THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 270, No. 8, Issue of February 24, pp. 3919–3925, 1995
Printed in U.S.A.

Effect of Caldesmon on the Assembly of Smooth Muscle Myosin*

(Received for publication, February 3, 1994, and in revised form, November 14, 1994)

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Smooth muscle myosin filaments are much less stable than the skeletal muscle counterpart. Smooth myosin requires higher concentration of Mg^{2+} than skeletal myosin to form thick filaments and addition of ATP disassembles the dephosphorylated smooth muscle myosin filaments into monomers but not phosphorylated ones. We found that the addition of caldesmon to dephosphorylated myosin induced the formation of the filaments under the conditions where myosin by itself is soluble or disassembled. Although the induced filaments were short at 1 mM Mg^{2+}, they became medium sized and seemed like side polar filaments with prominent 14 nm periodicity at higher Mg^{2+} conditions (8 mM). In the presence of F-actin, myosin filaments induced by caldesmon were associated along actin filaments to form large structures. The association of actin and myosin filaments was observed only in the presence of caldesmon, suggesting that caldesmon cross-linked actin and myosin filaments. This cross-linking was disrupted by the addition of calmodulin. Caldesmon-induced filament formation of dephosphorylated myosin in the presence of Mg^{2+}-ATP may explain the existence of myosin filaments in relaxed smooth muscle fibers. A similar effect of telokin on myosin filament assembly was also examined and is discussed.

It is well accepted that the initial regulation of the smooth muscle contractile machinery is through the reversible phosphorylation of the 20-kDa light chain of myosin. This is catalyzed by Ca^{2+}/calmodulin-dependent myosin light chain kinase and myosin light chain phosphatase (Hartshorne, 1987; Sellers and Adelstein, 1987; Kamm and Stull, 1989). Although phosphorylation of myosin is necessary and sufficient for the initiation of muscle shortening (Ito *et al.*, 1989b), the response of smooth muscle to various stimulants is complex. It has been suggested that secondary regulatory systems might be involved in smooth muscle regulation in addition to myosin phosphorylation (Hartshorne, 1987; Marston and Redwood, 1991).

Because of its ability to modify actomyosin ATPase, caldesmon has been implicated as a potential regulator of contraction, secondary to myosin phosphorylation. In support of a modulatory role for caldesmon, Katsuyama *et al.* (1992) reported that a synthetic peptide of caldesmon (Gly^{651}–Ser^{667}) which can bind to calmodulin and actin raises the basic tone of skinned smooth muscle cells.

Caldesmon is an actin and calmodulin-binding protein isolated from smooth and non-muscle cells. In addition to actin and calmodulin (Sobue *et al.*, 1981), caldesmon also has a specific affinity to tropomyosin (Graceffa, 1987; Fujii *et al.*, 1987) and myosin (Ikebe and Reardon, 1988).

Three actin binding regions were mapped to the COOH-terminal domain (Mornet *et al.*, 1988; Wang *et al.*, 1991), and the calmodulin binding region lies between residues 659–665 (Bartegi *et al.*, 1990; Wang *et al.*, 1991; Hayashi *et al.*, 1991). The COOH-terminal domain of caldesmon is also involved in the binding to tropomyosin (Fujii *et al.*, 1987; Dabrowska *et al.*, 1985; Katayama *et al.*, 1989; Hayashi *et al.*, 1991), whereas myosin binding is restricted to the amino-terminal domain of caldesmon (Sutherland and Walsh, 1989; Katayama *et al.*, 1989; Katayama, 1989a; Hemric and Chalovich, 1990). Although the primary structure of caldesmon and its domain mapping has been studied in detail, the physiological functions of caldesmon have not yet been clarified.

A number of studies have shown that caldesmon can inhibit actomyosin ATPase activity *in vitro*, suggesting its involvement in the regulation of cross-bridge cycling (Marston and Redwood, 1991; Sobue and Sellers, 1991). Ca^{2+}/calmodulin not only reverses the inhibition induced by caldesmon but also reduces the binding of caldesmon to actin (Sobue *et al.*, 1981; Bretcher, 1984; Furst *et al.*, 1986; Dingus *et al.*, 1986). However, a much higher calmodulin concentration is required for the dissociation of caldesmon from actin than that for the reversal of inhibition. These results suggest that the reversal of the inhibition might not be due to the dissociation of caldesmon from F-actin but rather due to the change in the interaction of caldesmon with actin induced by Ca^{2+}/calmodulin.

The physiological significance of caldesmon-myosin interaction is not clear. Localization of caldesmon in smooth muscle cells was reported to be in the thin filament-thick filament domain but not at the thin filament-intermediate filament domain (Furst *et al.*, 1986), suggesting a role for caldesmon interaction with the contractile machinery.

Under physiological salt conditions *in vitro* dephosphorylated monomeric myosin adopts an unconventional conformation in which the tail portion of myosin is bent back toward the head-rod junction so as to form a folded structure (Trybus *et al.*, 1982; Onishi and Wakabayashi, 1982; Craig *et al.*, 1983). Upon phosphorylation, myosin changes to an extended conformation which is conceivably more suitable for filament formation. Dephosphorylated smooth muscle myosin can form thick filaments in the presence of high Mg^{2+} concentration; however, the filaments are readily disassembled by addition of ATP (Suzuki *et al.*, 1978). Phosphorylated myosin filaments are more stable and are not disassembled in the presence of Mg^{2+}-ATP (Suzuki *et al.*, 1978). Although this may suggest that in smooth muscle, myosin filament formation may be regulated by

* This work was supported by National Institutes of Health Grants AR38888, AR41653, and HL37117 and partly by grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (to E. K.) and by the Alberta Heritage Foundation for Medical Research (to G. S. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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phosphorylation, *in vivo* studies using quick-freeze electron microscopy have demonstrated that thick filaments are unambiguously present in resting smooth muscle cells where the majority of myosin molecules are assumed to be dephosphorylated (Somlyo *et al.*, 1981). This discrepancy in the stability of smooth muscle myosin thick filaments *in vitro* and *in vivo* is unresolved. It has been suggested that high concentrations of myosin such as those found in smooth muscle cells could be sufficient to produce thick filaments even though myosin is dephosphorylated (Kendrick-Jones *et al.*, 1987). Another possibility is that myosin-binding proteins might stabilize myosin thick filaments. We have explored this latter possibility and in this study, we show that caldesmon induces filaments of smooth muscle myosin.

MATERIALS AND METHODS

Smooth muscle myosin was prepared from turkey gizzard as described previously (Ikebe and Hartshorne, 1985b). Caldesmon was prepared according to Bretcher (1984). Actin was prepared from rabbit skeletal muscle acetone powder by the method of Spudich and Watt (1971). Telokin was prepared according to Ito *et al.* (1989a). Tropomyosin was prepared from turkey gizzard as follows. Muscle mince was homogenized with 4 volumes of 0.1 M KCl, 0.2 mM DTT,[1] and 30 mM Tris-HCl, pH 7.5, then centrifuged for 5 min at 4000 × *g*. The pellet was washed four times with the same buffer. To the pellet, 4 volumes of 0.3 M KCl, 0.15 M KP_i, pH 6.5, was added and homogenized. After 30 min at 4 °C, the homogenate was centrifuged at 10,000 × *g* for 15 min. The pellet was washed five times with 4 volumes of 1 mM $NaHCO_3$. Following the homogenization with 3 volumes of 2 mM $NaHCO_3$, pH was adjusted to 8.3 with Tris base. Tropomyosin was extracted for 2 h at room temperature. The extract was collected by centrifugation at 10,000 × *g* for 5 min, then subjected to ammonium sulfate fractionation. 250–500 g/liter ammonium sulfate fraction was collected, suspended with and dialyzed against (50 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5, and 0.2 mM DTT). The produced pellet was dissolved in 5 volumes of 1 M KCl. Tropomyosin was precipitated by adjusting the pH to 4.6 with acetic acid. After standing for 1 h, the precipitate was collected and resuspended in 1 M KCl, and the pH was adjusted to 7.5. Any undissolved materials were removed by centrifugation, and the final supernatant was dialyzed against 10 mM Tris-HCl, pH 7.5, and 0.5 mM DTT and stored at −80 °C. The final product contained no contaminant proteins as judged by SDS-PAGE analysis.

Myosin Mg^{2+}-ATPase activity was measured in solution containing 1 mM $MgCl_2$, 0.5 mg/ml dephosphorylated myosin, 30 mM Tris-HCl, pH 7.5, and 0.2 mM [γ-^{32}P]ATP at 25 °C. The ATPase activity was also measured in the presence and absence of 0.5 mg/ml F-actin and 0.2 mg/ml caldesmon. The liberated ^{32}P was quantitated as described previously (Ikebe and Hartshorne, 1985a).

SDS-gel electrophoresis was carried out in a 7.5–20% polyacrylamide gradient slab gel using the discontinuous buffer system of Laemmli (1970).

Various combinations of protein mixture samples were examined by negative staining electron microscopy. To obtain clear images of small protein aggregates, samples of various protein mixtures (0.5 mg/ml myosin, 0.14 mg/ml caldesmon, and/or 0.1 mg/ml telokin or 0.14 mg/ml myosin 0.2 mg/ml actin, 0.07 mg/ml tropomyosin, 0.15 mg/ml caldesmon) in buffer (50 mM KCl, 1 or 8 mM $MgCl_2$, 0.5 mM ATP, 1 mM EGTA, 5 mM sodium phosphate, pH 7.5) were applied to uncoated number 400 copper grids followed by 1% uranyl acetate containing bacitracin as described previously (Katayama, 1989b). When the sample was too thick, it was diluted five times with the same buffer prior to the negative staining. Electron micrographs were taken by JEOL 100CX or JEOL 2000ES with an acceleration voltage of 80 kV. The advantages and disadvantages for the use of this staining method have been described previously (Katayama, 1989b). Myosin-caldesmon-actin complexes in the presence of ATP formed large aggregates of thick and thin filaments, and the structural details were difficult to resolve by negative staining. In order to visualize the structural components of such a complex, we subjected the mixture to harsh mechanical mixing or ultrasonic agitation followed by quick dilution and immediate negative staining. Optical transforms of thick filaments were taken using Luzex-F real-time image analyzer (Nireco, Japan). The statistics on the distribution of the filament length was done with the same equipment.

[1] The abbreviations used are: DTT, dithiothreitol; S-2, myosin subfragment 2; PAGE, polyacrylamide gel electrophoresis.

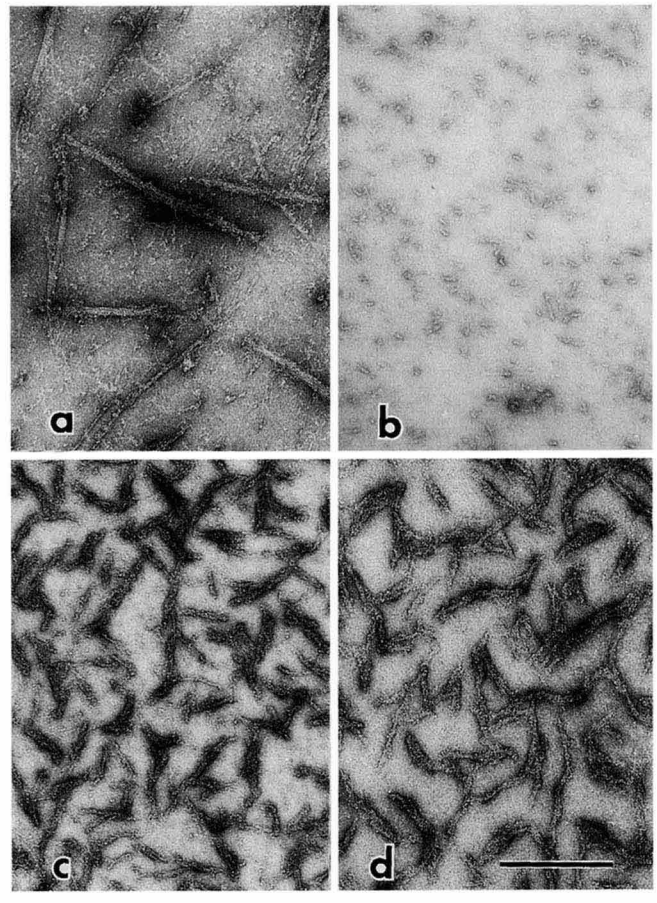

FIG. 1. **Negatively stained images of dephosphorylated myosin.** *a*, under 10 mM $MgCl_2$ in the absence of ATP, many side polar filaments were observed in the field; *b*, under 1 mM $MgCl_2$ in the presence of ATP myosin molecules remained soluble without forming any filaments; *c*, under 1 mM $MgCl_2$ in the absence of ATP, addition of caldesmon induced numerous filament-like aggregates, whereas myosin by itself was soluble as in *b*; *d*, under 1 mM $MgCl_2$ but with ATP, many filament-like aggregates were observed when caldesmon was added. The size of the aggregates was almost the same or slightly larger than that in the absence of ATP. *Scale bars* indicate 0.2 μm throughout all electron micrographs.

RESULTS

Myosin with phosphorylated light chain has high Mg^{2+}-ATPase activity and can form thick filaments in the presence of ATP, initiating superprecipitation with actin filaments (Ikebe *et al.*, 1977; Suzuki *et al.*, 1978). The structure of thick filaments formed by phosphorylated myosin is stable irrespective of the presence of ATP under high (10 mM Mg^{2+}) or low (1 mM) Mg^{2+} concentrations (data not shown). On the other hand, the activity of myosin with dephosphorylated light chain is kept low in terms not only of ATPase but also its ability to form filaments. Although dephosphorylated myosin forms side polar filaments (Craig and Meyerman, 1977) at high Mg^{2+} concentration (Fig. 1*a*), addition of small amounts of ATP readily disassembles the filaments almost completely to its soluble form (data not shown).

Although caldesmon was first described as a thin filament component, it was later reported that caldesmon binds to myosin at S-2 region (Ikebe and Reardon, 1988). We examined the effects of caldesmon on the assembly properties of myosin. As shown in Fig. 1*c*, many short filament-like aggregates were observed upon addition of caldesmon, under the conditions where myosin by itself hardly formed filaments. Very similar

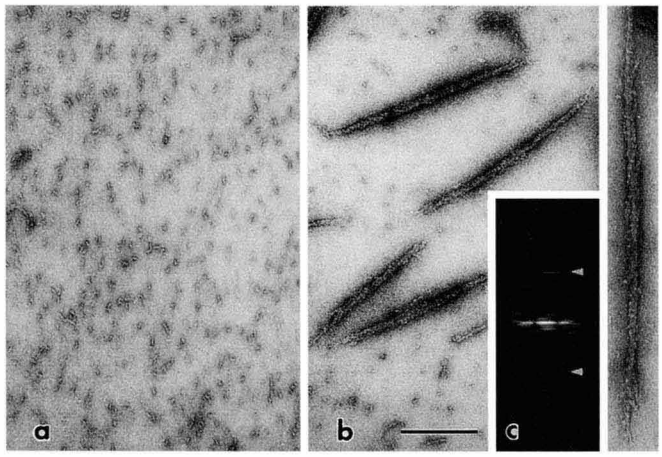

FIG. 2. **Negatively stained images of dephosphorylated myosin under 8 mM $MgCl_2$ conditions in the presence of 0.5 mM ATP.** *a*, most myosin molecules remain disassembled even under such high Mg^{2+} conditions; *b*, when caldesmon was added, myosin forms rigid medium sized filaments with apparent 14 nm axial periodicity throughout its whole length. The *inset* in *c* is an optical transform of one of such filaments as enlarged on the *right* with prominent 14 nm banding pattern. *Arrows* indicate the meridional reflections for that periodicity.

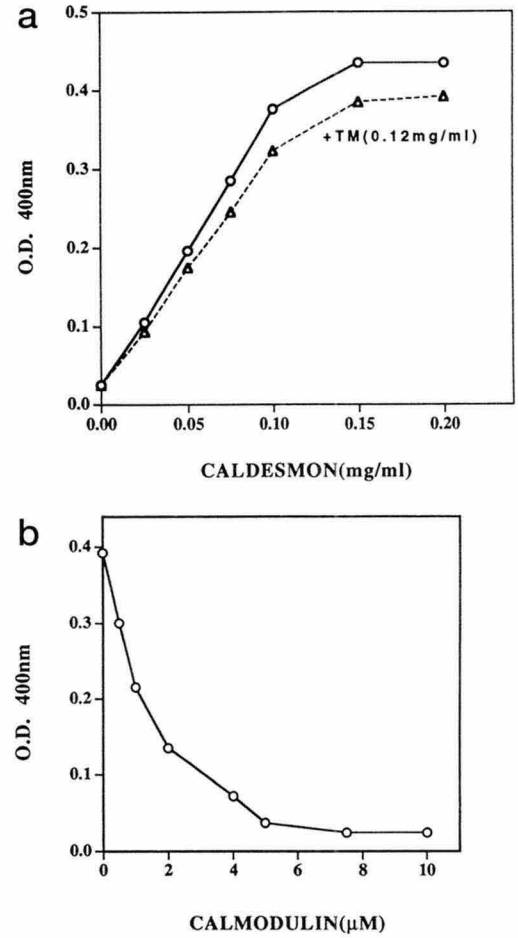

FIG. 3. **Increase in the turbidity of smooth muscle actomyosin by caldesmon and the reversal effect by calmodulin.** *a*, various concentrations of caldesmon were added to a solution containing 0.5 mg/ml myosin, 0.5 mg/ml actin, 5 mM sodium phosphate, pH 7.5, 1 mM EGTA, 1 mM $MgCl_2$, 50 mM KCl, and 0.5 mM ATP. The mixture was incubated for 5 min at 25 °C, and the turbidity was measured by Perkin-Elmer UV/VIS spectrophotometer at a wavelength of 400 nm. The turbidity was stable for at least 30 min at room temperature. ○, without tropomyosin; △, with 0.12 mg/ml gizzard tropomyosin. *b*, effect of calmodulin on the caldesmon-induced increase in the turbidity of smooth muscle actomyosin. Conditions are the same as for *a*, except that 0.15 mg/ml caldesmon and 0.1 mM $CaCl_2$ were used.

filamentous aggregates were also induced when caldesmon was added to myosin solution in the presence of Mg-ATP (Fig. 1*d*), although their length distribution seemed somewhat larger than those in the absence of ATP. Under 8 mM Mg^{2+} concentration, dephosphorylated myosin could not yet form any filamentous structure if ATP was present (Fig. 2*a*). However caldesmon induced decent medium sized filaments (Fig. 2*b*) well comparable with those produced with phosphorylated myosin or with dephosphorylated myosin in the absence of ATP. The filaments seemed straight and stiff showing prominent axial periodicity corresponding to 14 nm throughout the whole length (Fig. 2*c*) and characteristic of side polar filaments. Because caldesmon is known to associate with thin filaments, we examined the effects of caldesmon-F-actin complex on myosin filament formation.

Fig. 3*a* shows that the turbidity of the solution increased upon the addition of caldesmon to actomyosin in the presence of Mg^{2+}-ATP. The marked increase in the turbidity was not observed in the absence of myosin (data not shown). Similar turbidity increase was also observed when F-actin was added to myosin/caldesmon solution (data not shown). The increase in turbidity was dependent on caldesmon concentration and reached a plateau at 0.15 mg/ml caldesmon if actin concentration was fixed at 0.5 mg/ml. This corresponds to approximately 1 caldesmon/16 actin monomers.

The results suggest that caldesmon can cross-link myosin and actin and supports our earlier report (Ikebe and Reardon, 1988). Tropomyosin which is also known to bind caldesmon did not significantly affect the increase in the turbidity of actin/myosin solution induced by caldesmon (Fig. 3*a*). Fig. 3*b* shows that the turbidity of actomyosin induced by caldesmon declined by a further addition of Ca^{2+}/calmodulin. The decrease in the turbidity occurred in calmodulin dose-dependent manner and more than 6 μM was required to completely reverse the caldesmon-induced increase in turbidity. This was consistent with the notion that calmodulin can compete with the binding of caldesmon to actin.

The structure responsible for the large increase in turbidity was examined by electron microscopy (Fig. 4). In the absence of caldesmon the majority of myosin was dissociated from actin filaments and did not form filamentous aggregates (Fig. 4*a*). Addition of caldesmon induced filamentous aggregates of myosin as described above and these aggregates were associated along F-actin filaments to form large structures (Fig. 4*b*). This large actin-caldesmon-myosin filament complex tended to become entangled with each other and harsh mechanical mixing of the solution was needed to obtain clear image of each structural component by negative staining.

To examine further the constitution of these complexes, actin, myosin, caldesmon, and tropomyosin were mixed in various combinations and were spun by low speed centrifugation at 10,000 × *g* for 5 min. The protein composition of the supernatant and the pellets was analyzed by SDS-PAGE (Fig. 5). Caldesmon, myosin, and actin coprecipitated. The molar amounts of myosin and actin which coprecipitated with caldesmon were higher than that of caldesmon. This is consistent with the observation by electron microscopy that the formation of large aggregates is not due to the cross-linking of F-actin and individual myosin molecules by caldesmon, but involves myosin in aggregated form. Caldesmon and myosin coprecipitated under the conditions where myosin by itself did not precipitate, although the amount of precipitated proteins were less than in the presence of F-actin (Fig. 5). Under these conditions the smaller size of each myosin filamentous aggregate was similar

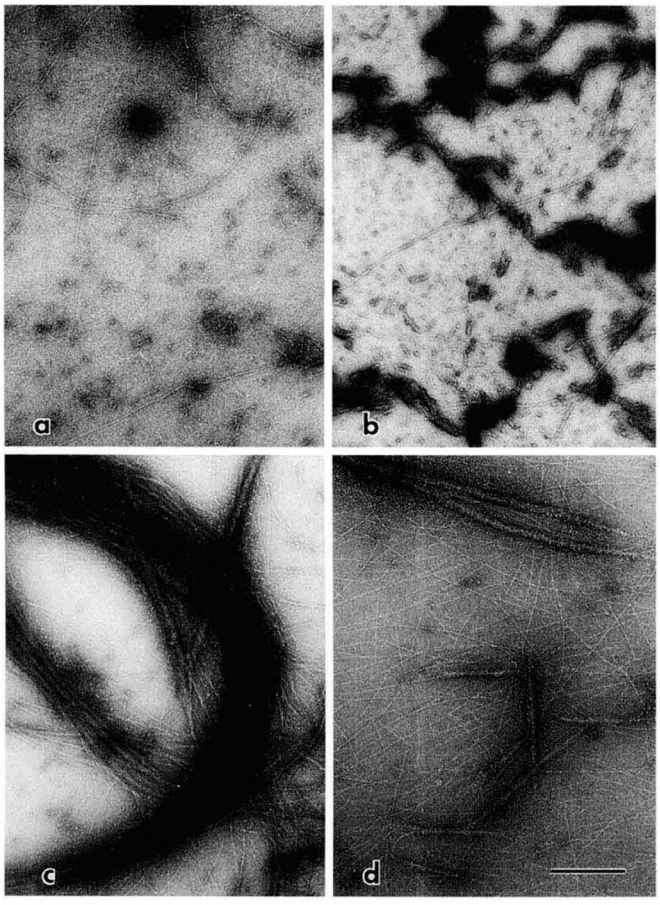

FIG. 4. **Negatively stained images of the mixture of unphosphorylated myosin and F-actin in the presence of ATP.** *a*, most myosin oligomers were dissociated from actin-filaments; *b*, under 1 mM $MgCl_2$ conditions, filamentous myosin aggregates induced by caldesmon were all closely associated to actin filaments forming large light-scattering clumps. Micrograph was taken after vigorous mechanical agitation; *c*, under 8 mM $MgCl_2$ in the presence of ATP, actomyosin with added caldesmon formed very solid filament bundles whose appearance was similar to stress fibers. Micrograph shows the association of filaments in the complex which was so tight that a substantial fraction remained still in bundles even after ultrasonic treatment; *d*, on the same specimen grid was found an image showing the structural constituents of filament bundles which was dispersed by the above treatment. Several thick filaments were observed together with many actin filaments. Note the presence of clear periodic banding pattern throughout the thick filament surface, suggesting that thick filaments are side polar myosin filaments.

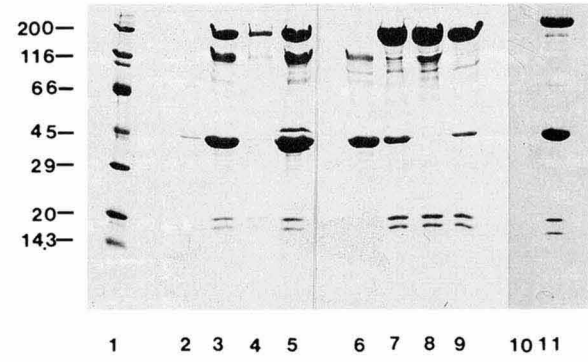

FIG. 5. **Cosedimentation analysis of myosin-actin-caldesmon complex.** Various combination of myosin (0.5 mg/ml), actin (0.5 mg/ml), caldesmon (0.15 mg/ml), and tropomyosin (0.12 mg/ml) were mixed in the buffer containing 5 mM sodium phosphate, pH 7.5, 1 mM EGTA, 1 mM $MgCl_2$, 0.5 mM ATP, and 50 mM KCl and then centrifuged at low speed (10,000 × *g*) for 5 min. The precipitate (*lanes 2–5* and *10*) and the supernatant (*lanes 6–9* and *11*) were analyzed by SDS-PAGE: *lanes 2* and *6*, actin and caldesmon; *lanes 3* and *7*, myosin, actin, and caldesmon; *lanes 4* and *8*, myosin and caldesmon; *lanes 5* and *9*, myosin, actin, caldesmon, and tropomyosin; *lanes 10* and *11*, actin and myosin; *lane 1*, molecular mass standards, molecular masses in kilodaltons are indicated on the *far left*.

to aggregates formed under low Mg^{2+} conditions in the presence of ATP. If caldesmon was added to the mixture of actin and dephosphorylated myosin in the presence of ATP but with 8 mM $MgCl_2$, very thick bundles composed of a parallel array of thick and thin filaments were formed and were reminiscent of stress fiber in the cell. The filaments involved in such bundles associated with each other so tightly that the harsh mixing, which was effective under 1 mM Mg^{2+} conditions, hardly altered the final image of the complex. It was necessary to subject the entire mixture to ultrasonic agitation to effect partial dissociation (Fig. 4, *c* and *d*). Negatively stained images of forcibly dissociated bundles showed the parallel array of actin filaments together with myosin filaments with periodic banding pattern throughout the length, indicating a side polar assembly.

Monomeric smooth muscle myosin forms a folded (10 S myosin) or an extended structure (6 S myosin), depending on its environment. Thick filaments are formed only from the latter conformers (Hartshorne, 1987). Since the two conformations are characterized by distinct enzyme activities (Ikebe *et al.*, 1983), we examined the effect of caldesmon on the equilibrium between 10 S and 6 S myosins. The KCl dependence of Mg^{2+}-ATPase of dephosphorylated myosin was measured in the presence and absence of caldesmon and/or actin (Fig. 6). The depression of the Mg^{2+}-ATPase activity below 0.3 M KCl, which reflects the myosin conformational transition from 6 to 10 S, was unaffected by caldesmon and/or actin. At lower KCl conditions where caldesmon induced formation of filamentous myosin as well as actin-myosin filament cross-linking, the Mg^{2+}-ATPase activity was not influenced by caldesmon. These results suggest that the formation of the small myosin filamentous aggregate might not be due to the change in the myosin conformation.

It was recently reported that telokin, a protein whose primary amino acid sequence is identical to the tail part of myosin light chain kinase, might contribute to the stabilization of the thick filament structure of dephosphorylated myosin in the presence of ATP (Shirinsky *et al.*, 1993). We have found that dephosphorylated myosin filaments were induced by the addition of telokin. However, the size and shapes of the filaments observed in our hands were less homogenous than those induced by caldesmon, including the slender ones and some aggregates under 1 mM Mg^{2+} conditions (Fig. 7*b*). In the presence of 8 mM Mg^{2+}, myosin aggregates consisted of a mixture of very long and medium to small sized filaments. The filaments in the very long population often showed a curved contour with a tendency to merge with each other giving a somewhat fragile appearance. When caldesmon and telokin were simultaneously added to the solution of dephosphorylated myosin, the observed filaments were shorter than the longest population in the latter case but were more homogenous in shape and size (length distribution was 0.95 + 0.25 μm for ~100 filaments in some selected fields) as compared with telokin alone. The tendency for filaments to merge was less, although they sometimes aligned themselves side-by-side. The density of myosin molecules in the background seemed less than the other cases, indicating that most molecules were efficiently taken up into filamentous form. These filaments also showed prominent 14 nm periodicity, indicating an ordered structure. Myosin light chain kinase did not induce thick filament formation at all, in spite of the existence of the amino acid sequence identical to telokin at its COOH-terminal domain (data not shown).

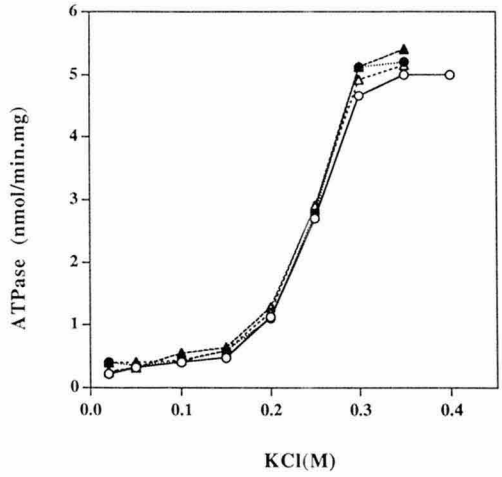

FIG. 6. **KCl dependence of Mg^{2+}-ATPase activity of myosin in the presence of caldesmon.** ATPase activity was measured as described under "Materials and Methods." ○, myosin alone; ●, with 0.2 mg/ml caldesmon; △, with 0.5 mg/ml actin; ▲, with 0.2 mg/ml caldesmon and 0.5 mg/ml actin.

DISCUSSION

Caldesmon can bind various smooth muscle contractile proteins such as actin (Sobue *et al.*, 1981), tropomyosin (Graceffa *et al.*, 1987), and myosin (Ikebe and Reardon, 1988) within distinct domains (Marston and Redwood, 1991; Sobue and Sellers, 1991). It was suggested previously that caldesmon can cross-link F-actin and myosin (Ikebe and Reardon, 1988; Marston *et al.*, 1992; Hemric and Chalovich, 1988), but detailed structures of such myosin aggregates have not been investigated. The present study confirmed the cross-linking of F-actin and myosin by caldesmon, but more importantly, this study demonstrated that caldesmon induced the assembly of myosin filaments in the presence of Mg^{2+}-ATP.

Smooth muscle myosin requires a higher Mg^{2+} concentration (≃8~15 mM) for thick filament formation as compared with the concentration required by skeletal myosin. Even at high Mg^{2+} concentration the dephosphorylated myosin filaments are disassembled by the addition of Mg^{2+}-ATP (Suzuki *et al.*, 1978). Under these conditions, myosin has been shown to be in a monomeric, 10 S conformation as determined by analytical ultracentrifugation (Trybus *et al.*, 1982; Ikebe *et al.*, 1983; Suzuki *et al.*, 1978; Onishi and Wakabayashi, 1982). In the present study we confirmed that freshly isolated smooth muscle dephosphorylated myosin did not form filaments under lower Mg^{2+} conditions (≃1 mM). At higher Mg^{2+} concentration (≃8 mM) dephosphorylated myosin formed thick filaments (Fig. 1*a*), but this was readily disassembled by the addition of Mg^{2+}-ATP (Fig. 1*b*). By raising Mg^{2+} concentration to 8 mM, such small aggregates of myosin grew into the filaments whose size was comparable with that of dephosphorylated myosin in the absence of ATP or phosphorylated myosin in the absence or presence of ATP. The small aggregates of myosin induced by caldesmon at low Mg^{2+} condition might act as a precursor or "seed." Larger myosin filaments induced by caldesmon can be produced either from small myosin oligomer or monomer, but in either case caldesmon seems to shift the equilibrium between monomeric myosin and filamentous myosin toward the formation of filaments.

Under low Mg^{2+} conditions in which myosin alone exists as monomer or small oligomers but not large filaments, caldesmon induced formation of filamentous aggregates of myosin though these filaments were smaller than that observed with myosin alone at higher Mg^{2+} concentration. It is known that caldesmon itself forms aggregate due to oxidation. However, we do not think that caldesmon aggregation is involved in the observed myosin filament formation because: 1) caldesmon was treated with 5 mM DTT to reduce any oxidized species of caldesmon; 2) caldesmon by itself was not precipitated during the sedimentation analysis; 3) electron microscopic observation failed to detect a large caldesmon aggregate; 4) the effect of caldesmon to induce the turbidity increase of actin/myosin mixture was saturated within the range of expected molar ratio of actin/caldesmon/myosin. It remains obscure as to how caldesmon stabilizes myosin filaments in the presence of Mg^{2+}-ATP where dephosphorylated myosin alone is in the 10 S conformation and does not form filaments. One possibility is that a folded conformation of myosin is destabilized by binding of caldesmon at S-2 moiety (Ikebe and Reardon, 1988), and the

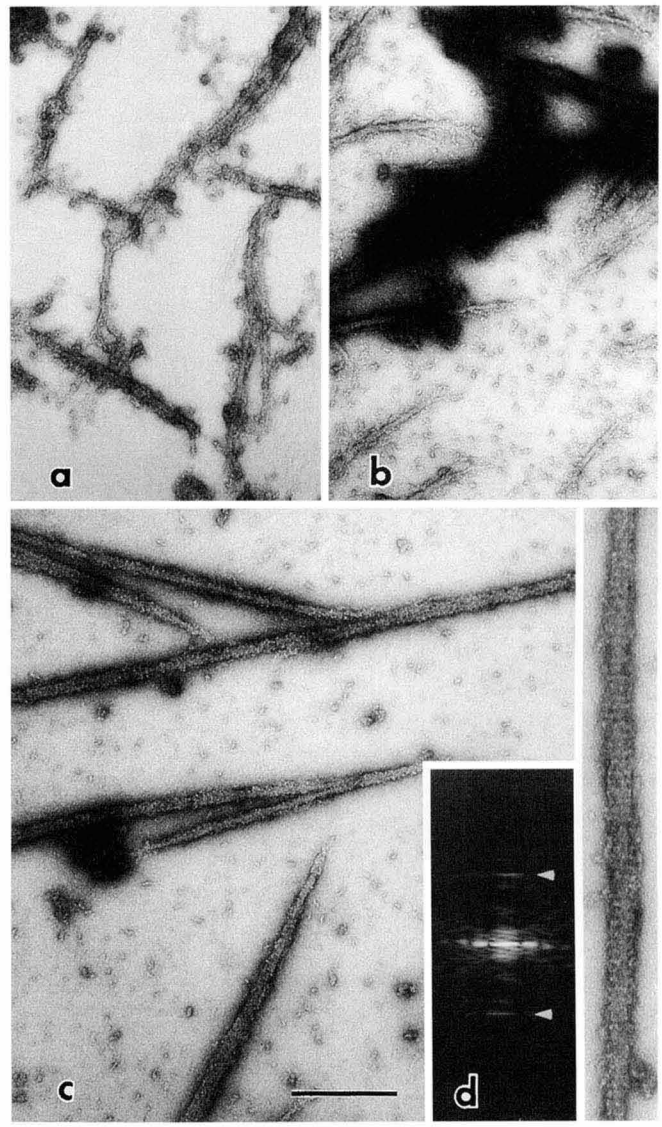

FIG. 7. **Negatively stained images showing the effect of telokin on the assembly of dephosphorylated myosin.** *a*, addition of telokin induces slender filaments under 1 mM $MgCl_2$ and 0.5 mM ATP conditions, whereas control myosin by itself did not form any filaments; *b*, when telokin alone was added to dephosphorylated myosin under 8 mM $MgCl_2$ and 0.5 mM ATP conditions, induced filaments are variable in size and shape, including very long ones (densely stained across the field) and medium sized but flexible filaments. Some periodic banding pattern was recognized in very long filaments; *c*, by addition of caldesmon together with telokin, the size and shape of thick filaments became less variable and each component showed prominent 14 nm periodicity. The inset of *d* indicates the optical transform of one of such filaments as enlarged on the *right*. *Arrows* indicate the meridional reflections for that periodicity.

tail portion is consequently extended and induces filament formation. Supporting this notion, we recently observed that native smooth muscle thin filaments show thin whisker like projections which are labeled with an antibody recognizing the NH_2-terminal myosin binding domain of caldesmon.[2] It is plausible, therefore, that the NH_2-terminal domain of caldesmon protrudes from the thin filaments and binds the S-2 portion of myosin so as to stabilize the myosin filaments.

The results of cosedimentation experiment where much more myosin molecules coprecipitated with caldesmon molecules suggests that caldesmon may exert a cooperative effect on filament formation by inducing myosin into some conformation that will polymerize. This may be less likely since 10 S myosin does not polymerize with 6 S myosin to form copolymer (Trybus and Lowey, 1987). Alternatively, caldesmon may bind to the S-2 region of several myosin molecules. This hypothesis is supported by previous findings by Katayama and co-workers (Katayama *et al.*, 1989; Katayama, 1989a) that caldesmon contains more than two S-2 binding sites within the NH_2-terminal and central domains. It has also been shown that the caldesmon molecule can bind several molecules of heavy meromyosin (Marston, 1989). Recent reports on the properties of caldesmon's COOH-terminal domain (Huber *et al.*, 1993) also indicated its affinity to myosin. We also observed by electron microscopy that clusters of heavy meromyosin bind periodically to the thin filaments via their S-2 region.[2] These results support the idea that caldesmon can bind several myosin molecules and stabilize its filamentous structure. It has been shown in two different types of smooth muscle tissue that the thick filaments are present in relaxed smooth muscle fiber (Somlyo *et al.*, 1981; Tsukita *et al.*, 1982), even though isolated dephosphorylated myosin fails to form stable thick filaments under physiological ionic conditions. As shown in the present study, caldesmon may help stabilize myosin filaments under physiological ionic conditions and resolve the apparent discrepancy between *in vitro* and *in vivo* reports.

After completion of our experiments on the effects of caldesmon on myosin filament formation, a paper appeared concerning very similar issues to ours (Shirinsky *et al.*, 1993). They attributed the stability of dephosphorylated myosin filaments in relaxed muscle, to the presence of telokin, an interesting protein whose function is not fully elucidated. We confirmed their results except in the distribution of the filaments in terms of size and shape. These differences may be attributed to the differences in our experimental conditions from theirs. In addition, we checked the effect of simultaneous addition of caldesmon and telokin on dephosphorylated myosin filament formation. Under such conditions, thick filaments were shorter but seemed more homogenous in size and shape as compared with those with telokin alone. It is notable that these long filaments showed clear 14 nm periodicity, giving rise to the appearance of native thick filaments (Cooke *et al.*, 1989). Caldesmon and telokin are both the abundant constituents of smooth muscle cells. These results suggest they might work cooperatively to stabilize the organization of dephosphorylated myosin filament under relaxed conditions. It has been reported that telokin is not necessarily a universal component of all smooth muscle tissues and is deficient in aorta, trachea, and non-muscle cells (Gallagher and Herring, 1991). Caldesmon, on the other hand, distributes more widely among various tissues, including those above. Therefore, caldesmon might be a more universal factor in stabilizing dephosphorylated myosin thick filament structure in relaxed smooth muscle cells. Furthermore, we observed that caldesmon-induced myosin filaments were tethered to actin filaments in the presence of Mg^{2+}-ATP. This is consistent with the intracellular localization of caldesmon in the actomyosin domain (Furst *et al.*, 1986) and suggests an important structural role in smooth muscle organization.

In non-muscle cells, myosin colocalizes with actin in stress fibers. During mitosis, microfilament organization changes dramatically and stress fibers are disassembled during prophase. It was shown recently (Yamashiro *et al.*, 1990, 1991) that caldesmon dissociates from microfilaments during mitosis probably due to the phosphorylation by cdc2 kinase. These results suggest that caldesmon may play an important role in stabilizing stress fibers in non-muscle cells. The present results show that caldesmon stabilizes myosin filaments and furthermore cross-links actin and myosin filaments to form actomyosin bundles that resemble stress fibers (Fig. 4*c*). These findings predict roles for caldesmon in the stabilization of stress fibers consisting of actin filament bundles and myosin molecules.

Acknowledgments—E. Katayama expresses his cordial thanks to Prof. K. Hirosawa for encouraging the international collaboration. He is also indebted greatly to T. Shiraishi for her technical assistance in photography.

[2] E. Katayama and M. Ikebe, unpublished observation.

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