with IFs near or at a dense body was clearly observed (Fig. 6C).

**DISCUSSION**

The impetus of this work was to seek independent evidence that might substantiate our earlier IEM findings that CP appears to be associated with IF (desmin) in certain areas of the chicken gizzard SMC (17). We found that at physiological ionic strengths, CP interacts with synthetic desmin IFs only weakly, so that it is not likely that CP is associated with IFs via direct binding. We noted that this is in agreement with Wang and Gusev (27), who reported that CP binds to IFs only at low ionic strengths. Instead, we found that CP binds strongly to tetrameric desmin and that the primary binding site is likely to be at one end of the IF polymerization subunit. This raises the possibility that CP is capable of being involved in IF assembly.

To examine this possibility, we mixed CP and desmin in 6 M urea followed by stepwise dialysis into a buffer known to promote IF polymerization. By this procedure, we produced a co-polymer of CP and desmin as judged by co-sedimentation and IEM examination. These *in vitro* studies clearly show that although it does not associate with IFs directly, CP is capable of forming a co-polymer with desmin in IFs. We noted that these findings are consistent with those of Wang and Gusev (27), who reported that CP binds to IFs only at low ionic strengths and that CP inhibits the rate of desmin polymerization in a solution of 50 mM NaCl and 5 mM MgCl₂ at pH 8.3.

Further, we examined the association of CP with various purification fractions starting with homogenates of chicken gizzards. As is well known, thorough washing followed by extraction with a solution containing ATP, EGTA, and MgCl₂ removes most of the soluble proteins, leaving a residue that is primarily composed of dense body-like structures linked by the IF network. We found that some CP is associated with this dense body/IF-enriched fraction as judged by SDS-PAGE and IEM. This fraction was further extracted with the ATP/EGTA solution containing 0.15–0.4 M NaCl to compare the salt extractability of CaD and Tm with that of CP. We found that whereas Tm and CaD were nearly completely removed at 0.15 and 0.3 M NaCl, respectively, CP remained with the dense body/IF fraction even after the 0.4 M NaCl wash. IEM exami-
of IFs that seems to be at the boundary of dense bodies. However, we do find labeled IFs that are not attached to dense bodies and some IFs that are not labeled at all. This may be due to disruption of the original contact between IFs and dense bodies by the high salt that was used for the extraction. Since the dense body contains many other proteins, it is also possible that other cytoskeletal proteins might be involved in the connection between IFs and dense bodies. It should be stressed that the proposed involvement of CP in cross-linking IFs to dense bodies appears to be only one of the in vivo functions of CP. This is because a high proportion of CP is extractable together with actin filaments with low salt ATP/EGTA/MgCl2 solution. Unfortunately, it is not clear whether or not CP is associated with IFs in areas other than the vicinity of dense bodies because only the dense body-CP complexes remain intact after the extensive extraction of actin and myosin filaments, which most likely cause the loss of structural integrity of the cytoskeletal channels. It is clear that much more work is still needed before the function of CP can be understood at the molecular level.

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Fig. 6. Localization of CP in the dense body/IF-enriched fraction*. Panel A, negative staining electron micrograph of the dense body/IF-enriched fraction showing the presence of dark, dense body-like objects connected by IFs. Panel B, same as A, with the addition of gold-conjugated anti-anti-CP. Panel C, same as B, using the residue after the 0.3 M NaCl wash (Fig. 5, lane 9) as specimen. See “Results” for analysis.