Characterization of 83-kilodalton Nonmuscle Caldesmon from Cultured Rat Cells: Stimulation of Actin Binding of Nonmuscle Tropomyosin and Periodic Localization along Microfilaments Like Tropomyosin

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Abstract. Nonmuscle caldesmon purified from cultured rat cells shows a molecular weight of 83,000 on SDS gels, Stokes radius of 60.5 Å, and sedimentation coefficient ($S_{20\,w}$) of 3.5 in the presence of reducing agents. These values give a native molecular weight of 87,000 and a frictional ratio of 2.04, suggesting that the molecule is a monomeric, asymmetric protein. In the absence of reducing agents, the protein is self-associated, through disulfide bonds, into oligomers with a molecular weight of 230,000 on SDS gels. These S-S oligomers appear to be responsible for the actin-bundling activity of nonmuscle caldesmon in the absence of reducing agents. Actin binding is saturated at a molar ratio of one 83-kD protein to six actins with an apparent binding constant of $5 \times 10^6$ M$^{-1}$.

Because 83-kD nonmuscle caldesmon and tropomyosin are colocalized in stress fibers of cultured cells, we have examined effects of 83-kD protein on the actin binding of cultured cell tropomyosin. Of five isoforms of cultured rat cell tropomyosin, tropomyosin isoforms with high molecular weight values (40,000 and 36,500) show higher affinity to actin than do tropomyosin isoforms with low molecular weight values (32,400 and 32,000) (Matsumura, F., and S. Yamashiro-Matsumura. 1986. J. Biol. Chem. 260:13851-13859). At physiological concentration of KCl (100 mM), 83-kD nonmuscle caldesmon stimulates binding of low molecular weight tropomyosins to actin and increases the apparent binding constant ($K_b$) from $4.4 \times 10^5$ to $1.5 \times 10^6$ M$^{-1}$. In contrast, 83-kD protein has slight stimulation of actin binding of high molecular weight tropomyosins because high molecular weight tropomyosins bind to actin strongly in this condition. As the binding of 83-kD protein to actin is regulated by calcium/calmodulin, 83-kD protein regulates the binding of low molecular weight tropomyosins to actin in a calcium/calmodulin-dependent way. Using monoclonal antibodies to visualize nonmuscle caldesmon along microfilaments or actin filaments reconstituted with purified 83-kD protein, we demonstrate that 83-kD nonmuscle caldesmon is localized periodically along microfilaments or actin filaments with similar periodicity (36 ± 4 nm) as tropomyosin. These results suggest that 83-kD protein plays an important role in the organization of microfilaments, as well as the control of the motility, through the regulation of the binding of tropomyosin to actin.

Caldesmon, first identified in smooth muscle, is a unique actin-binding protein whose binding to actin is regulated by Ca$^{2+}$/calmodulin (41). In the presence of micromolar concentrations of Ca$^{2+}$, caldesmon binds to calmodulin and such caldesmon/calmodulin complexes do not bind to actin. On the other hand, caldesmon does not bind to calmodulin in the absence of Ca$^{2+}$, and calmodulin-free caldesmon binds to actin.

Recent immunological studies have demonstrated the widespread occurrence of caldesmon-like proteins in a variety of nonmuscle tissues and cells, suggesting their important roles in the microfilament organization and regulation of cell motility. These nonmuscle caldesmons have been shown to have lower molecular weights on SDS gels than that of smooth muscle caldesmon (ranging from 70,000 to 80,000 vs. 140,000 of smooth muscle caldesmon) (5, 20). These studies have also revealed that nonmuscle caldesmon is localized in stress fibers as well as in membrane ruffles. Subsequent biochemical studies have demonstrated that the actin binding of purified proteins is also regulated by Ca$^{2+}$/calmodulin like smooth muscle caldesmon (9, 44). Although the properties of smooth muscle caldesmon have been relatively well studied, the properties of nonmuscle caldesmon, as well as its functions, are yet unclear.

The distribution of nonmuscle caldesmon in stress fibers is periodic and appears, at the light microscopic level, to be coincidental with that of tropomyosin (5). We have shown that a heat-stable protein with a molecular weight of 83,000, which is later identified as a nonmuscle caldesmon (51), is enriched in tropomyosin (TM)-containing microfilament fractions isolated from cultured rat cells using anti-TM monoclonal antibody (33). These observations have led us
to examine interrelation between 83-kD nonmuscle caldesmon and cultured cell tropomyosin. We have found that the 83-kD nonmuscle caldesmon can stimulate the binding of nonmuscle TMs to actin and that it regulates the binding of TMs to actin in a Ca²⁺/calmodulin-dependent manner. Furthermore, we have demonstrated that 83-kD nonmuscle caldesmon can be seen, at the electron microscopic level, to localize along microfilaments with similar periodicity as TM.

In addition, we have examined some physical chemical properties of nonmuscle caldesmon from cultured rat cells to help us understand its functions. We show evidence that nonmuscle caldesmon is monomeric asymmetric protein with a native molecular weight of 87,000. The protein is self-associated into oligomers through disulfide bonds, which appears responsible for the actin-bundling activity of 83-kD nonmuscle caldesmon. These properties are similar to those of smooth muscle caldesmon reported by Lynch et al. (26) but inconsistent with those of smooth and nonmuscle caldesmon previously reported by Kakuchi and co-workers (42, 44).

Materials and Methods

Protein Purification

83-kD nonmuscle caldesmon was purified from cultured rat cells (REF-4A) as described in detail elsewhere. Briefly, cultured cells (REF-4A cells) in wet weight of 120 g were homogenized in 500 ml of 20 mM Tris/HCl buffer of pH 8.0 containing 2.5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiothreitol (DTT), and 0.1 M NaCl. After centrifugation, the supernatants were heated in a boiling water bath for 15 min, cooled on ice for 30 min, and then centrifuged. The heat-stable supernatant was fractionated by adding ammonium sulfate powder between 0 and 28 g per 100 ml of the supernatant. After dialysis, the ammonium sulfate fraction was applied on a DEAE cellulose ion-exchange column equilibrated in 20 mM Tris/HCl buffer of pH 8.0 containing 2.5 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT. The column was developed with a linear gradient of NaCl from 0 to 500 mM. The 83-kD protein was eluted at about 100 mM NaCl. After addition of 1.3 mM CaCl₂, the pooled fractions were directly applied to a calmodulin-Sepharose column. The 83-kD protein was eluted with 50 mM Tris acetic buffer of pH 7.5 containing 3 mM MgCl₂, 1 mM DTT, 0.2 mM PMSF, and 4 mM EGTA. The final purification was done with direct loading of calmodulin column fractions on a hydroxyapatite column. The column was developed with a linear gradient from 10 to 250 mM sodium phosphate buffer of pH 7.0 containing 0.5 mM DTT and 0.2 mM PMSF. The proteins eluted at about 200 mM phosphate were dialyzed against 20 mM imidazole buffer of pH 7.0 containing 100 mM KCl and 0.5 mM DTT, and concentrated by a Centricon 30 (Amicon Corp., Danvers, MA). The purity was found to be >94% when checked by SDS-PAGE.

Chicken gizzard caldesmon was purified after Bretscher (3). A mixture of five nonmuscle TM isoforms was isolated from tissue culture rat cells (REF 4A cells), and separated into TM isoforms with lower molecular weight values (low mol wt TMs; a mixture of TM-1 (40,000) and TM-2 (36,500)) as described (31). PMSE The proteins eluted at about 200 mM phosphate were dialyzed against 20 mM imidazole buffer of pH 7.0 containing 100 mM KCl and 0.5 mM DTT, and concentrated by a Centricon 30 (Amicon Corp., Danvers, MA) at 38,000 rpm for 20 h at 4°C in a L8 Beckman preparative ultracentrifuge. Protein standards were purchased from Pharmacia Fine Chemicals (Piscataway, NJ) and included chymotrypsinogen (S₂₀ₒ = 2.6) and aldolase (S₂₀ₒ = 7.4). The Stokes radius of the 83-kD protein was determined by gel filtration with Sephacryl-300 (0.6 × 80 cm, Pharmacia Fine Chemicals) according to the method of Siegel and Monty (38). The column was equilibrated with the 20 mM imidazole buffer (pH 7.0), 0.1 M KCl, 0.5 mM DTT. Standards used to prepare a calibration curve were purchased from Pharmacia Fine Chemicals and included blue dextran (mol wt 2,000,000), thyroglobulin (85.0 Å), ferritin (64 Å), catalase (52.2 Å), Aldolase (48.1 Å), albumin (35.5 Å) and ovalbumin (30.5 Å). In some experiments, these standard proteins were loaded on the same column together with 83-kD protein, however, blue dextran was excluded because 83-kD protein was found to bind to blue dextran.

Actin-binding Assay

We have analyzed actin binding of 83-kD protein, as well as that of cultured cell TMs, in the following three conditions. (a) F-actin (final concentration, 0.5 mg/ml) was first incubated for 1.5 h at room temperature with either high or low mol wt TMs or without TMs, and then varying amounts (final concentrations, 0-0.3 mg/ml) of the 83-kD protein were added. The salt condition was 20 mM imidazole buffer of pH 7.0, 100 mM KCl, with or without 0.5 mM DTT. After incubation at room temperature for 1.5 h, the mixtures were centrifuged in a Beckman Airfuge (140,000 g [28 psi] for 20 min). (b) F-actin (final concentration, 0.5 mg/ml) was first incubated for 1 h at room temperature with or without 83-kD protein (final concentration, 0.17 mg/ml), and then varying amounts (final concentrations, 0-0.3 mg/ml) of low mol wt TMs were added. The salt condition was the same as those of (a). (c) The effect of calmodulin on the stimulation of actin binding of TMs by 83-kD protein was examined in the following conditions; 0.5 mg/ml of F-actin, 0.05 mg/ml of low mol wt TMs, 0.08 mg/ml of 83-kD protein, 0.1 mg/ml of calmodulin in 20 mM imidazole buffer of pH 7.0, 100 mM KCl, and 0.5 mM DTT. The concentrations of free Ca²⁺ were regulated using 2 mM EGTA-Ca²⁺ buffer calculated according to Amos et al. (4).

Densitometry was used to determine the concentrations of 83-kD protein, TMs and actin essentially as described (49). Both pellets and supernatants are suspended in an equal volume of SDS sample buffer, run on 12.5% SDS-PAGE, stained with Coomassie Brilliant Blue R-250 and scanned with a densitometer (model GS 300, Hoefer Scientific Instruments, San Francisco, CA) or Joyce Loebl Chromoscan 3 (Vickers Instruments Inc., Malden, MA). The densitometric absorbance was used to prepare a calibration curve. All samples were loaded within these limits of linear range of dye binding. The percentage of the amounts of the protein present in pellets and supernatants was determined and used to calculate the concentrations of bound and free proteins, respectively. Apparent binding constants, Kₐ, for 83-kD protein and for TMs were estimated as 1/(free 83-kD protein or TM concentration) at the point where the bound 83-kD protein or TM/actin concentration is half its maximal value.

Monoclonal Antibody

Mouse hybridoma production was performed essentially as described (19, 50). Positive clones were screened by immunofluorescence of cultured cells to see stress fiber staining. Clone SM12 was isolated to a single clone by agarose cloning (37) using gerbil fibroma cells as a feeder layer. The specificity of the antibody was examined by Western blot (47). As Fig. 1 shows, SM12 antibody reacted only with 83-kD nonmuscle caldesmon when total cell lysates of cultured rat cells were examined (lane 1 of panel B). It also reacted with smooth muscle caldesmon from chicken gizzard (lane 3 of panel B). Furthermore, SM12 antibody stained stress fibers as well as membrane ruffles of cultured cells, like the polyclonal antibody against smooth muscle caldesmon or nonmuscle caldesmon (5, 9, 20, 51).

Monoclonal Antibody-induced Aggregation of Microfilaments or Reconstituted Actin Filaments

We have previously demonstrated the periodic localization of nonmuscle TM along microfilaments using anti-TM monoclonal antibodies (33). The anti-TM monoclonal antibodies aggregate TM-containing microfilaments into ordered bundles that show periodic cross-striations due to the antibody binding to TM molecules within microfilaments. In a similar way, we have used anti-83-kD protein monoclonal antibody (SM12) to visualize the localization of 83-kD protein.
Other Procedures

Protein concentration was determined by the method of Lowry et al. (25) using bovine serum albumin as a standard. SDS-PAGE was performed essentially as described by Blatter et al. (2) using 12.5% polyacrylamide except that the buffer system of Laemmli (21) was used. Samples were dissolved in equal volumes of 2× SDS sample buffer at a final concentration of 1% SDS, 50 mM DTT, 40 mM Tris-HCl, pH 6.8, 7.5% glycerol, and 0.005% bromphenol blue. SDS-PAGE was also carried out in the absence of reducing agents where 2× SDS sample buffer without DTT was used to dissolve samples. Western blot was performed according to the method of Towbin et al. (47). To prepare total cell lysates, cells (one 100-mm culture dish, 10⁶ to 10⁷ cells) were quickly washed three times with PBS and extracted by addition of 300 μl of hot SDS sample buffer. The extracts were homogenized with a syringe by several passages through a needle (gauge no. 28). The homogenates were heated for 3 min at 100°C and used immediately for SDS-PAGE and immunoblotting. Cultured cells used were REF-52, REF-4A, and normal rat kidney cells, which were maintained in DME containing 10% FCS in an atmosphere of 5% CO₂ and 95% air at 37°C.

Results

Physical-Chemical Properties of 83-kD Protein

To determine the native molecular mass for the purified 83-kD protein, we measured both its Stokes radius and sedimentation coefficient according to the method of Siegel and Monty (38). As described later, about half of 83-kD protein was present in the sample.

Figure 1. Specificity of the monoclonal antibody (clone SM12) against 83-kD protein. Total cell lysates of normal rat kidney cells (lane 2 of A and lane 1 of B), purified 83-kD protein (lane 3 of A and lane 2 of B), and purified chicken gizzard smooth muscle caldesmon (lane 4 of A and lane 3 of B) were electrophoresed, transferred to nitrocellulose paper, and stained with Amido Black (A) or stained for immunoreactivity with the monoclonal antibodies against an 83-kD protein (B). Although the monoclonal antibody reacted both with smooth muscle caldesmon (lane 3 of B) and 83-kD protein (lane 2 of B), it reacted only with a polypeptide corresponding to 83-kD protein when total cell lysates were tested (lane 1 of B). Lane 1 of A includes molecular mass markers (from top to bottom: 200, 117, 94, 68, 43, 30, 21, and 14 kD).

Briefly, cultured cells were treated for 2 min at room temperature with Triton/glycerol solution (0.1 M Pipes, 5 mM MgCl₂, 0.2 mM EGTA, 0.05% Triton X-100, 4 M glycerol, pH 6.9) and residual cytoskeleton was homogenized in the presence of 5 mM Mg-ATP. After centrifugation with an Eppendorf centrifuge, a supernatant containing dispersed microfilaments was incubated with 0.05 vol of ascites fluids of SM12, anti-83-kD protein monoclonal antibodies. Resultant bundles of microfilaments were collected with an Eppendorf centrifuge, negatively stained with uranyl acetate, and observed with an electron microscope.

Reconstitution of actin filaments containing 83-kD protein was made by mixing 0.5 mg/ml of purified F-actin with 0.3 mg/ml of 83-kD protein in 0.1 M KCl, 0.2 mM DTT and 20 mM imidazole buffer of pH 7.0. In this condition, F-actin was saturated with 83-kD protein at a molar ratio of 6:1 as described later. The reconstituted actin filaments were then incubated with the same monoclonal antibodies against 83-kD protein (SM12). Resultant bundles were collected by low-speed centrifugation and observed as described above.

Figure 2. Actin binding of 83-kD protein in the absence or presence of TM. F-actin (final concentration 0.5 mg/ml) was first mixed with either high mol wt TMs (a; final concentration, 0.12 mg/ml) or low mol wt TMs (c; final concentration, 0.12 mg/ml) or buffer alone (a), and then incubated with varying concentrations (0-0.3 mg/ml) of 83-kD protein. The salt conditions were 100 mM KCl, 20 mM imidazole buffer of pH 7.0, and 0.5 mM DTT. After centrifugation at 140,000 g (28 psi) for 20 min in a Beckman Airfuge, both supernatant and pellets were adjusted to equivalent volumes of SDS sample buffer and run on 12.5% acrylamide gels. The bound 83-kD protein per actin concentration was determined by densitometry and plotted vs. free 83-kD protein concentrations. Apparent binding constant, Kₐ, was estimated to be ~5 × 10⁶ M⁻¹ in the absence of TM and 2 × 10⁷ M⁻¹ in the presence of TM. Saturation of binding is observed at a molar ratio of one 83-kD protein to six actin molecules either in the presence or absence of TMs.
Figure 3. (A) Electron micrograph and (B and C) phase-contrast micrograph of F-actin bundles caused by 83-kD protein in the absence of reducing agents. 83-kD protein (0.3 mg/ml) was first extensively dialyzed against 20 mM imidazole buffer of pH 7.0 and 0.1 M KCl and mixed with F-actin (0.5 mg/ml) in the same buffer. While 83-kD protein does not change the morphology of F-actin in the presence of reducing agents, it causes aggregation of F-actin into bundles (A) in the absence of reducing agents. These bundles can also be observed by a phase-contrast light microscopy (B). The bundle formation is reversible. The addition of 1 mM DTT makes these bundles disappear as shown in C. Bundles of F-actin reappear when reducing agents are dialyzed out.

The 83-kD protein forms oligomers in the absence of reducing agents, which makes it difficult to determine its Stokes radius and sedimentation coefficient accurately. We have, therefore, determined these values in the presence of 0.5 mM DTT. The 83-kD protein eluted upon gel filtration in a position very similar to that (61 Å) of ferritin, and its Stokes radius was calculated to be 60.5 Å. A sedimentation coefficient (S_{20,w}) of 3.5 was determined by sucrose density-gradient centrifugation for the 83-kD protein. These values give native molecular weight of 87,000 and a frictional ratio of 2.04, sug-
Figure 4. SDS-PAGE analyses of 83-kD protein oligomers formed in the absence of reducing agents. Purified 83-kD protein in volume of 500 μl was extensively dialyzed for 24 h with several changes against 1 liter of 20 mM imidazole and 0.1 M KCl to remove DTT. Then varying concentrations (0-1 mM) of DTT were added to dialyzed 83-kD protein. The mixtures were resolved with the same volume of SDS sample buffer without DTT and analyzed by SDS-PAGE. Lane 1, 1 mM DTT; lane 2, 500 μM DTT; lane 3, 100 μM DTT; lane 4, 50 μM DTT; lane 5, 10 μM DTT; lane 6, 5 μM DTT; lane 7, 1 μM DTT; lane 8, no DTT added. Below 100 μM of DTT, 83-kD protein makes oligomers through disulfide bonds. These S-S oligomers show a slower mobility on SDS gels whose molecular weight is calculated to be 230,000. Densitometry has shown that ~50% of 83-kD protein makes oligomers in the absence of reducing agents.

Actin-binding Properties

We have measured stoichiometry of the actin binding of monomeric 83-kD protein by high-speed centrifugation (150,000 g for 20 min). As Fig. 2 shows, saturation is achieved at an approximate molar ratio of one 83-kD protein to six actin monomers. The ratio of saturation remains the same even if reducing agents such as DTT are omitted.

The apparent binding constant, $K_a$, was estimated as $1/(\text{free 83-kD protein concentration})$ at the point where the (bound 83-kD protein)/(actin concentration) is half its maximal value. The range of $K_a$ estimated from four independent experiments is about $3-8 \times 10^6$ M$^{-1}$.

Actin binding of 83-kD protein is enhanced by the presence of TM, as Fig. 2 shows. The apparent binding constant, $K_a$, is increased to $2-3 \times 10^7$ M$^{-1}$ when either high or low mol wt TMs bind to actin. The saturation ratio (1:6) of 83-kD protein to actin, however, remained unchanged. Smooth muscle caldesmon has been reported to show similar enhancement of actin binding by tropomyosin (39).

Like smooth muscle caldesmon, the binding of 83-kD protein to actin is regulated by Ca$^{2+}$/calmodulin. Similar to the case of smooth muscle caldesmon, high concentrations of calmodulin, more than 10 molar excess of calmodulin over 83-kD protein are required for the complete release of 83-kD protein from actin.

Relationship between Actin-bundling Activity and Oligomer Formation of 83-kD Protein in the Absence of Reducing Agents

The morphology of 83-kD protein/F-actin complex was examined by an electron microscopy using negative staining technique. In the presence of 0.2 mM DTT, the structure of F-actin complexed with a saturating amount of 83-kD protein appears the same as that of F-actin.

In the absence of reducing agents, on the other hand, 83-kD nonmuscle caldesmon makes F-actin aggregate into bundles as shown in Fig. 3 A. These bundles can also be observed by a phase-contrast microscopy (Fig. 3 B). The formation of bundles by 83-kD protein is reversible. The addition of 1 mM DTT caused F-actin bundles to disappear (Fig. 3 C), and the bundles reappeared when DTT was dialyzed out.

These effects of reducing agents on actin-bundling activity of 83-kD protein have led us to examine if 83-kD protein forms oligomers through disulfide bonds in the absence of reducing agents. Such S–S oligomers should give slower mobilities when analyzed by SDS-PAGE in the absence of reducing agents.

As Fig. 4 shows, 83-kD protein gives two bands on SDS gels in the absence of reducing agents. While one band with a faster mobility corresponds to that of original 83-kD protein in the presence of DTT, the other shows a much slower mobility corresponding to the molecular weight of 230,000. The amounts of the slower moving band are decreased as the increase in the concentration of DTT, although >100 μM of DTT is necessary for complete elimination of the slower moving band. The amounts of slower moving band are not increased even if 50 mM CuCl$_2$ are included to facilitate oxidation of 83-kD protein. Densitometry of gels has revealed that ~50% of the total mass of 83-kD protein show slower moving band in the absence of reducing agents. Because the bundle formation is well correlated with the self-association of 83-kD protein through disulfide bonds, these S–S oligomers are likely to be responsible for the formation of F-actin bundles. Lynch et al. (26) have recently reported similar effect of reducing agents on self-association and actin-bundling activity of smooth muscle caldesmon.

Stimulation of Actin Binding of Nonmuscle Tropomyosin by 83-kD Protein

TM stimulates the binding of 83-kD nonmuscle caldesmon as shown in Fig. 2. We have now examined effects of 83-kD protein on actin binding of low or high mol wt TMs, and found that the 83-kD protein greatly stimulates the binding of low mol wt TMs to actin (Fig 5, A and B).
Figure 5. Stimulation of binding of low mol wt TMs by 83-kD protein. F-actin (0.5 mg/ml) was incubated for 1 h at room temperature with either 0.12 mg/ml (A) or 0.06 mg/ml (B) of low mol wt TMs in 20 mM imidazole buffer of pH 7.0, 0.1 M KCl, and 0.5 mM DTT, and then indicated amounts of 83-kD protein were added. After incubation for 1.5 h at room temperature, mixtures were centrifuged with a Beckman Airfuge (28 psi for 30 min), and both supernatants and pellets were analyzed on SDS gels. Actin binding of low mol wt TMs (●, a mixture of TM-4 and TM-5), and that of 83-kD protein (▲) were quantified by densitometry of Coomassie Brilliant Blue-stained gels, and plotted against total concentrations of 83-kD protein. Actin binding of 83-kD protein without TMs (●) was also measured for comparison.

In the absence of the 83-kD protein, the binding of low mol wt TMs to actin is very weak as described previously (31). At concentrations of 0.12 mg/ml of low mol wt TMs and 0.5 mg/ml of actin, only one-fifth of the mass of the low mol wt TMs are bound to actin; namely the molar ratio of low mol wt TMs to sedimentable actin is 1:29. Under these conditions addition of 0.83 μmol of the 83-kD protein promotes the binding of the TMs to actin by as much as threefold (Fig. 5 A) and the molar ratio is increased to 1:9. Accordingly, free TM concentrations are decreased from 1.68 to 0.8 μmol.

Further addition of 83-kD protein, however, does not increase the binding of low mol wt TMs to actin. The full saturation of actin with low mol wt TMs (1:6 molar ratio) can be achieved by the increase in the total concentration of low mol wt TMs as described later.

This stimulating effect is more obvious when the concentration of TM is halved to 0.06 mg/ml (Fig. 5 B). At this TM concentration only 5-7% of the mass of low mol wt TMs bind to actin and addition of the 83-kD protein increases the binding of the TMs to actin by as much as 11-fold. The molar ratio of low mol wt TMs to sedimentable actin is increased from 1:157 to 1:14 in this case.

We have next estimated the change in the apparent binding constants, \( K_a \), of actin binding for low mol wt TMs by 83-kD nonmuscle caldesmon. Fig. 6 shows a plot of bound amounts of low mol wt TMs vs. free concentrations of low mol wt TMs in the presence or absence of 83-kD protein. \( K_a \) is increased from \( 4.4 \times 10^5 \) to \( 1.5 \times 10^6 \) M\(^{-1}\) by the addition of 83-kD protein. It is worthwhile to note, however, that the increased \( K_a \) for low mol wt TMs is still lower than that \( (6.7 \times 10^6 \) M\(^{-1}\)) of actin binding for high mol wt TMs.

In contrast to low mol wt TMs, high mol wt TMs show high affinity for actin by themselves under physiological salt concentrations (31). Thus, the 83-kD protein has only slight effects on the binding of high mol wt TMs to actin (Fig. 7 A). At 0.12 mg/ml of high mol wt TMs, 78% of the high mol wt TMs already bind to actin in the absence of the 83-kD protein, giving 1:8 for a molar ratio of high mol wt TM to sedimentable actin. The addition of the 83-kD protein in-
increases the binding of high mol wt TMs up to a saturation ratio of 1:7 (Fig. 7 A).

We have further tested if 83-kD protein stimulates the actin binding of high mol wt TMs at lower KCI concentration (50 mM), where <10% of the mass of the high mol wt TMs bind to actin in the absence of the 83-kD protein. As Fig. 7 B shows, the 83-kD protein stimulates the binding of high mol wt TMs to actin by as much as 10-fold, so that almost 100% of actin becomes saturated with the high mol wt TMs. These results indicate that the 83-kD protein can promote the actin binding of either high or low mol wt TMs, although the effect is much more prominent on low mol wt TMs at physiological salt concentrations (100 mM KCl).

**Ca<sup>2+</sup> Regulation of Actin Binding of Tropomyosin by 83-kD Protein**

Actin binding of the 83-kD protein is regulated by Ca<sup>2+</sup>/calmodulin like that of smooth muscle caldesmon. We have, therefore, examined if the 83-kD protein coupled with calmodulin regulates the binding of low mol wt TMs to actin in a Ca<sup>2+</sup>-dependent way. As Fig. 8 shows, the binding of low mol wt TMs to actin in the presence of micromolar Ca<sup>2+</sup> is reduced to about half of that in the absence of Ca<sup>2+</sup>, while the actin binding of low mol wt TM alone is not regulated by Ca<sup>2+</sup> in the absence of 83-kD nonmuscle caldesmon and calmodulin.

**Periodic Localization of 83-kD Protein along Microfilaments**

We have previously reported the use of monoclonal antibodies to TM as a means of visualizing, at the electron microscopic level, the periodic localization of nonmuscle TM along microfilaments (33). We have now examined the localization of the 83-kD protein along microfilaments by similar means. An anti-83-kD protein monoclonal antibody (clone SM12) appears to cross-link a juxtaposed antigenic determinant of 83-kD protein molecules along adjacent microfilaments. This causes the microfilaments to align into ordered bundles with obvious cross-striations. The regularity of the
cross-striations indicates that the 83-kD protein molecules localize periodically along microfilaments. The periodicity is estimated to be $36 \pm 4$ nm ($n = 32$). This is very similar to the periodicity ($34 \pm 2$ nm) of cross-striations seen using anti-TM monoclonal antibodies (33).

It should be noted, however, that the microfilament bundles caused by the anti-83-kD protein antibody are thinner in width and fewer in number than the microfilament bundles caused by anti-TM antibody. Furthermore, loose bundles or meshworks of microfilaments without obvious cross-striations are also observed together with ordered bundles. This is in contrast to the microfilament aggregation caused by anti-TM monoclonal antibodies where many ordered bundles with obvious cross-striations are almost exclusively observed. These observations suggest that some of 83-kD protein is not regularly localized along microfilaments unlike TM. This is probably due to the lesser content of 83-kD protein (about one-sixth of total amounts of TMs) than that of TMs in cultured rat cells.

We have then tested if 83-kD protein localizes periodically along reconstituted actin filaments when enough amounts of 83-kD protein are present. Actin filaments were first mixed with a saturating amount of 83-kD protein, and SM12 monoclonal antibody was added. In this case, anti-83-kD protein monoclonal antibody makes almost all actin filaments aggregate into similar, but much thicker, well-ordered bundles with the same cross-striation pattern (Fig. 9 B). These observations suggest that the 83-kD protein can localize along microfilaments in vivo or along actin filaments in vitro, with a periodicity similar to that of TM.

We have also examined localization of smooth muscle caldesmon by similar means because SM12 monoclonal antibody cross-reacts with smooth muscle caldesmon from chicken gizzard (Fig. 1). Thin filaments were prepared from chicken gizzard as described previously (29) and incubated with either anti-83-kD monoclonal antibodies or anti-TM monoclonal antibodies. While anti-83-kD protein monoclonal antibodies again caused thin filaments to aggregate into bundles, cross-striations were not obvious (Fig. 10 A). The antibodies were, however, clearly observed as electron dense spots to bind along certain lengths of the thin-filament bundles (arrows in Fig. 10 A).

This sparse localization may be again due to lesser amounts of smooth muscle caldesmon in thin filaments. The molar ratio of caldesmon to actin in smooth muscle was reported 1:26 while that of TM to actin is 1:7 (27). We have, therefore, again examined if smooth muscle caldesmon can localize periodically along actin filaments when saturating amounts of smooth muscle caldesmon are allowed to bind to actin. As Fig. 10 B shows, SM12 monoclonal antibodies clearly make reconstituted actin filaments aggregate into ordered bundles with obvious cross striations. The periodicity is measured to be $55-57$ nm, which is $\sim 50\%$ longer than those of 83-kD nonmuscle caldesmon or smooth muscle TM (Fig. 10 C). The longer periodicity shown by smooth muscle caldesmon may suggest that 83-kD nonmuscle caldesmon is a shorter molecule than smooth muscle caldesmon. The differences in their Stokes radii ($61 \, \text{Å}$ of 83-kD protein vs. $91 \, \text{Å}$ of smooth muscle caldesmon) may support this notion.

**Discussion**

**Physical Properties**

Our results have elucidated some similarities and differences in properties between 83-kD nonmuscle caldesmon, smooth muscle caldesmon, and other nonmuscle caldesmon already reported.

First, the measurement of both Stokes radius and sedimen-
tion coefficient has elucidated that 83-kD nonmuscle caldesmon is a monomeric, asymmetric protein with a native molecular weight of 87,000. This is inconsistent with the previous report by Sobue et al. (44), who claimed that nonmuscle caldesmon from bovine adrenal medulla is a tetramer in solution with a native molecular weight of 300,000 (each subunit has a molecular weight of 77,000). Similar discrepancy is also reported on smooth muscle caldesmon. While Bretscher and co-workers (3, 26) have shown that smooth muscle caldesmon is a monomeric, elongated protein, Kakiuchi and co-workers (41, 42) reported that smooth muscle caldesmon is a heterodimer based on gel filtration. It appears likely that the determination of molecular weights based only on gel filtration leads to overestimation when the protein is rod-shaped.

Secondly, the estimated apparent binding constant ($K_a$) of $3-8 \times 10^6 \text{ M}^{-1}$ for 83-kD nonmuscle caldesmon is between those of high affinity ($K_a = 10^7$) and low affinity ($K_a = 10^5$) sites of smooth muscle caldesmon (39). It should be also noted that actin binding of 83-kD protein is enhanced by TM as seen in the case of smooth muscle caldesmon. However, it remains to be studied if 83-kD protein has two classes of actin-binding sites as reported for smooth muscle caldesmon (39). More accurate measurement using radioactive proteins may be needed to this end.

Third, both 83-kD nonmuscle caldesmon and smooth muscle caldesmon form S-S oligomers in the absence of reducing agents, which are likely to be responsible for the formation of actin bundles (Figs. 3 and 4; reference 26). In both cases, relatively high concentrations of reducing agents (>0.1 mM DTT) are necessary to dissociate these S-S oligomers, and its physiological meaning remains to be elucidated. It should be noted, however, that 83-kD nonmuscle caldesmon makes only one species of cross-linked oligomers with molecular weight of 230,000, while smooth muscle caldesmon forms many cross-linked species of oligomers with no upper limit of molecular weights (26).

Our data indicate that 83-kD protein and smooth muscle caldesmon share most of the properties including actin binding, calmodulin binding, molecular shape, self-association, and actin bundling, although the size of the 83-kD protein is about half of smooth muscle caldesmon. This is not surprising as judged from recent studies on domain mapping of smooth muscle caldesmon from several laboratories (12, 36, 45). These laboratories have shown that 20- to 40-kD fragments include both actin-binding and calmodulin-binding sites. Fuji et al. (12) have further suggested that the actin-binding and calmodulin-binding domain is located in the carboxyl-terminal half of smooth muscle caldesmon, and that nonmuscle caldesmon with molecular weight of 77,000 from platelets is homologous to the carboxyl-terminal of smooth muscle caldesmon. It is, therefore, likely that 83-kD non-

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**Figure 10.** Electron micrographs of periodic localization of smooth muscle caldesmon along thin filaments prepared from chick gizzard (A), along reconstituted actin filaments (B) and electron micrograph of periodic localization of smooth muscle TMs along thin filaments (C). Smooth muscle caldesmon localizes periodically with a 55-57-nm repeat (indicated by arrows in B) whereas smooth muscle TMs localizes periodically with a 38-nm repeat (indicated by arrows in C).
muscle caldesmon is also homologous with the carboxy-terminal half of smooth muscle caldesmon. At present, we neither know why nonmuscle caldesmon lacks the amino-terminal half of smooth muscle caldesmon, nor understand the function of the amino-terminal half of smooth muscle caldesmon. Future comparative studies on smooth muscle and nonmuscle caldesmon should have insight into the functional differences between these muscle and nonmuscle caldesmon.

**Stimulation of Actin Binding of TM by 83-kD Protein**

What is the molecular mechanism by which the 83-kD protein promotes the binding of nonmuscle TMs? Of muscle contractile proteins, myosin subfragment 1 (S-I) and tropomycin are known to stimulate the binding of TM to actin (10, 11). Because these two proteins appear to be unrelated, there are likely more than one mechanism for the enhancement of TM-binding to actin.

Two possibilities are present though they are not exclusive of each other. One is the direct interaction between 83-kD nonmuscle caldesmon and nonmuscle TM from cultured cells as observed in the case of the binding between tropomyosin and tropomycin. The other possibility is that 83-kD protein may change the conformation of F-actin, which favors more binding of TM to actin. S-I may act in this way since there is no evidence that S-I and TM interact directly.

Several other laboratories have reported the binding between smooth muscle TM and smooth muscle caldesmon using immobilized proteins to Sepharose CL-4B (12, 36, 39). We, however, could not show the binding between 83-kD protein and nonmuscle tropomyosin under the same salt conditions where we observed the stimulation of actin binding of TM by 83-kD protein. For example, both proteins behaved independently in gel filtration, as well as in sucrose density-gradient centrifugation. Furthermore, we could not show the binding of cultured cell TMs to 83-kD protein attached to Sepharose CL-4B. It is possible, however, that these methods may not be appropriate to detect the direct binding if the equilibrium is too fast.

It is worthwhile to note that full enhancement of TM-binding to actin by 83-kD protein is observed at molar ratios (1:12 to 1:20) of 83-kD protein to actin much lower than the saturation (1:6). For example, at 0.12 mg/ml (2.1 μmol) of low mol wt TMs (Fig. 5 A), ~0.9 μmol of 83-kD protein increase actin binding of TM from 0.4 to 1.2 μmol. Further binding of 83-kD protein up to saturation (1.88 μmol of 83-kD protein) does not increase TM binding to actin although 40% of actin is not saturated and considerable amounts (0.8 μmol) of TM still remain as free TM. Likewise, the actin binding of 83-kD protein at a molar ratio as little as 1:24 can fully stimulate the actin binding of high mol wt TMs as shown in Fig. 7 B. These results may not be simply explained by the direct binding between 83-kD protein and TM, but rather easily explained by conformational changes of F-actin induced by the substoichiometric binding of 83-kD protein.

**Periodic Localization of 83-kD Nonmuscle Caldesmon along Microfilaments**

The periodical localization of 83-kD protein with 36 ± 4 nm repeats suggests that, like TM, one 83-kD protein molecule binds per six or seven actin monomers, assuming that the 83-kD protein has one antigenic site to the monoclonal anti-body per one 83-kD protein molecule. This seems likely and is consistent with biochemical data indicating that 83-kD protein binds to actin with a saturating molar ratio of one 83-kD protein molecule to six actin monomers (Fig. 2). Because 83-kD protein is a rodlike protein as is TM, it seems likely that the 83-kD protein binds along the length of actin filaments as TM does.

**Possible Functions of 83-kD Nonmuscle Caldesmon in the Reorganization of Microfilaments upon Cell Transformation**

The biological functions of nonmuscle caldesmon, as well as those of nonmuscle TM, are still unclear. Smooth muscle caldesmon has been suggested to play a regulatory role in smooth muscle contraction (3, 4, 8, 13, 17, 27, 35, 39, 40, 43). As 83-kD protein appears to be functionally similar to smooth muscle caldesmon, it may also play a similar role in the motility and contraction of nonmuscle cells.

We (24, 30, 33), as well as others (6, 14-16, 22, 23), have suggested that TMs are involved in the reorganization of microfilaments upon oncogenic transformation of cultured cells. In transformed rat cells, we have shown that the expression of one or two of the high mol wt TMs is suppressed and the low mol wt TMs become predominant (30). The low mol wt TMs show a lower affinity to actin than do the high mol wt TMs (31). These results suggest that changes in the extents of actin binding of TM are intimately involved in the alteration of microfilament patterns in cell transformation.

It is tempting to speculate the function of 83-kD nonmuscle caldesmon in the reorganization of microfilaments in cell transformation because the 83-kD protein can regulate the binding of cultured cell TMs to actin as described in this paper. In normal cells, 83-kD protein may increase the stability of microfilaments through its enhancement of actin binding of TMs. In transformed cells, levels of nonmuscle caldesmon appear to be reduced as reported by Koji-Owada et al. (20). In addition, Criss and Kakuichi (7) have shown that levels of both total calcium and calmodulin are increased in transformed cells. Thus, 83-kD nonmuscle caldesmon may not stimulate the binding of TMs to actin in transformed cells as it may in normal cells.

Furthermore, we have previously demonstrated that another actin-binding protein with mol wt of 55,000 (55-kD protein) inhibits binding of low mol wt TMs, but not of high mol wt TMs, to actin (32). Our preliminary data have shown that levels of 55-kD protein, in contrast to those of 83-kD protein, are increased in cell transformation. Therefore, all these changes in levels of 83-kD protein, 55-kD protein and calcium, coupled with the replacement of high mol wt TMs with low mol wt TMs are likely to reduce binding of TMs to actin. Because the binding of TM to actin stabilizes the structure of actin filaments (18, 34, 46, 48), these changes may result in the formation of unstable microfilaments in transformed cells, that may cause, at least in part, the morphological alterations observed in many transformed cells.

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