

# Mode of Caldesmon Binding to Smooth Muscle Thin Filament: Possible Projection of the Amino-Terminal Domain of Caldesmon from Native Thin Filament

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**ABSTRACT** The structure of smooth muscle thin filament was examined by various electron microscopy techniques, with special attention to the mode of caldesmon binding. Chemical cross-linking was positively used to avoid the dissociation of accessory proteins upon dilution. Caldesmon in reconstituted thin filament was observed as fine filamentous projections from thin filament. Native thin filament isolated from smooth muscle showed similarly numerous fine whisker-like projections by all the techniques employed here. Antibody against the amino-terminus of caldesmon labeled the end of such projections indicating the possibility that the amino-terminal myosin binding moiety might stick out from the shaft of the thin filament. Such whiskers are often projected out as a cluster to the same side of native thin filament. Further, we could visualize the assembly of dephosphorylated heavy meromyosin (HMM) with native or reconstituted thin filament forming “nonproductive” complex in the presence of ATP. The association of HMM to the shaft of thin filament was through subfragment-2 moiety, in accordance with biochemical studies. Some HMM particles bound closer to the thin filament shaft, possibly suggesting the presence of the second myosin-binding site on caldesmon. Occasionally two kinds of HMM association as such coexisted at a single site on thin filament in tandem. Thus, we constructed a structural model of thin filament. The proposed molecular arrangement is not only compatible with all the biochemical results but also provides additional support for our recent findings (E. Katayama, G. C. Scott-Woo, and M. Ikebe (1995) *J. Biol. Chem.* 270, 3919–3925) regarding the capability of caldesmon to induce dephosphorylated myosin filament, which explains the existence of thick filaments in relaxed smooth muscle cells.

## INTRODUCTION

It has been well established that  $\text{Ca}^{2+}$  regulation of smooth muscle contraction operates mostly through the phosphorylation-dephosphorylation cycle of myosin light-chain catalyzed by myosin light chain kinase and phosphatase system, the former of which is activated by the binding of  $\text{Ca}^{2+}$ /calmodulin (Hartshorne, 1987; Sellers and Adelstein, 1987; Kamm and Stull, 1989). The discovery of caldesmon (Sobue et al., 1981), an actin-binding protein having the ability to prohibit actomyosin superprecipitation, accelerated the re-evaluation of the role of thin filaments as independent regulatory factors, because that inhibition was reversibly abolished by the addition of  $\text{Ca}^{2+}$ /calmodulin, which might suggest the capability of that protein system to control the motility. It was shown later that caldesmon also has a specific affinity to the subfragment-2 (S2) moiety of myosin (Ikebe and Reardon, 1988), indicating another possible role to cross-link actin-containing thin filament with myosin-containing thick filament.

One of the well known properties of caldesmon is that its domain structure is directly related to its functional organization. Studies on the function of each domain were performed mainly using enzymatically or chemically produced

fragments. It was found that the carboxy-terminal fragments are responsible for most of the major functions of caldesmon (see reviews by Marston and Redwood, 1991; Sobue and Sellers, 1991); actin-binding, tropomyosin-binding, calmodulin-binding and inhibition of actomyosin superprecipitation, whereas the amino-terminal fragments have no affinity to actin and are responsible for the binding of myosin S2 (Katayama et al., 1989; Katayama, 1989a; Sutherland and Walsh, 1989; Velaz et al., 1990). In parallel with such functional studies, structural examination of caldesmon by itself or as a constituent of thin filament was carried out mainly by observing rotary-shadowed or negatively stained samples with electron microscopy (Furst et al., 1986; Lynch et al., 1987; Lehman et al., 1989; Moody et al., 1990; Mabuchi and Wang, 1991; Mabuchi et al., 1993). Numerous studies appear to agree that caldesmon is a fine filamentous protein in association with the actin-filament together with tropomyosin. If the characteristic behavior of each fragment as observed in biochemical studies related to its affinity to other protein constituents is reflected simply by the structure, the expected configuration of the total caldesmon molecule should be that the carboxy-terminal moiety is tightly bound with acto-tropomyosin, the main shaft of the thin filament, whereas the amino-terminal part is released from the shaft and associates with the S2 portion of myosin molecule. From the actual structural studies of native thin filaments, however, such a conceptual scheme was not necessarily substantiated. Although Moody et al. (1990) noticed the presence of very fine whisker-like projections in their negatively stained image of native thin filaments, their final

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conclusion was that caldesmon is in contact with thin filament throughout its total length. An essentially similar conclusion was deduced by Mabuchi et al. (1993) from the image of native thin filament complexed with monoclonal antibody, but using the novel rotary-shadowing technique they have devised. If the amino-terminal part of caldesmon is tightly attached to actin-filament as interpreted, the possibility is that the access of myosin S2 may not be as easy as indicated by *in vitro* affinity studies. Marston et al. (1992), on the other hand, showed that either reconstituted or native thin filaments containing caldesmon can collect, along its length, numerous thick filaments made of myosin rod, suggesting a possible role of caldesmon in organizing "contractile domain" in smooth muscle cells. Recently, we showed that caldesmon can induce the formation of thick filaments under the conditions where dephosphorylated myosin molecules by themselves exist in non-polymerizable 10S-conformation and that such thick filaments are tethered to thin filaments by cross-linking through caldesmon (Katayama, et al., 1995).

In the present study, attempts were made to clarify further the structure of reconstituted and native thin filaments, with special attention to the configuration of amino-terminal myosin binding moiety of caldesmon molecule. For that specific purpose, chemical cross-linking was positively used to prepare the specimens for electron microscopy, because Marston (1990) found that accessory proteins of thin filaments tend to detach easily upon dilution of the protein stock. By pretreating the protein samples with bifunctional reagents, we could get the clear images to indicate the mode of binding of caldesmon to thin filaments. The association of the S2 part of heavy meromyosin (HMM) with native and reconstituted thin filament was successfully visualized as well.

## MATERIALS AND METHODS

### Protein samples

Smooth muscle dephosphorylated myosin, HMM, tropomyosin, caldesmon, and skeletal muscle actin were prepared and purified according to a previous paper (Katayama et al., 1989). Thiol groups of caldesmon cysteinyl residues were modified with biotin maleimide as described (Katayama, 1989a). Native thin filament was prepared from chicken gizzard after the improved protocol of Marston and Lehman (1985), which includes the temperature-controlled purification steps. In some experiments, potassium iodide (KI) extract from the acetone powder of gizzard tissue was fractionated by the same procedure as for skeletal tissue (Szent-Gyorgyi, 1951) and was used as a substitute for reconstituted thin filament, because a relative abundance of each protein constituent was analogous to that of native thin filament preparation as above. Monoclonal antibody, CDg1, which recognized amino-terminal 25-kDa fragment of smooth muscle caldesmon (Scott-Woo et al., 1990), was produced as described previously (Araki and Ikebe, 1991; Higashihara et al., 1989) using isolated chicken gizzard caldesmon as an antigen.

### Sample preparation for electron microscopy

Reconstituted thin filament was prepared by mixing actin (1 mg/ml), tropomyosin (0.6 mg/ml), unmodified or biotin-modified caldesmon (0.42 mg/ml), in the buffer (15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 50 mM KCl, 2 mM MgCl<sub>2</sub>, and 0.05% NaN<sub>3</sub>, pH 7.5). The mixture was treated with 0.07% glutaraldehyde for 8 min at room tem-

perature immediately after 20-fold dilution with the buffer. Reaction was stopped by adding 20 mM Tris-HCl (pH 8) together with 5 mM dithiothreitol to the solution. KCl was supplemented to 150 mM and 0.05 mg/ml of succinyl-avidin (Sigma Chemical Co., St. Louis, MO) was further added when labeling was needed. Total mixture was spun for 1 h at 36,000 rpm and the pellet suspended in the buffer (30 mM KCl, 2 mM MgCl<sub>2</sub>, 5 mM potassium phosphate, 0.02% NaN<sub>3</sub>, pH 7.5) for negative staining.

To the stock solution of native thin filament was added the buffer (20 mM triethanolamine HCl, 3 mM MgCl<sub>2</sub>, 0.02% NaN<sub>3</sub>, pH 8.3) containing freshly dissolved disuccinimidyl suberate (DSS) (Pierce, Rockford, IL) at 10 mM final concentration. After 12–30 min incubation period at room temperature, 20 mM Tris-HCl (pH 8) with 5 mM dithiothreitol was added to terminate cross-linking reaction. Native thin filaments thus cross-linked were diluted with the buffer (0.1 M ammonium acetate, 5 mM MgCl<sub>2</sub>, pH 7.8), absorbed on mica surface, rinsed with the buffer, stabilized further with uranyl acetate, and washed with glycerol-containing solution before low-angle rotary-shadowing (Mabuchi, 1991) from an elevation angle of 4°. To label the amino-terminal of caldesmon, monoclonal antibody was mixed with cross-linked native thin filament, and the sample was processed in the same manner.

The binding of smooth muscle dephosphorylated HMM with native thin filament was visualized essentially in the same manner as for the antibody, except including 0.1 mM ATP to the dilution and rinsing buffer.

For negative-staining of HMM-thin filament complex, native thin filament (~0.3 mg/ml) or reconstituted thin filament from KI extracts was mixed with dephosphorylated HMM (0.4 mg/ml) in the buffer (10 mM imidazole, 30 mM KCl, 3 mM MgCl<sub>2</sub>, 2.5 mM ATP, 0.5 mM ethylene-glycol-bis(β-aminoethylether) *N,N'*-tetraacetic acid, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, pH 7.0), and was treated with 0.05% glutaraldehyde for 6 min at room temperature. Further processing was done as described above.

Negative staining of protein samples was done using a high-resolution protocol described in detail by Katayama (1989b). Conventional rotary shadowing of avidin-tagged caldesmon was made from the elevation angle of 10° after spraying protein solution (containing 40% glycerol and 0.3 M ammonium formate) onto a freshly cleaved mica surface. Rotary shadowing of native thin filament and its complex with monoclonal antibody or HMM was carried out as described above, according to the procedure devised by Mabuchi (1991). Quick-freeze deep-etch replication was performed after absorbing the sample onto the surface of mica flakes as described previously (Katayama, 1989b; Heuser, 1983).

Electron micrographs were taken usually as a stereo-pair (10°) by a goniometer equipped in JEOL-2000ES electron microscope.

## RESULTS

### Need for chemical cross-linking of protein samples for electron microscopy

According to Marston (1990), native thin filaments isolated from smooth muscle tissue easily release caldesmon (and tropomyosin, although to a lesser extent) upon dilution of the stock solution. As a matter of fact, preliminary observation of native thin filament preparation using a quick-freeze deep-etch rotary-replica technique with mica flakes (Heuser, 1993; Katayama, 1989b) gave clear images of the helical structure of actin filament (not shown), but also indicated that a substantial fraction of caldesmon (and some tropomyosin) dissociated from actin filamentous upon dilution of the original protein stock solution to the concentration suitable for electron microscopy. To avoid complications due to such partial detachment, a part of the protein solution was treated with bifunctional reagents (glutaraldehyde or DSS). Glutaraldehyde, the most frequently used fixative in electron microscopy, generally gives more extensive cross-linking with less

specificity to various amino-acid residues, as compared with amino-specific reagents such as dimethylimidioester or DSS. It was used mostly to prepare the samples for negative staining, because uranyl stain might penetrate into the cleft between residues even after heavy cross-linking, though the actual extent of reaction was kept minimal. On the other hand, glutaraldehyde did not seem to be the best choice for rotary shadowing of actin filament, especially with HMM. The resultant image was less conspicuous after cross-linking, possibly because the decoration with reagent groups on the protein might cover up the surface details or loss of positive charge of the protein could weaken its adsorption onto mica surface. Further, a pair of heads in each HMM molecule were prone to be cross-linked readily by glutaraldehyde, giving rise to fused heads, which are not appropriate for unambiguous recognition by shadowing. Hence, most of the rotary-shadowed images of thin filaments exhibited in this paper were obtained after pretreatment with DSS, an amino-specific bifunctional reagent, which is known as a mild cross-linker hardly affecting the intrinsic function of actin filament (Prochniewicz et al., 1993).

### Mode of caldesmon binding in reconstituted thin filament

Caldesmon is known as a fine filamentous protein, the central part of which consists of a single  $\alpha$ -helix (Graceffa et al., 1988; Wang et al., 1991). To assess the mode of its binding to actin filament in the absence or presence of tropomyosin, the other filamentous constituent of thin filament, an attempt was made to observe directly by electron microscopy the avidin labels on caldesmon molecules in association with actin filament.

The negatively stained image of reconstituted thin filaments after cross-linking with glutaraldehyde (Fig. 1 *b*) showed small but somewhat periodical protrusions along the side of actin filaments, which were seen neither in purified actin (Fig. 1 *a*) nor acto-tropomyosin filaments without caldesmon (not shown). To confirm further that the protrusion was due to the presence of caldesmon in the filament, another sample was prepared with caldesmon specifically modified with biotin maleimide at cysteinyl residues. The rotary-shadowed image of avidin-tagged caldesmon through biotinyl groups indicated unambiguously the location of two avidin labels at both ends of the molecules (Fig. 1 *c*), reflecting quite reasonably the positions of two cysteinyl residues in amino acid sequence of caldesmon (Bryan et al., 1989; Hayashi et al., 1989; Katayama, 1989a; Sutherland and Walsh, 1989). Mabuchi and Wang (1991) labeled caldesmon at the same positions but with calmodulin. Reconstituted thin filaments with and without tropomyosin were cross-linked by glutaraldehyde, and each sample was later labeled with avidin for comparison. It is known from biochemical studies that caldesmon binds to thin filament whether tropomyosin is present or not, but with greater affinity in its presence (Velaz et al., 1989). The negatively stained images of avidin-labeled thin filaments indicated this difference clearly. The

labeling was less dense when tropomyosin was not included in the reconstitution (Fig. 1 *d*), but there was an apparent periodic binding of avidin along the filament. In some cases, filamentous projections connecting actin filament and avidin particles were recognized (Fig. 1 *e*). Although the attempt to determine the precise periodicity was not done, the distance between the two labels seemed to be close to twice the length of the putative tropomyosin repeat on thin filament. According to the recent report (Graceffa et al., 1993), carboxy-terminal side cysteinyl residue of caldesmon can easily make disulfide cross-linking with the penultimate cysteinyl group of actin molecule, suggesting that those two thiols may face each other in reconstituted and native thin filaments. This result may explain the likely appearance that only one of two biotinyl groups of caldesmon was subject to decoration with avidin in these filaments. When tropomyosin was included in reconstitution, avidin labeled the filaments more heavily with apparent lateral projections (Fig. 1 *f*). The axial periodicity of such structures in the reconstituted thin filaments was almost the same as the tropomyosin repeat length. We do not think, however, that the molecular organization observed here directly applies to that of native thin filament, since an excess amount of caldesmon was added to prepare the reconstituted thin filaments. In fact, caldesmon seems to stick out from only a single side of native thin filament, as described in the following section (see Fig. 2), which implies that the actual stoichiometry might be close to half of reconstituted thin filament.

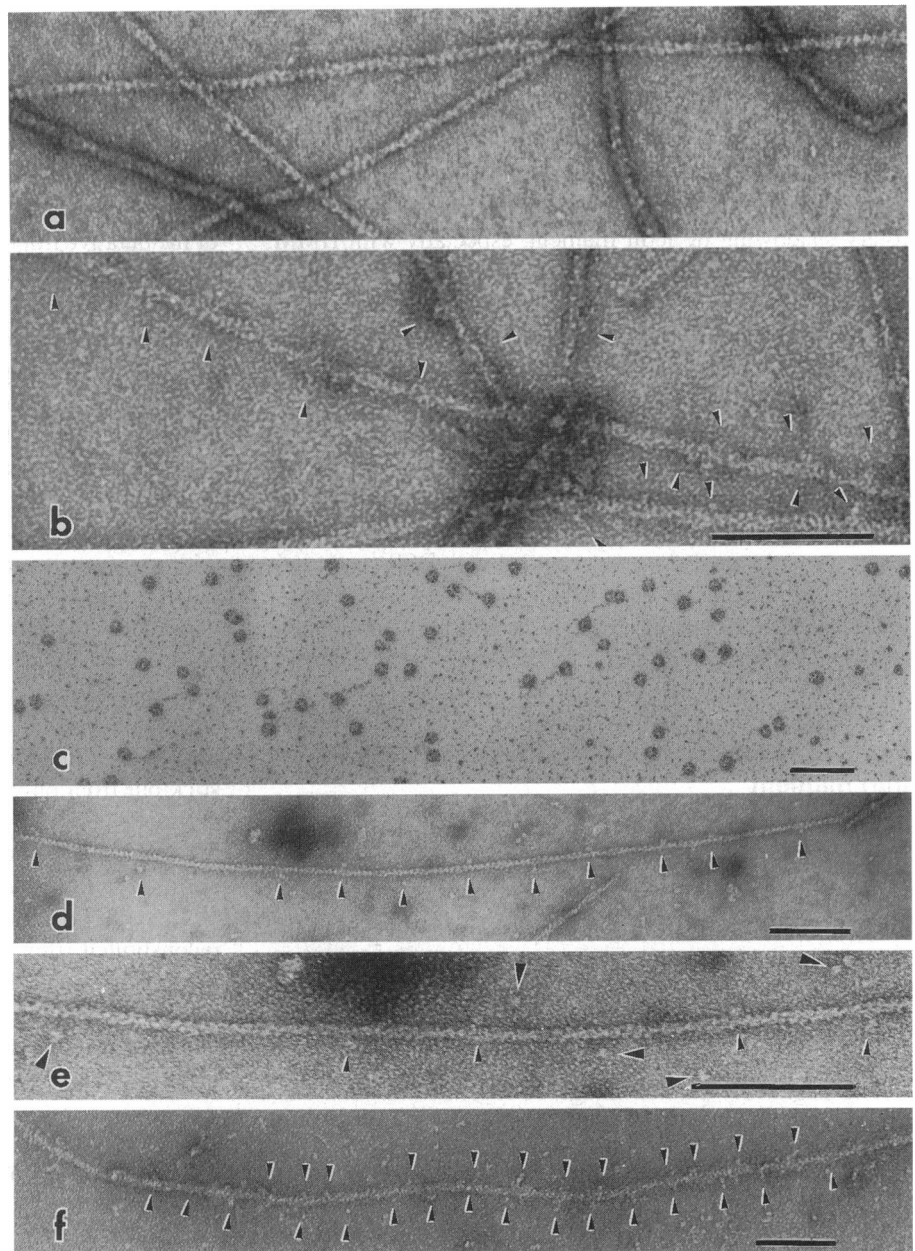
Thus, the observed distribution of avidin markers along reconstituted thin filament confirmed that filamentous caldesmon molecules are associated along with actin filament but indicated the possibility that some portion of the molecule can be released out and tethered to the main-body of the actin filament.

### Mode of caldesmon binding in native thin filament

The structure of native thin filament isolated from chicken gizzard tissue was examined by electron microscopy. Negatively stained image of uncross-linked native thin filament (Fig. 2a) showed a clear but less conspicuous helical repeat compared with the image of purified actin (see Fig. 1a with the same magnification) probably due to the association of accessory proteins along the filament. Careful examination of high power electron micrograph revealed the presence of a number of very fine whisker-like projections with a thickness close to the resolution limit of negative staining. This structure seemed to correspond to the one which was seen in Fig. 1e as to connect avidin particles to thin filament. Projections were similarly observed in negatively stained image of DSS-treated native thin filament (not shown).

The structure of native thin filament after glutaraldehyde cross-linking was observed on mica surface after quick-freeze and deep-etch replication (Fig. 2, *b* and *c*). The striation pattern due to actin's genetic helix was, again, less conspicuous than of pure actin (not shown). The surface of the

**FIGURE 1** (a) Negatively stained image of pure actin filament with conspicuous helical structure. Scale bars indicate 100 nm in all the following electron micrographs. (b) Negatively stained image of glutaraldehyde-treated thin filament, which was reconstituted from actin, tropomyosin, and caldesmon. Arrowheads indicate the presence of small protrusions or filamentous projections. (c) Rotary-shadowed image of caldesmon whose thiol groups were modified with biotin-maleimide and tagged with succinyl avidin. Fine filamentous molecules with two globules on both ends are apparent. (d) Negatively stained image of glutaraldehyde-treated actin-biotinylated caldesmon complex. Succinyl avidin was added later as a marker for electron microscopy. Note the periodic binding of marker particles along the filament (*arrowheads*). (e) High power image of the central part of (d). Arrowheads indicate avidin markers, and the large arrowheads exhibit the same markers but tethered to the shaft of the filament by fine filamentous structure. (f) Negatively stained image of glutaraldehyde-treated actin-tropomyosin-caldesmon complex coupled with avidin markers. Note the clear axial periodicity of the projections along the filament (*arrowheads*).



filament was almost periodically decorated with small protrusions or with thin whisker-like projections whose end(s) sometimes swelled, presumably representing the globular domain structure at the end(s) of the molecules.

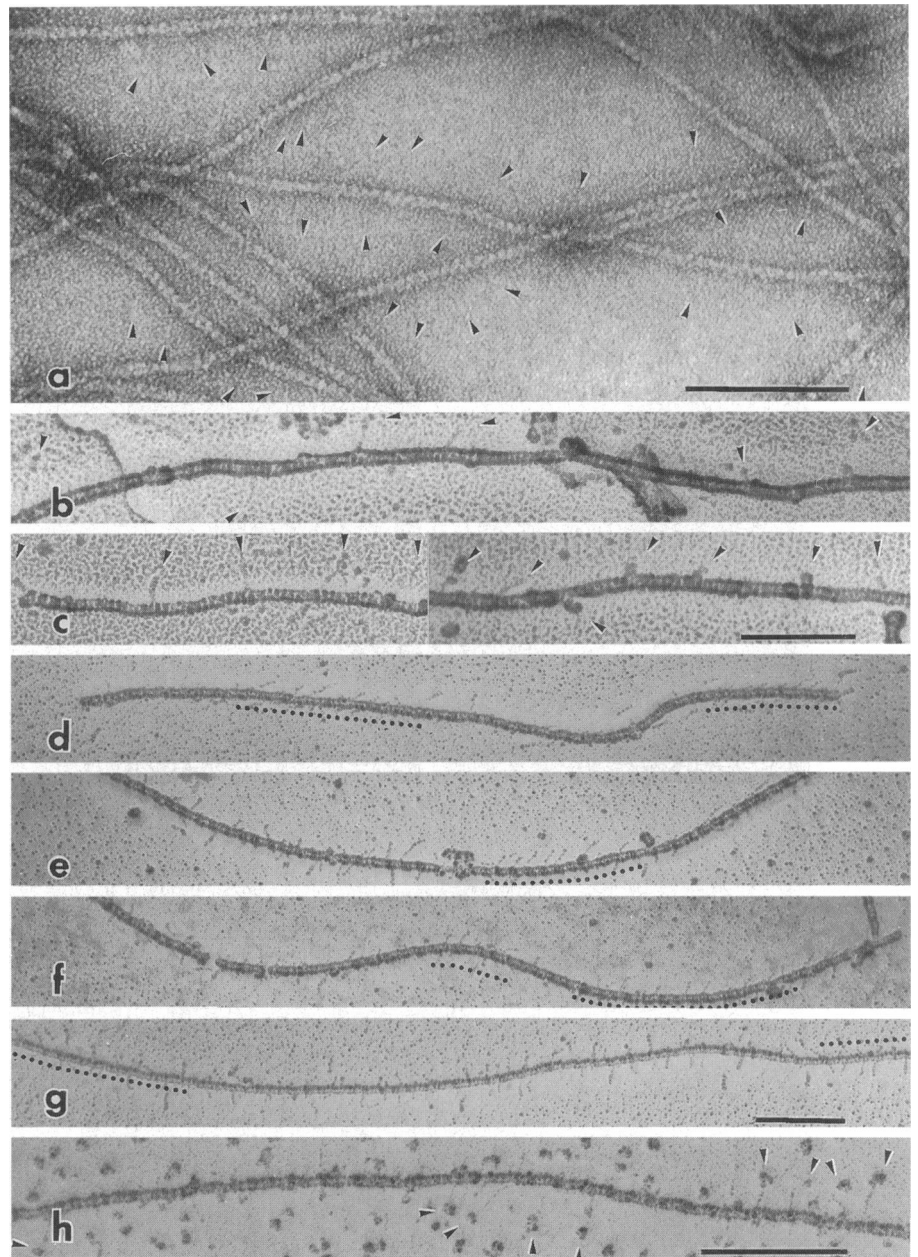
The presence of such filamentous projections was further and more strikingly demonstrated by rotary-shadowing DSS-pretreated native thin filament after uranyl acetate stabilization. Examples of such images are exhibited in Fig. 2 *d-g*. Many fine whiskers were evident and their length was, in most cases, longer than the tropomyosin repeat or the long pitch of actin helix. There was a significant tendency for a cluster of such filamentous projections to come out from one side of the actin filament and to align parallel to each other. When such filaments were preincubated with monoclonal antibody against the amino-terminus of caldesmon, the tip of the filamentous projection was selectively decorated with the

antibody molecule (Fig. 2 *h*). These results, combined together, strongly suggest that most of the observed whisker-like projections correspond to the amino-terminal through the central portion of caldesmon and that the caldesmon molecule is associated with actin filament, having distinct polarity both axially and azimuthally.

#### Binding of HMM onto native and reconstituted thin filament

One of the most distinctive properties of the interaction between smooth muscle thin filament and smooth muscle myosin is the presence of so-called "nonproductive" binding (Lash et al., 1986; Hemric and Chalovich, 1988, 1990; Marston 1989), which does not accompany the hydrolysis of ATP. It is now almost certain that such binding would be due

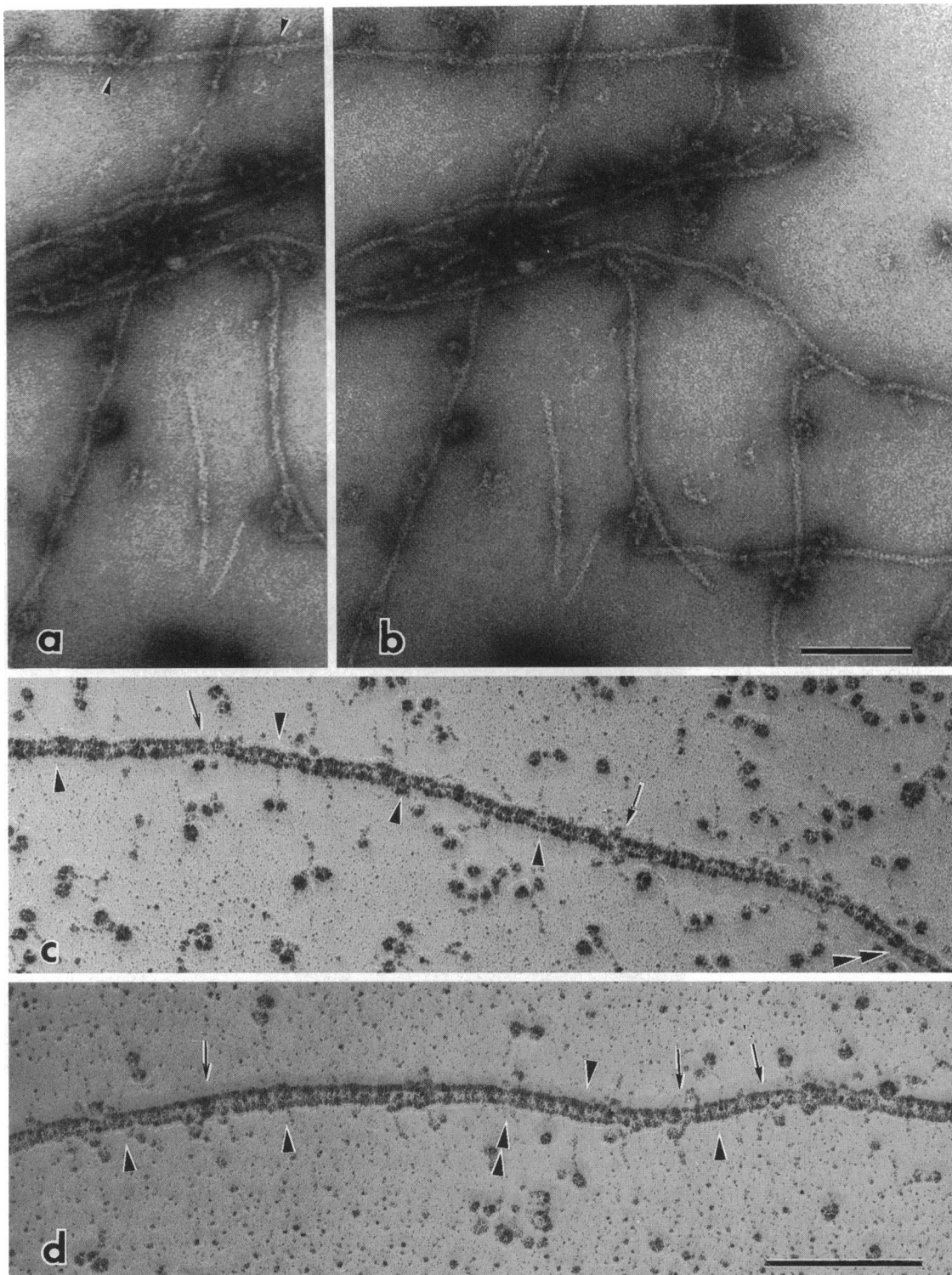
FIGURE 2 (a) Negatively stained image of native thin filament isolated from chicken gizzard. Note the presence of many fine projections along the filament. Helical structure is not as conspicuous as that of pure actin filament. (b and c) Quick-freeze deep-etch replica images of native thin filament lightly fixed with glutaraldehyde. Striation pattern due to left-handed genetic helix of actin is apparently observed, although not as clearly as in purified F-actin. Fine filamentous projections or sometimes small globular protrusions stick out periodically from the shaft. (d–g) Rotary-shadowed images of DSS-pretreated native thin filament after absorption on mica sheet and stabilization with uranyl acetate treatment. Numerous fine whiskers along the side of actin filament are evident. Note the prominent tendency of clusters of parallel whiskers projecting out to the same side of the filament shaft (·····). (g) Example with more randomized caldesmon arrangement, although some clusters still remain at both ends. (h) Rotary-shadowed image of native thin filament similarly processed but preincubated with monoclonal antibody against the amino-terminal portion of caldesmon. Whisker-like projections are labeled with antibody molecules at the tip (arrowheads).



to the association of the S2 region of myosin to caldesmon (Ikebe and Reardon, 1988), more specifically to its amino-terminal part (Katayama et al., 1989; Katayama, 1989a; Sutherland and Walsh, 1989). Although this unique interaction was well characterized biochemically, structural studies have been hampered by its putative sensitivity to uranyl acetate staining. Marston et al. (1992) tried to visualize the interaction of HMM with native thin filament but failed for that reason. They also examined the structure of the assembly of myosin filaments with actin-caldesmon complex or with native thin filament, but could not obtain clear enough images, this time because of the large clumps formed. So, they tactically used myosin-rod filament as an alternative of total myosin filament and finally got the images exhibiting thick filaments heavily aggregated close to and along thin filaments. In an attempt to visualize the mode of interaction

between myosin molecule with caldesmon bound to thin filament, we used chemical cross-linking as a pretreatment of thin filaments before or after mixing with HMM in the presence of ATP, and examined those samples with negative staining or rotary shadowing. Negatively stained images of glutaraldehyde-treated mixture of native thin filament and dephosphorylated HMM showed clearly the attachment of lateral protrusions on thin filaments (Fig. 3, a and b; shown partly as a stereo-pair). Each protrusion had a narrow stalk and a thick tip, reminiscent of the shape of HMM, but was completely distinguishable from the typical arrowhead appearance in the absence of ATP. Some of the protrusions seemed to consist of two or more HMM molecules possibly in tandem or in a parallel arrangement. The axial distribution of such protrusions were not random but showed a hint of periodicity. When a similar mixture of dephosphorylated





**FIGURE 3** (*a* and *b*) Negatively stained image of glutaraldehyde-fixed mixture of native thin filament and dephosphorylated HMM in the presence of ATP. Left side half of (*b*) is paired with (*a*) for stereoexamination. HMM particles forming nonproductive binding to thin filament are identified by mushroom-shaped protrusions along thin filament, which are sometimes aligned in tandem (*arrowheads*). The attachment sites of the protrusions do not seem randomly distributed but with some periodicity. (*c* and *d*) Rotary-shadowed image of the mixture of dephosphorylated HMM and DSS-treated native thin filament in the presence of ATP, and after stabilization by uranyl acetate treatment. HMM particles (*arrowheads*) are attached to thin filament shaft through S2 portion, clearly in a different manner from the rigor binding. Small arrows indicate HMM particles associating more closely to the thin filament, which might represent the second mode of nonproductive binding. Double arrowheads point out the particles in which two HMM molecules appear to be tethered to the same site on thin filament. Such particles might correspond to those aligned in tandem as shown above. Control images with purified F-actin with HMM did not include HMM molecules associated to actin filaments in such a manner.

HMM and DSS-treated native thin filament was rotary shadowed, the shape of each HMM was unambiguously recognized by two heads and the S2 portion connecting them (Fig. 3, *c* and *d*). A significant number of HMM molecules were found attached to thin filament through S2 moiety. Although there are unbound HMM molecules in the background of the images in Fig. 3 *c*, we think that this is within a reasonable range considering the extremely low protein concentration used for electron microscopy, since the  $K_d$  value of caldesmon and HMM is of the order of  $10^{-6} M^{-1}$ . Furthermore, such binding of HMM to actin filament was not observed under the same conditions but for the absence of caldesmon. The length of the whiskers bridging the HMM heads and the shaft of the thin filament was almost the same as or slightly longer than the S2 portion of the unbound HMM. There were some HMM particles that appeared to associate closer to the thin filament shaft than the former species but not like the rigor one. Such configuration might represent another mode of nonproductive interaction through a second myosin-binding site, although we cannot completely rule out the possibility that they could be “weakly bound” HMM whose affinity is by far weaker (Lash et al., 1986). Taking some overlapping length necessary for the physical contact of those two constituents into consideration, it is possible that at least some part of amino-terminal portion of caldesmon might protrude from the shaft of the native thin filament, with which S2 segment of myosin is tethered to thin filament. The negatively stained image of the mixture of reconstituted thin filaments with dephosphorylated HMM after glutaraldehyde treatment (Fig. 4 *a*) showed a number of bouquet-like HMM clusters in association with thin filaments through narrow stalks and with axial periodicity, which often approached to the tropomyosin repeat length along actin filament. Occasionally bundles of thin filaments were formed by cross-connecting HMM clusters giving rise to the bead-like appearance (Fig. 4 *b*). Averaged distance between each bead ranged from about 80 to 85 nm, close to two tropomyosin repeats or the putative molecular length of caldesmon (Graceffa et al., 1988; Mabuchi and Wang, 1991).

On the basis of the images observed in this study, we constructed the structural model of smooth muscle native thin filament with HMM molecules bound through S2. Fig. 4 *c* depicts our model schematically. The molecular arrangement of actin, tropomyosin, and caldesmon is essentially the same as that described by Marston and Redwood (1991). However, our model includes some additional features, including partial detachment of the amino-terminal portion of caldesmon from the shaft of the thin filament and the association of HMM molecules to this released structure producing nonproductive binding.

## DISCUSSION

In this study, the structure of reconstituted and native thin filaments was examined by various electron microscopy techniques. Concomitant use of chemical cross-linking reagents as a pretreatment of the sample made it possible to

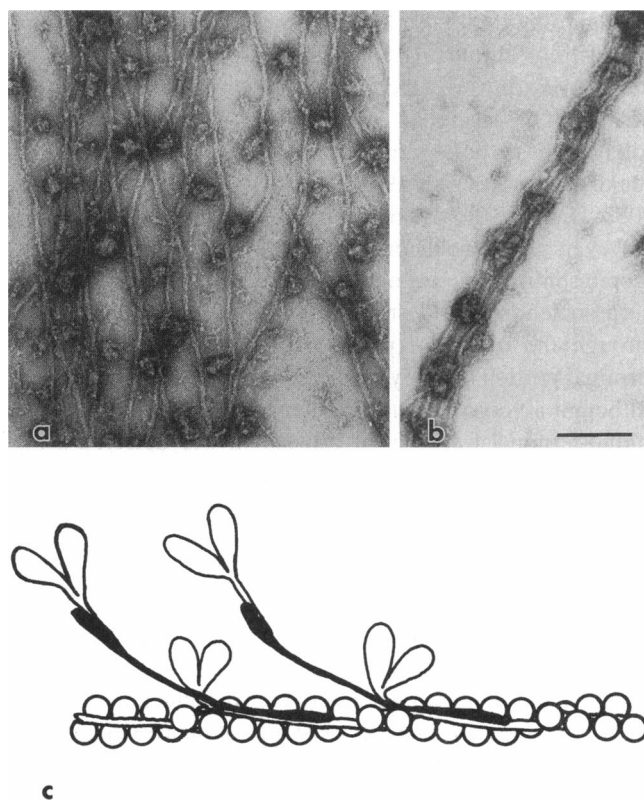


FIGURE 4 (*a* and *b*) Negatively stained image of glutaraldehyde-fixed mixture of reconstituted thin filament (from KI extract) and dephosphorylated HMM in the presence of ATP. Numerous bouquet-like protrusions are tethered to thin filaments through thin stalks. This structure is similar to that seen in dephosphorylated HMM-native thin filament complex, but the size of each protrusion seems somewhat larger than the former, possibly because the larger number of HMM might be involved. These aggregates of HMM occasionally connect thin filaments side by side to form bundles with a bead-like appearance. The repeating distance between HMM aggregates is close to the length of one caldesmon or two tropomyosin molecules. (*c*) Structural model of smooth muscle native thin filament schematically depicted from our observation. Tropomyosin (*light rod*) runs along the groove of actin filament. Filamentous caldesmon molecule (*dark rod*) associates with actin and tropomyosin at its carboxy-terminus, forming a ternary complex with them. The amino-terminus of caldesmon is away from the shaft of the thin filament and rather contributes to the association with the S2 portion of HMM. Central  $\alpha$ -helical domain or a part of the carboxy-terminal domain may work to tether the other HMM molecule to thin filament.

prevent the accessory proteins from detachment upon dilution for electron microscopy. We could thus obtain the images with which the realistic structural model of smooth muscle native thin filament might be constructed. Several models have already been proposed mainly from the studies using antibodies against various epitopes of caldesmon (Lehman et al., 1989, 1990; Marston and Redwood, 1991). The points of argument were focused on the mode of association of caldesmon to the acto-tropomyosin shaft of thin filament, more specifically, 1) whether the amino-terminal portion is tightly attached to the shaft, 2) whether each domain of caldesmon molecule faces the same side of the filament, and 3) how caldesmon connects the S2 portion of myosin to thin filament. The results of the present study might, at least partly, give the answers to those questions.

Regarding the first question, biochemical studies including affinity chromatography and cosedimentation experiments by various research groups have shown that the tight binding of caldesmon to actin filament is attributable almost solely to the carboxy-terminal moiety of the molecule (Szpacenko and Dabrowska, 1986; Fujii et al., 1987; Yazawa et al., 1987; Dingus et al., 1986). As mentioned in the introduction, however, the conclusion of the previous electron microscopic studies have been rather biased toward the view that the association of caldesmon to thin filament occurs not only through the carboxy-terminal part but also with amino-terminal portion (Moody et al., 1990; Mabuchi et al., 1993). Although a very weak association of tropomyosin with the amino-terminal fragment of caldesmon was detected using the material that was overexpressed by bacteria, that fragment did not cosediment with acto-tropomyosin (Redwood and Marston, 1993). Our cosedimentation experiments with caldesmon fragments (E. Katayama, unpublished data) indicated that the stronger binding of caldesmon to acto-tropomyosin as compared with actin alone is attributable to the enhanced association of the same carboxy-terminal fragments to thin filaments and not to the additional binding site on tropomyosin. Further, the existence of many whisker-like projections representing the amino-terminal of caldesmon was recognized by all of the electron microscopic methodologies employed here, whether the samples were cross-linked or not. Hence, on the basis of this experimental data, we cannot help inclining to the view that the firm association of caldesmon to the shaft of native thin filament would occur mainly through its carboxy-terminal moiety, and the contribution of the amino-terminal portion seems much smaller, if it exists at all. Another possibility is that the configuration of the amino-terminal portion could be in dynamic equilibrium between bound and unbound states. Considering the increased binding affinity of the same fragments of caldesmon to actin in the presence of tropomyosin, the carboxy-terminal binding site presumably forms the ternary complex with actin and tropomyosin (Horiuchi and Chacko, 1988).

The answer to the second question seems to be "yes." Lehman et al. (1989) proposed several structural models of native thin filament that explain the periodicity observed in the image of the complex made of thin filaments and anti-caldesmon antibody. Marston and Redwood (1991) pointed out more explicitly the characteristic feature of the proposed model, that each part of caldesmon molecule faces the same side of thin filament. Some of our images clearly showed the tendency of whisker-like projections to come out from the same side of actin filament and to align parallel. The partly randomized arrangement may be explained by cutting and annealing of the filaments during and after the isolation procedure (Lehman et al., 1989).

Based on our observations, we proposed here a model analogous to the one described in Marston and Redwood (1991) as to depict the most realistic molecular arrangement in native thin filament. Vibert et al. (1993) reconstructed the three-dimensional images of smooth muscle native thin filament from its helical projection in the absence and presence

of  $\text{Ca}^{2+}$ /calmodulin and compared them with each other as related to the regulation of motility. They concluded that the filamentous strands they observed along the grooves of actin filament would reflect mostly the presence of tropomyosin, because the similar strands were seen shifted, when caldesmon was almost completely removed from the actin filament. They discussed, in various ways, the reasons why they could not visualize caldesmon in their reconstructed image. However, the extreme difficulty of such an attempt seems quite natural, if the lack of azimuthal symmetry as proposed here is taken into consideration together with the fact that caldesmon consists of a single  $\alpha$ -helix (Wang et al., 1991), with only half the mass of coiled coil structure in tropomyosin.

Despite many studies including affinity chromatography and cosedimentation experiments (Ikebe and Reardon, 1988; Katayama et al., 1989; Katayama, 1989a; Sutherland and Walsh, 1989; Marston, 1989; Hemric and Chalovich, 1988) showing the specific affinity of the amino-terminus of caldesmon to the S2 part of HMM, there has been no direct electron microscopic evidence for the binding of two components. Our images for the first time indicate clearly the mode of association of dephosphorylated HMM with thin filament in the presence of ATP, so-called nonproductive interaction (Hemric and Chalovich, 1988) under relaxed conditions. Both negatively stained and rotary-shadowed images include some particles presumably indicating the presence of a tandem arrangement of two HMM particles on a single caldesmon molecule. We showed recently (Katayama et al., 1995) that caldesmon, probably its amino-terminus, induces the filament formation of dephosphorylated myosin under the conditions where purified myosin assumes nonpolymerizable 10S-conformation. Although this property may reasonably explain the observed presence of myosin filament in relaxed smooth muscle cells (Somlyo et al. 1981; Tsukita et al., 1982), the molecular origin for such capability was not clear. In our previous studies (Katayama et al., 1989; Katayama, 1989a) using affinity chromatography of caldesmon fragments, we showed that several fragments derived from the central domain have some affinity to S2 portion of HMM. Huber et al. (1993) also reported the presence of a second myosin-binding site at the carboxy-terminal side of the molecule. These additional sites may operate to induce dephosphorylated myosin molecules to form stable filaments even under relaxed conditions. In smooth muscle cell, it is thought that myosin molecules assemble into side-polar filament (Craig and Megerman, 1977; Cooke et al., 1989), in which myosin heads might align parallel with each other. Provided that some part of caldesmon molecules projecting to the same side of thin filament as a cluster, myosin heads arranged in such a manner could face straight to clustered caldesmon, facilitating easier interaction between them. Hence, the molecular arrangement proposed here is quite compatible with the functional aspect of smooth muscle cell, the tension development, and its regulation.

Whatever the case, the participation of a fine filamentous protein caldesmon to the molecular organization of smooth



muscle thin filament makes it a greatly different protein system both in structure and function from the counterpart in striated muscle. Rather, it might be more appropriate to state that the molecular constitution of striated muscle is unique, because caldesmon is known to distribute widely in almost any motile cells other than striated muscle, if its lower molecular weight form is taken into account (see review by Sobue and Sellers, 1991).

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