Three-Dimensional Image Reconstruction of Reconstituted Smooth Muscle Thin Filaments: Effects of Caldesmon

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ABSTRACT Caldesmon inhibits actomyosin ATPase and filament sliding in vitro, and therefore may play a role in modulating smooth and non-muscle motile activities. A bacterially expressed caldesmon fragment, 606C, which consists of the C-terminal 150 amino acids of the intact molecule, possesses the same inhibitory properties as full-length caldesmon and was used in our structural studies to examine caldesmon function. Three-dimensional image reconstruction was carried out from electron micrographs of negatively stained, reconstituted thin filaments consisting of actin and smooth muscle tropomyosin both with and without added 606C. Helically arranged actin monomers and tropomyosin strands were observed in both cases. In the absence of 606C, tropomyosin adopted a position on the inner edge of the outer domain of actin monomers, with an apparent connection to sub-domain 1 of actin. In 606C-containing filaments that inhibited acto-HMM ATPase activity, tropomyosin was found in a different position, in association with the inner domain of actin, away from the majority of strong myosin binding sites. The effect of caldesmon on tropomyosin position therefore differs from that of troponin on skeletal muscle filaments, implying that caldesmon and troponin act by different structural mechanisms.

INTRODUCTION

The main trigger for switching-on actomyosin ATPase, and therefore contraction in smooth muscle, is myosin-phosphorylation (for reviews see Kamm and Stull, 1985; Sellers and Adelstein, 1986). There is also evidence for thin filament-based regulation in smooth muscle (see Marston and Smith, 1985, for a review). Caldesmon, which is localized in the contractile apparatus of smooth muscle cells (Fuerst et al., 1986; North et al., 1994; Mabuchi et al., 1996), is generally considered to participate in this regulation. This is supported by observations that caldesmon, at ratios thought to occur in thin filaments in situ, inhibits actomyosin ATPase (Smith et al., 1987; Chalovich, 1992) and filament sliding in vitro (Okagaki et al., 1991; Haeberle et al., 1992; Shirinsky et al., 1992; Fraser and Marston, 1995), and this inhibition can be reversed in the presence of Ca^{2+} and calcium binding proteins such as calmodulin.

The biochemical mechanism of inhibition of actomyosin ATPase by caldesmon has been investigated extensively, yet remains controversial (see reviews by Chalovich, 1992, and Marston and Huber, 1996). To gain a better understanding about the role of caldesmon and its mechanism of action, detailed information about its structure and the structural basis for its effects is needed. Structural studies to date have shown that caldesmon molecules are long, thin (80 nm × 2 nm), and flexible (Fuerst et al., 1986; Mabuchi and Wang, 1991). They appear to be made up of several rigid rodlike domains joined by more flexible regions (Levine et al., 1990; Mabuchi and Wang, 1991). Several studies indicate that the elongated protein is bound longitudinally to thin filaments with a periodicity that is defined by tropomyosin (Lehman et al., 1989; Moody et al., 1990). Even though the molecule is very long, the inhibitory and actin binding region is seemingly compact and restricted to the C-terminal 150 amino acids, representing <20% of the entire protein (Szpacenko and Dabrowska, 1986). Caldesmon inhibition of actomyosin ATPase is enhanced by tropomyosin, suggesting an inhibitory mechanism similar to that of troponin in skeletal muscle (Fraser and Marston, 1995; Marston et al., 1994; Marston and Redwood, 1993), in which caldesmon, like troponin, fixes tropomyosin over sites of strong myosin binding to interfere with actin-myosin interaction. However, despite such similarities, Vibert et al. (1993) suggested that an analogous steric mechanism cannot operate on smooth muscle thin filaments. Using three-dimensional image reconstruction of caldesmon-containing native thin filaments from smooth muscle, they observed that, in fact, tropomyosin in smooth muscle was located away from the myosin binding sites, and therefore direct steric blocking by tropomyosin was improbable. However, since native thin filaments are not necessarily a homogeneous system and contain small amounts of other actin binding proteins, e.g., filamin, α-actinin and calponin, as well as myosin, it is difficult to explicitly ascribe the effect obtained by Vibert et al. (1993) to caldesmon. Moreover, since both caldesmon and tropomyosin are elongated molecules, it was not possible to determine unequivocally which protein(s) were responsible for the elongated density interpreted as tropomyosin.

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In the present study, we explore the effect of bound caldesmon on thin filament structure using a more defined system. We test the influence of a 16-kDa bacterially expressed C-terminal fragment of chicken gizzard caldesmon, 606C, comprising the sequence 606–756 (Redwood and Marston, 1993) on actin-tropomyosin structure using purified proteins and reconstituted filaments. The fragment inhibits actomyosin ATPase at low stoichiometry. Moreover, since it lacks the extended central α-helical region as well as N-terminal domain of intact caldesmon, it is unlikely to extend over many adjacent actin monomers along F-actin. Therefore, any filament strand density observed is most likely to originate from tropomyosin and not caldesmon. In the studies presented here, we successfully reconstituted actin-tropomyosin filaments in the presence of 606C-caldesmon, and resolved tropomyosin by three-dimensional reconstruction of the negatively stained filaments. The position of tropomyosin in this reconstituted system is the same as that originally observed by Vibert et al. (1993). Comparing actin-tropomyosin in the presence and absence of 606C-caldesmon, we show tropomyosin movement for the first time in reconstituted filaments, and again confirm results obtained originally on native thin filaments (Vibert et al., 1993).

MATERIALS AND METHODS

Protein preparation

Skeletal muscle F-actin was prepared from rabbit back muscle using the Drabikowski and Gergely (1964) modification of the original Straub (1942) method and smooth muscle tropomyosin was prepared from sheep aorta by the Eisenberg and Kielley (1974) adaptation of the Bailey (1948) protocol. Rabbit skeletal muscle HMM was prepared according to Margossian and Lowy (1982). Recombinant caldesmon fragment 606C (amino acids 606–756) of chicken caldesmon heavy isoform was produced according to Redwood and Marston (1993).

Electron microscopy and helical reconstruction

Thin filaments were reconstituted by combining 11 μM actin and 2.5 μM tropomyosin in the presence and absence of 7 μM 606C. The samples were gently mixed at room temperature in 5 mM PIPES dipotassium salt, 5 mM KCl, 5 mM $MgCl_2$, 10 mM NaN_3, 1 mM DTT, pH 7.0 (ATPase buffer), and left to incubate for 20 min at room temperature before rapid dilution 12.5-fold with additional buffer containing 7 μM 606C. Diluted samples were then immediately applied to grids for electron microscopy. Low ionic strength buffers and excess 606C in the dilution buffer were needed to prevent dissociation of the fragment from F-actin-tropomyosin at the protein concentrations used for electron microscopy. The binding of tropomyosin was sufficient to saturate F-actin at these dilutions and adding excess tropomyosin was not required. Grids were negatively stained using 1% uranyl acetate (Moody et al., 1990) and electron micrograph images recorded at 60,000× magnification under minimal dosage conditions ($12^{e^-}/Å^2$) on a Philips CM120 microscope. Micrographs were digitized on an Eikonix model 1412 CCD camera and displayed at a pixel size corresponding to ~0.67 nm in the filaments (Vibert, 1992). Regions of filaments suitable for helical reconstruction were selected on the basis of uniformity of staining, freedom from astigmatism, straightness, and diameter. Only filaments with diameters of ~15 nm were chosen, since narrower ones often do not show tropomyosin in reconstructions (Lehman et al., 1995). Slightly curved filaments were straightened by fitting a cubic spline and then re-interpolating the image (Egelman, 1986). Helical reconstruction was carried out using standard methods (DeRosier and Moore, 1970; Amos and Klug, 1975) as described previously (Vibert et al., 1993, 1997). The statistical significance of densities in maps was computed from the standard deviations associated with each contributing point using Student's *t*-test (Milligan and Flicker, 1987; Trachtenberg and DeRosier, 1987). Fitting of the atomic resolution actin monomer into our reconstructions was carried out according to McGough and Way (1995) using the program O (Jones et al., 1991).

Binding and ATPase assays

Activation of rabbit skeletal muscle heavy meromyosin (HMM) MgATPase by the thin filament preparations was measured in ATPase buffer at 25°C using 1.5 μM HMM and 5 mM MgATP by our standard methods (Smith et al., 1987; Marston and Redwood, 1992) before filaments were diluted for electron microscopy. After dilution, the protein content of filament samples, collected by sedimentation (80,000 × *g* for 20 min) in ATPase buffer, was estimated by SDS-PAGE. Gels were stained in Coomassie Brilliant Blue-R and the quantity of actin, tropomyosin, and 606C in filaments determined by densitometric scanning and comparison to known standards run on the same gel.

RESULTS

The ratio of tropomyosin and 606C bound to the F-actin used in our studies was estimated by SDS-PAGE (Table 1). The relative amount of actin and tropomyosin in our reconstituted thin filament preparations was comparable to that found in native thin filaments (Lehman et al., 1989; Marston, 1990). Under our set of conditions, one 606C was bound for approximately every three actin subunits (Table 1). This 606C content was high relative to that used in other studies (Redwood and Marston, 1993) but was needed to guarantee saturation of the high affinity binding sites on actin-tropomyosin (Smith et al., 1987) at the low filament concentrations required for electron microscopy.

Electron micrographs of actin-tropomyosin filaments and actin tropomyosin-606C-containing filaments showed typical actin subunit structure and, occasionally, thin strands following the helical array of actin molecules (Fig. 1).

TABLE 1 Thin filament composition and activation of acto-HMM ATPase

Sample	Tm/Actin (mol/mol)	606c/Actin (mol/mol)	ATPase (s^{-1})
F-Actin	—	—	1.423 ± 0.206
F-Actin-TM	0.143 ± 0.038	—	1.113 ± 0.0115
F-Actin-TM-606C	0.147 ± 0.025	0.387 ± 0.055	0.236 ± 0.055

Values given are mol ATP/mol HMM active sites/s^{-1}. Molar ratios of proteins bound to F-actin in samples used for electron microscopy were estimated by SDS-PAGE and gel densitometry, assuming molecular weights of 42, 70, and 16 for actin, tropomyosin, and the 606C-fragment of caldesmon resp. The actin-activation of HMM ATPase by thin filament preparations at 25°C is also shown (see Materials and Methods section for details). Approximately 20% inhibition of acto-HMM ATPase was noted for F-actin saturated with tropomyosin; by adding 606C, inhibition increased steadily up to ~80% of the acto-TM-HMM ATPase. Further addition of 606C, beyond what was used to prepare our samples, had no appreciable additional inhibitory effect. For all standard deviations $n = 3$.

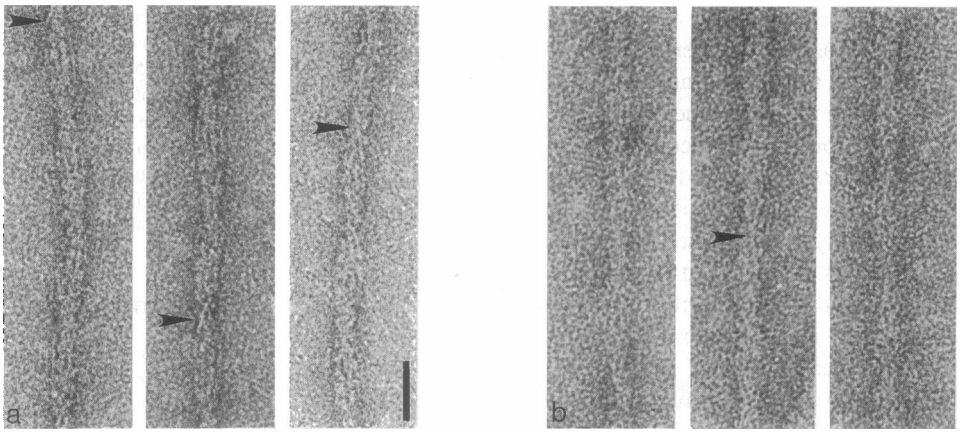

FIGURE 1 Electron micrographs of negatively stained reconstituted F-actin-tropomyosin filaments. (*A*) F-actin-tropomyosin; (*B*) F-actin-tropomyosin plus caldesmon C-terminal fragment, 606C. In both cases, tropomyosin strands were occasionally observed, indicated by arrows. Scale bar is 20 nm.

Background from excess unbound 606C, in samples containing the peptide, did not obscure these structural details (Fig. 1 *b*). No evidence of bound 606C density was obvious when micrographs were directly inspected.

The average amplitudes and phases along the layer lines of Fourier transforms were calculated for F-actin-tropomyosin (derived from 16 filaments) and F-actin-tropomyosin-606C (derived from 18 filaments), data not shown. The reconstructed densities from averaged layer line data showed characteristically bilobed actin monomers (Figs. 2 and 3) with continuous strands that closely followed the long-pitch actin helix (Fig. 2). These strands have been previously attributed to tropomyosin (Vibert et al., 1993).

In reconstructions of acto-tropomyosin controls, the tropomyosin was located over the outer domain of actin with strongest connectivity to the inner portion of sub-domain 1 of actin (Figs. 2 *a* and 3 *b*). The presence of 606C caused a change in the position of tropomyosin to the inner domain of actin exposing the shallow groove between the inner and outer domains. Here the major connectivity of tropomyosin was to the outer aspect of sub-domain 3 of actin (Figs. 2 *b* and 3 *c*). No obvious difference in strand density or diameter was observed between the two reconstructions. However, weak positive density, possibly attributable to 606C, was observed on sub-domain 1 in difference maps in which actin-tropomyosin density was subtracted from actin-tropomyosin-606C density. In contrast to the consistent position and shape of actin and tropomyosin densities in reconstructions of sets of individual (unaveraged) filaments, these extra minor densities were very variable (data not shown).

Good overall fit of our reconstructions to the Lorenz et al. (1993) atomic model of F-actin was achieved (Fig. 4). Fitting reconstructed densities derived from the control actin-tropomyosin filaments to the atomic model (Fig. 4 *a*) revealed that tropomyosin covered clusters of amino acids on actin thought to participate in strong myosin binding (Rayment et al., 1993). Sites apparently involved in weak actin-myosin binding remained accessible (Fig. 4 *a*). In contrast, when the atomic model was fitted to the 606C-containing filaments (Fig. 4 *b*), tropomyosin was then located further away from the majority of the strong myosin binding sites. However, one strong binding cluster, amino acids 332–334 of the actin sequence, may still be covered by the edge of tropomyosin, although whether direct contact between the cluster and tropomyosin is established is uncertain.

DISCUSSION

We have been able to reconstitute actin-smooth muscle tropomyosin filaments, which consistently show well-defined tropomyosin strands in reconstructions. Electron micrographs showed filaments comparable in structure to the native smooth muscle thin filaments of Vibert et al. (1993). While tropomyosin was only visible intermittently in micrographs, all our reconstructed images showed well-defined tropomyosin strands. The structure of actin monomers and their connectivity was comparable to that in reconstructions of other negatively stained and frozen-hydrated thin filaments (Vibert et al., 1993; Lehman et al., 1994, 1995; Milligan et al., 1990). The radial position of tropomyosin on filaments matched that found for native smooth and skeletal muscle thin filaments (Vibert et al., 1993, 1997; Lehman et al., 1994, 1995).

In the present studies, we used the 606C fragment of caldesmon, which consists only of the inhibitory actin binding region of the molecule and lacks the elongated portion that might bind along side tropomyosin (Redwood and Marston, 1993). This was done to ensure that strand density seen in corresponding reconstructions was derived from tropomyosin and had no major caldesmon component. Strand density and positions, in fact, were very similar to those observed by Vibert et al. (1993), who studied the position of tropomyosin in the presence and absence of whole caldesmon on native filaments. Thus we conclude that the central helix and the N-terminal domain of the caldesmon molecule (domains 1–3) were unlikely to represent or contribute significantly to strand density.

It is not surprising that the densities derived from 606C itself were very weak and quite variable between filaments. Helical reconstruction methods treat densities associated

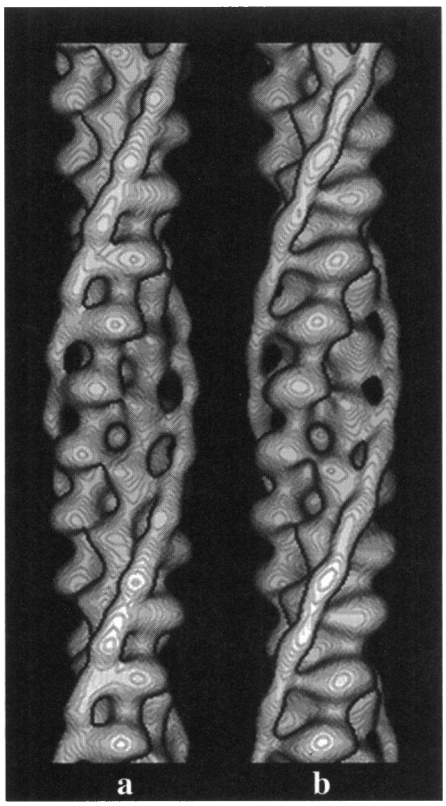

FIGURE 2 Surface views of reconstructed densities from negatively stained, reconstituted thin filaments. Filaments show characteristic bilobed actin monomers and well-ordered continuous tropomyosin strands in both cases. (*a*) F-actin-tropomyosin controls in the absence of caldesmon. Here, tropomyosin adopts a position on F-actin on the inner edge of the outer domain, with a distinct connection to sub-domain 1 of actin. (*b*) F-actin-tropomyosin-606C filaments (acto-myosin ATPase inhibited by 80% or more, see Table 1). Here, tropomyosin is found in a position in contact with the inner domain of actin. The average amplitudes and phases along the layer lines of Fourier transforms were calculated for F-actin-tropomyosin (derived from 16 filaments) and F-actin-tropomyosin-606C (derived from 18 filaments) and used to generate the above maps. The total number of constituent actin molecules averaged for each 3D map was 567 and 1020, respectively. The up-down phase residuals (±SD), a measure of filament polarity, were 34.5 ± 5.6° and 27.6 ± 5.6°, respectively. Phase residuals between layer line data sets and the corresponding average, a measure of filament alignment, were 46.4 ± 4.9° and 52.7 ± 5.8°, respectively. Layer line data for averaged filaments extended to a resolution of ~1/2.5–3.0 nm in both radial and axial directions. The major difference between data sets was magnitude of peaks along the second layer line (l = 2) which were larger in the pattern associated with the 606C-treated filaments than in the controls.

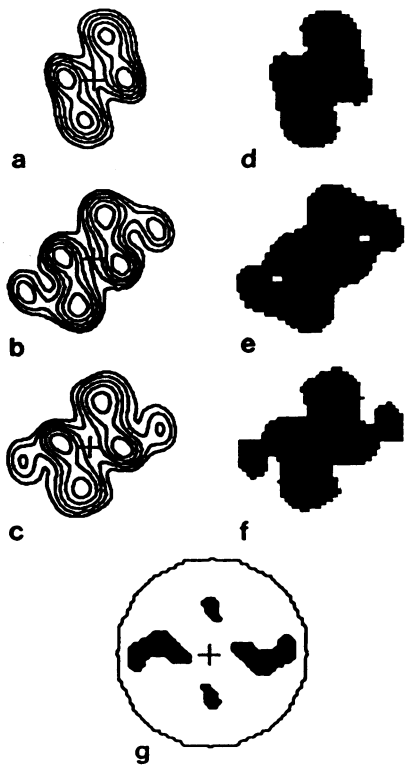

FIGURE 3 (*a–c*) Helical projections formed by projecting densities in our maps along their helical tracks onto a plane perpendicular to the filament axis. (*a*) F-actin (free of tropomyosin and 606C) used as a reference for comparison with *b* and *c*. (*b*) F-actin-tropomyosin; (*c*) F-actin-tropomyosin-606C. Note the additional density associated with actin in *b* and *c* due to the presence of tropomyosin; also note the positional differences of the tropomyosin density in *b* and *c* dependent on 606C. (*d–f*) maps showing the statistical significance of the contributing densities in the helical projections *a–c*, respectively, and indicating those that are significantly different from zero at a confidence level of 99.95%. Each map pair (*a, d*; *b, e*; *c, f*) shows a near perfect fit demonstrating the reliability of the data. The statistical significance of the differences between F-actin-tropomyosin-606C and F-actin tropomyosin (maps in *c* and *b*, respectively) was computed by point-by-point comparison; a difference map (not shown) was calculated by subtracting densities associated with map *b* from those in map *c* and the significance of the difference was evaluated using a Student's *t*-test. (*g*) The densities associated with map *c* that are significantly different from those in map *b* at a confidence level >99.95% are shown. The major difference is associated with the tropomyosin strand relocation. Minor differences are noted at the junction between adjacent actin monomers along the genetic actin helix. Differences are also noted on the very peripheral edge of the outer domain of actin, which are possibly due to the presence of the poorly defined 606C mass, but these are only apparent at lower levels of confidence (data not shown).

with actin as if they were identical on each monomer. Since 606C and actin in our reconstituted filaments were not equimolar, it is likely that any 606C density detected may have been averaged over several actin monomers or otherwise lost during reconstruction. The peptide also is apparently very flexible (Mornet et al., 1995) making detection additionally difficult.

Fitting the atomic model of F-actin (Lorenz et al., 1993) into the envelope representing our actin-tropomyosin reconstruction indicates that in the absence of 606C tropomyosin is located over the strong myosin binding sequences on actin proposed by Rayment et al. (1993). This position of tropomyosin is indistinguishable from that considered to sterically block myosin-binding in native troponin-regulated filaments in the off-state (Lehman et al., 1995), even though these actin-tropomyosin filaments could activate HMM-ATPase (Table 1). This seemingly paradoxical result, however, may be easily explained since in the absence of regulatory proteins tropomyosin is not *fixed* over myosin-binding sites on actin. By binding to these sites strongly and stereo-specifically (Rayment et al., 1993), myosin may sim-

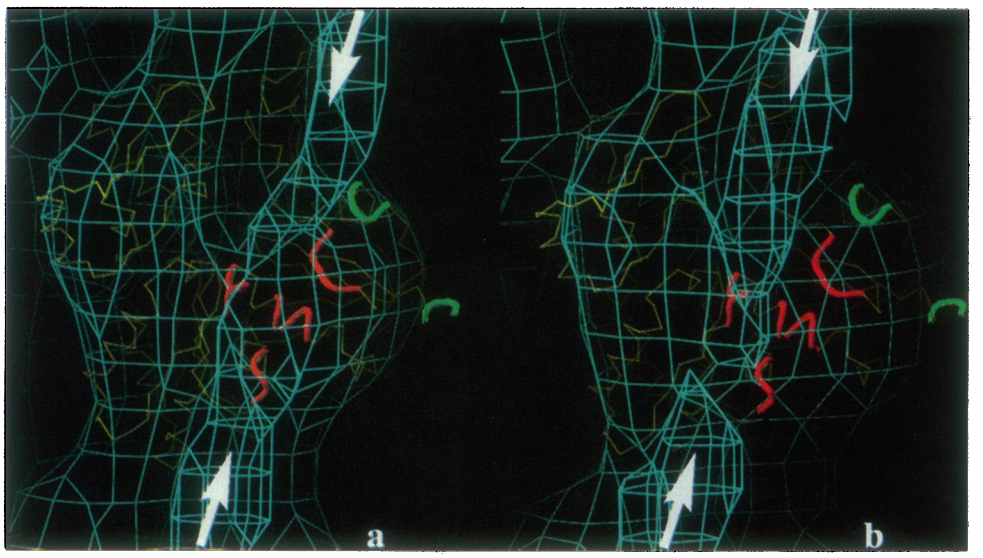

FIGURE 4 Fitting the atomic model of F-actin (Lorenz et al., 1993) into our maps obtained by electron microscopy. Views of single actin monomers in which α-carbon chains from the atomic model are depicted in yellow fitted into cyan wire-cage envelopes corresponding to our reconstructions. The polarities of the actin monomers are the same as those in Fig. 2. Clusters of amino acids thought to bind myosin strongly (Rayment et al., 1993) are highlighted in red, clusters of amino acids that may interact weakly with myosin are shown in green. Position of tropomyosin density is indicated by white arrows. (*a*) Actin-tropomyosin alone; tropomyosin appears to be located over the strong myosin binding clusters, whereas the weak binding clusters remain uncovered. (*b*) Actin-tropomyosin-606C; tropomyosin position is different, apparently covering only one remaining strong myosin binding cluster (residues 332–334).

ply compete and displace tropomyosin. This is supported by observation of cooperative myosin binding in both smooth and skeletal muscle actin-tropomyosin (Marston et al., 1994) and by several kinetic analyses (see Lehrer, 1994; Geeves and Halsall, 1987; Hill et al., 1980).

The C-terminal 150-amino acid sequence of caldesmon is known to inhibit actin-tropomyosin activation of myosin ATPase (Szpacenko and Dabrowska, 1986), and we confirmed that under the conditions used in this study 80% inhibition was obtained by addition of this fragment. In spite of the ATPase inhibition fitting the atomic structure of actin (Lorenz et al., 1993) into our reconstructed density map showed that the caldesmon fragment caused a relocation of the tropomyosin *away from* the majority of the strong myosin binding sites on actin. These results are consistent with those of Vibert et al. (1993) who studied a less well-defined preparation of isolated native smooth muscle thin filaments. However, these results are in sharp contrast with those on troponin-regulated thin filaments in which tropomyosin is located in a sterically blocking position over the strong myosin binding sites in the off-state, (Lehman et al., 1994, 1995) and can thereby inhibit the actomyosin ATPase directly. Caldesmon cannot therefore sterically inhibit actin-tropomyosin activation of myosin ATPase by the same mechanism as that which occurs in skeletal muscle.

We cannot determine on the basis of our own evidence alone how caldesmon interacts with tropomyosin or how caldesmon may inhibit actin filament activity. It is known that the inhibitory properties of caldesmon are greatly enhanced by tropomyosin (Dabrowska et al., 1985; Ngai and Walsh, 1984; Smith et al., 1987); for instance, under the conditions of our experiments, 7 μM 606C caused 80% inhibition of actin-tropomyosin activation compared with not more than 20% inhibition of actin activation (Redwood and Marston, 1993; Marston and Redwood, 1993). It has been suggested that caldesmon might act by preventing potentiation of acto-myosin ATPase activity by tropomyosin and, as pointed out (Vibert et al., 1993) such a mechanism would be compatible with the structural observations (Chacko and Eisenberg, 1990; Horiuchi and Chacko, 1989). However, we did not actually see potentiation by tropomyosin under our buffer conditions in the absence of caldesmon fragment. In fact, there was ~20% inhibition of the ATPase relative to actin alone, and the caldesmon fragment caused still greater inhibition of the ATPase activity (Table 1; Smith et al., 1987). An alternative proposal that caldesmon inhibits actin-tropomyosin by reducing strong interactions of myosin with actin can account for all the ATPase and motility assay observations (Marston et al., 1994; Fraser and Marston, 1995). We have noted that, following addition of 606C to reconstituted thin filaments, the postulated strong myosin binding residues 332–334 on actin may still remain obstructed by tropomyosin, but we have no basis on which to judge whether this is sufficient to interfere with strong myosin binding. Another possibility which should be considered is that a change in actin conformation may be associated with the binding of caldesmon. Propagation of an actin conformational change may be responsible for the effect of caldesmon on myosin binding, and hence regulation of smooth muscle thin filaments.

One or more sets of quasi-repeating motifs of charged amino acids are thought to be involved in specifying alter-

native locations of tropomyosin on skeletal muscle actin in on- and off-states (McLachlan and Stewart, 1976; Phillips et al., 1986). The equilibrium between the two positions is perturbed by troponin, Ca^{2+}, and myosin-binding. Smooth and skeletal tropomyosin have the same sets of heptad sequence repeats (Sanders and Smillie, 1984), and it is interesting to note that the two locations of smooth muscle tropomyosin in our reconstructions appear to correspond to the two positions observed in troponin-regulated thin filaments (Lehman et al., 1994, 1995; Vibert et al., 1993). Our results thus support predictions of the McLachlan and Stewart (1976) model that tropomyosin can adopt only a limited set of positions which are specified by conserved actin-tropomyosin charge interactions. The equilibrium between positions of tropomyosin on actin, however, may depend on tropomyosin and actin isoform interactions (Williams et al., 1984), and may also be affected by actin binding proteins. Regulatory proteins such as troponin and caldesmon may both act as a modulators of the equilibrium between these positions, but by different structural mechanisms.

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