Three-dimensional Reconstruction of Caldesmon-containing Smooth Muscle Thin Filaments

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Abstract. Caldesmon is known to inhibit actomyosin ATPase and filament sliding in vitro, and may play a role in modulating smooth muscle contraction as well as in diverse cellular processes including cytokinesis and exocytosis. However, the structural basis of caldesmon action has not previously been apparent. We have recorded electron microscope images of negatively stained thin filaments containing caldesmon and tropomyosin which were isolated from chicken gizzard smooth muscle in EGTA. Three-dimensional helical reconstructions of these filaments show actin monomers whose bilobed shape and connectivity are very similar to those previously seen in reconstructions of frozen-hydrated skeletal muscle thin filaments. In addition, a continuous thin strand of density follows the long-pitch actin helices, in contact with the inner domain of each actin monomer. Gizzard thin filaments treated with Ca^{2+}/calmodulin, which dissociated caldesmon but not tropomyosin, have also been reconstructed. Under these conditions, reconstructions also reveal a bilobed actin monomer, as well as a continuous surface strand that appears to have moved to a position closer to the outer domain of actin. The strands seen in both EGTA- and Ca^{2+}/calmodulin-treated filaments thus presumably represent tropomyosin. It appears that caldesmon can fix tropomyosin in a particular position on actin in the absence of calcium. An influence of caldesmon on tropomyosin position might, in principle, account for caldesmon's ability to modulate actomyosin interaction in both smooth muscles and non-muscle cells.

PRECISION in the control of many complex biological processes requires both regulatory switches and modulatory fine-tuning. The graded contraction displayed by different smooth muscles after neurohormonal stimulation no doubt involves fine control mechanisms governing the underlying actomyosin interaction which in turn determine the magnitude of contraction. It is well known that in vertebrate smooth muscles, contractile activity is stimulated by a Ca^{2+}-calmodulin-dependent cascade resulting in myosin phosphorylation (for reviews see Kamm and Stull, 1985; Sellers and Adelstein, 1986). In addition, thin filament-associated proteins may play a role in modulation of smooth muscle activity (Marston and Smith, 1984). Caldesmon appears to be a necessary component of this modulation (Marston et al., 1988) and is the only actin-binding protein other than tropomyosin that is known to be located on thin filaments in regions of smooth muscle cells that are replete with myosin (Fürst et al., 1986; North, A. J., and J. V. Small, personal communication). Caldesmon inhibits actomyosin ATPase and the motility of actin and myosin filaments in vitro (Sobue et al., 1981; Ngai and Walsh, 1984, 1985*a*; Dabrowska et al., 1985; Marston and Lehman, 1985; Okagaki et al., 1991; Haeberle et al., 1992; Shirinsky et al., 1992). Its stoichiometry on isolated thin filaments is comparable to that required for maximum inhibition (Lehman et al., 1989; Marston, 1990; Redwood and Marston, 1993). Inhibition by caldesmon is potentiated by tropomyosin, implying that the two proteins act in concert to modulate actin activation of myosin ATPase (Marston and Redwood, 1991). Ca^{2+}/calmodulin (CaCM)[1] relieves caldesmon inhibition in reconstituted systems, although other high-affinity Ca^{2+}-binding proteins may be responsible for this action during muscle activation in vivo (Sobue et al., 1981; Marston and Redwood, 1991; Mani et al., 1992). While most of the evidence suggesting a modulatory role for caldesmon stems from work on isolated proteins, parallel studies on intact preparations provide further support (Szpacenko et al., 1985; Taggart and Marston, 1988; Katsuyama et al., 1992; Pfitzer et al., 1992). Caldesmon, however, is not the only potential modulator of thin filament function; other smooth muscle proteins, such as calponin and SM 22, also may be associated with thin filaments, but the evidence for their involvement in regulation

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1. *Abbreviation used in this paper*: CaCM, Ca^{2+}/calmodulin.

of actomyosin ATPase is inconclusive (Lehman, 1991; Marston, 1991; North, A. J., and J. V. Small, personal communication).

Caldesmon is found not only in smooth muscles, but also in non-muscle tissues of vertebrates, and possibly in invertebrates as well (cf. Csizmadia, Bonet-Kerrache, Nyitray, and Mornet. 1993. *J. Muscle Res. Cell Motil.* 14:239), indicating that its function may be of general significance (Ngai and Walsh, 1985*b*; Sobue et al., 1988). In fact, several studies suggest that caldesmon may participate in modulating such diverse cellular processes as cytokinesis, exocytosis, and receptor capping (Burgoyne et al., 1986; Mizushima et al., 1987; Walker et al., 1989; Yamashiro et al., 1990, 1991; Matsumura and Yamashiro, 1993). Of particular interest are studies demonstrating a synchronization of non-muscle caldesmon dissociation from thin filaments and cytokinesis. Notably, caldesmon dissociation appears to be a result of its phosphorylation by cdc 2 kinase which may be necessary for actin to function in the cleavage furrow (Hosoya et al., 1993). Since phosphorylation of cytoplasmic myosin-II is also linked to some of these processes (Sellers and Adelstein, 1986; Mabuchi, 1990; Pollard et al., 1990; Hosoya et al., 1991; Satterwhite et al., 1992), a system of dual regulation analogous to that occurring in smooth muscle may operate in non-muscle tissues as well.

Assessing caldesmon's role and mechanism of action in smooth and non-muscle contractility is limited at present by uncertainties about the structural organization, interactions, and dynamics of the protein on thin filaments. Caldesmon molecules are long and asymmetric (~80 × 2 nm) (Graceffa et al., 1988; Mabuchi and Wang, 1991). They are regularly arranged on thin filaments (Lehman et al., 1989), probably attached longitudinally in end-to-end fashion (Moody et al., 1990; Mabuchi et al., 1993) with a periodicity that is defined by tropomyosin (Lehman et al., 1989; Moody et al., 1990). Electron microscopy of negatively stained filaments has revealed narrow protein strands running along the long-pitch actin helices of isolated native thin filaments (Moody et al., 1990). These strands may represent either caldesmon or tropomyosin, individually, or an association of the two; we now analyze them by image processing.

In this paper, we describe a three-dimensional helical reconstruction of smooth muscle thin filaments, isolated under relaxing conditions, which reveals the position of the strands relative to the domains of the actin monomers. We also present a three-dimensional reconstruction showing that selective removal of caldesmon from thin filaments brought about by the addition of CaCM causes a shift in strand position. Thus the position of the strand, which appears to be primarily composed of tropomyosin, can be altered by caldesmon, an effect that may be significant for understanding caldesmon function. Our results suggest that global changes in thin filament structure not only characterize regulation by troponin-tropomyosin in skeletal muscle, but possibly also modulation by caldesmon-tropomyosin in smooth muscle.

Materials and Methods

Protein Preparation

Chicken gizzard thin filaments were prepared according to Marston and Lehman (1985) with some modifications to minimize proteolysis and maximize caldesmon content. Leupeptin (2 μg/ml) was used throughout preparation and casein (10 mg/ml) included during homogenization and the washing of the crude muscle homogenate. Solution A, which is used during the entire protocol, was reduced in ionic strength and consisted of 55 mM NaCl, 5 mM $MgCl_2$, 3.75 mM sodium phosphate buffer (pH 7.0), 6.25 mM Pipes buffer (pH 7.0), 1 mM EGTA, 1 mM NaN_3, 1 mM DTT, and the ATP concentration for thin filament extraction was reduced to 2.5 mM. Thin filament yield was unaffected. The caldesmon content of isolated filaments, determined by gel densitometry (Lehman et al., 1989), was 1 mol caldesmon/23.9 mol actin. Caldesmon content of unpurified thin filaments is higher (1 mol/17.0 mol actin [Lehman et al., 1989]), suggesting that small amounts of caldesmon dissociation or proteolysis occurred during preparation or that admixture of cytoskeletal calponin-containing filaments took place; tropomyosin content of purified filaments and in unfractionated muscle is identical. Bovine brain calmodulin was prepared as described by Masure et al. (1984).

Electron Microscopy and Helical Reconstruction

Thin filaments at ~0.1 mg/ml were negatively stained as described by Moody et al. (1990). Electron micrographs, recorded using the Minimal Dose System of a JEOL 100CX electron microscope, were digitized on an Eikonix model 1412 scanner and displayed with a pixel size corresponding to ~7.5 Å in the filaments. Regions of the filament images that were suitable for helical reconstruction were selected on the basis of several criteria: uniform staining, freedom from drift and astigmatism, and straightness or only gentle curvature over a minimum of four crossover repeats of the actin helix. In addition, filaments that by eye revealed a continuous narrow strand of density running along the 360-Å-pitch actin helices were especially noted. Curved filaments were straightened by fitting a cubic spline to a visually chosen axis and then reinterpolating the image (Egelman, 1986). Fourier transforms (512 × 512 points) were calculated from filament regions no longer than four crossover repeats of the actin helix to minimize the effects of angular disorder (Egelman et al., 1982).

Helical reconstruction was done by standard methods (DeRosier and Moore, 1970; Amos and Klug, 1975). The helical symmetry of the thin filaments was close to 13 actin monomers in six turns of the left-handed 59 Å-pitch genetic helix, although the crossover repeat was generally a little more than six turns, so that Bessel function overlap between $n = 6$ and $n = -7$ terms on the third layer line did not occur. Exact 13/6 symmetry was enforced during averaging. For the thin filaments in EGTA, sets of layer line data to be averaged were selected by (*a*) computing a preliminary average from three sets taken from filaments in which a strand of density was visible in the images; (*b*) using this average set as a reference to select from among 30 single-side layer line sets those with low phase residuals; and (*c*) computing density maps from individual selected layer line sets, and choosing for averaging only those with distinct density in the "strand position." This procedure was necessary because phase residuals alone were not sufficiently sensitive to detect layer line sets that would give rise to strand density (which is only a small fraction of the total map density, corresponding to only small amplitude contributions on certain layer lines). For the thin filaments that were treated with Ca^{2+}/calmodulin, strands were also visible only in some images, and layer line sets were selected for averaging in a similar way.

The statistical significance of the densities in the average maps was assessed by performing a Student's *t* test at each pixel (cf. Trachtenberg and DeRosier, 1987). The regions whose average density differed from zero (the mean density in the map) at the 99% confidence level were identified. For comparison of the thin filament structures in EGTA and CaCM, a difference map was calculated from the densities in the respective average maps, and its significance was evaluated using a Student's *t* test at the 99.5% confidence level (cf. Milligan and Flicker, 1987).

Results

Structure of Thin Filaments in EGTA

The thin filaments from chicken gizzard smooth muscle used for electron microscopy were isolated by techniques that yielded samples with close to native component stoichiometry of caldesmon, tropomyosin and actin, and essentially no contaminants (Fig. 1; note the low levels of myosin, desmin, myoglobin, SM-22, calponin, and filamin). The filaments were maintained in EGTA-containing buffers until they were negatively stained.

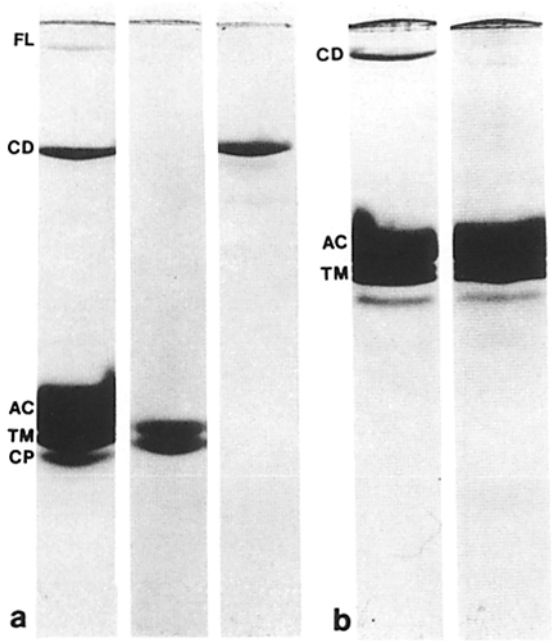

Figure 1. (*a*) 6% SDS-PAGE gels of (*left to right*) chicken gizzard thin filaments (47 μg), and of purified chicken gizzard tropomyosin (6 μg), and caldesmon (8 μg) standards. *CD*, *AC*, *TM*, *FL*, and *CP* designate caldesmon, actin, tropomyosin, filamin, and calponin bands. (*b*) 10% SDS-PAGE gel of (*left*) chicken gizzard thin filaments (23 μg; note caldesmon (*CD*), actin (*AC*), and tropomyosin (*TM*) bands; (*right*) thin filaments exposed to CaCM and collected by sedimentation (note the selective removal of caldesmon).

Electron micrographs showed evenly stained filaments on a low background (Fig. 2 *a*). In some cases, thin strands of protein aligned with the long-pitch actin helices could be seen running for short distances along the filaments (Moody et al., 1990). Three filaments of this type were selected for initial Fourier processing, and a preliminary average set of layer lines was made. These averaged layer line data were used to select from among a larger group of filaments (generally lacking visible strands) those with good phase agreement. Inspection of density maps showed that the selected filaments had well-preserved actin structure, but that only about half of the reconstructions also showed an axially oriented strand of density on the surface of the actin monomers. These filaments were chosen for further averaging, producing layer line data (Fig. 3 *a*, Table I) that extend to a resolution of ~1/23 $Å^{-1}$ radially and ~1/45 $Å^{-1}$ axially.

Density maps calculated from the averaged layer lines reveal bilobed actin monomers connected along both the right-handed 355 Å pitch helices and the left-handed 59 Å pitch genetic helix (Fig. 4 *a*). The inner and outer lobes of the monomers are of comparable size; their shape and position give the filament a distinct polarity. The outer diameter of the actin filament is ~95 Å. In contact with the surface of the inner actin domains is an axially continuous strand of density centered at a radius of ~45 Å which smoothly follows the 355 Å pitch helix (Figs. 4 *c* and 5, *a* and *b*). Maps of the variance in the densities were calculated, and statistical tests showed that the densities in both the actin monomer and strand regions of the map are significant at the 99% confidence level (Fig. 6, *a–c*).

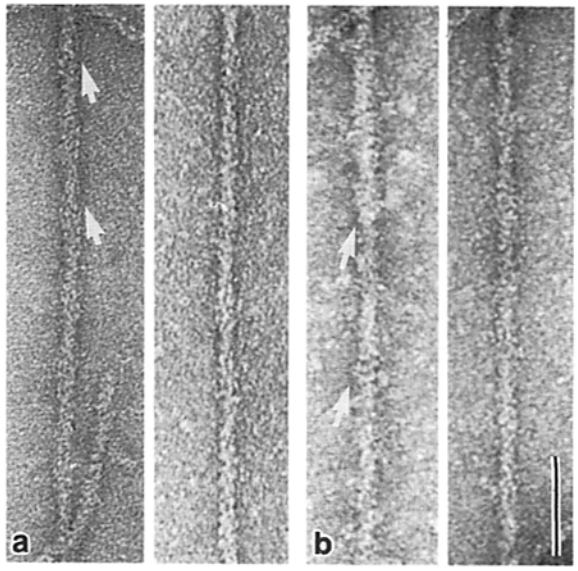

Figure 2. Electron micrographs of negatively stained chicken gizzard thin filaments (*a*) in EGTA; (*b*) treated with CaCM. In some cases, strands running along the actin helix are visible (*arrows*). In *b*, the higher background is due to the presence of CaCM and dissociated caldesmon. Bar, 0.05 μm.

Structure of Filaments Treated with Ca^{2+}/Calmodulin

At the low thin filament concentrations used for electron microscopy (typically 0.1 mg/ml), incubation with physiological amounts of calmodulin (20–30 μM) in the presence of Ca^{2+} lowers the affinity of caldesmon for actin-tropomyosin sufficiently to cause almost complete dissociation of the protein (Fig. 1 *b*). Densitometry of SDS-PAGE gels indicates a loss of ~93% of the caldesmon without any detectable dissociation of tropomyosin (Table II).

Electron micrographs of negatively stained filaments yielded images similar to those obtained in EGTA (Fig. 2 *b*). Strands of protein running along the actin helix were visible only in some filaments; nevertheless, the density maps calculated from individual filaments were less variable than for the EGTA-treated filaments and generally showed "strand density." Maps calculated from averaged layer lines (Fig. 3 *b*, Table I) revealed an actin monomer shape and connectivity very similar to those seen in EGTA-treated filaments (Fig. 4 *b*). By contrast, the strand now appeared to be in a different position relative to the actin monomer domains, closer to the outer domain in CaCM-treated filaments (Figs. 4 *d* and 5, *c* and *d*). The densities in both the actin and strand positions are statistically significant at the 99% confidence level (Fig. 6, *d–f*).

Comparison of the two maps shows that the strand position differs in the two kinds of filaments by an azimuthal rotation of ~20–25° about the helix axis. The linear movement, from a position in contact with the inner actin domain to one midway between the inner and outer domains, is ~15–20 Å, and there is some overlap between the two strand densities when the maps are superimposed. A "difference map," computed by point-by-point subtraction of densities, shows positive and negative peaks that reflect the strand rotation (Fig. 6 *g*). Statistical tests show that the two maps differ significantly in these regions at the 99.5% confidence level (Fig. 6, *h* and *i*).

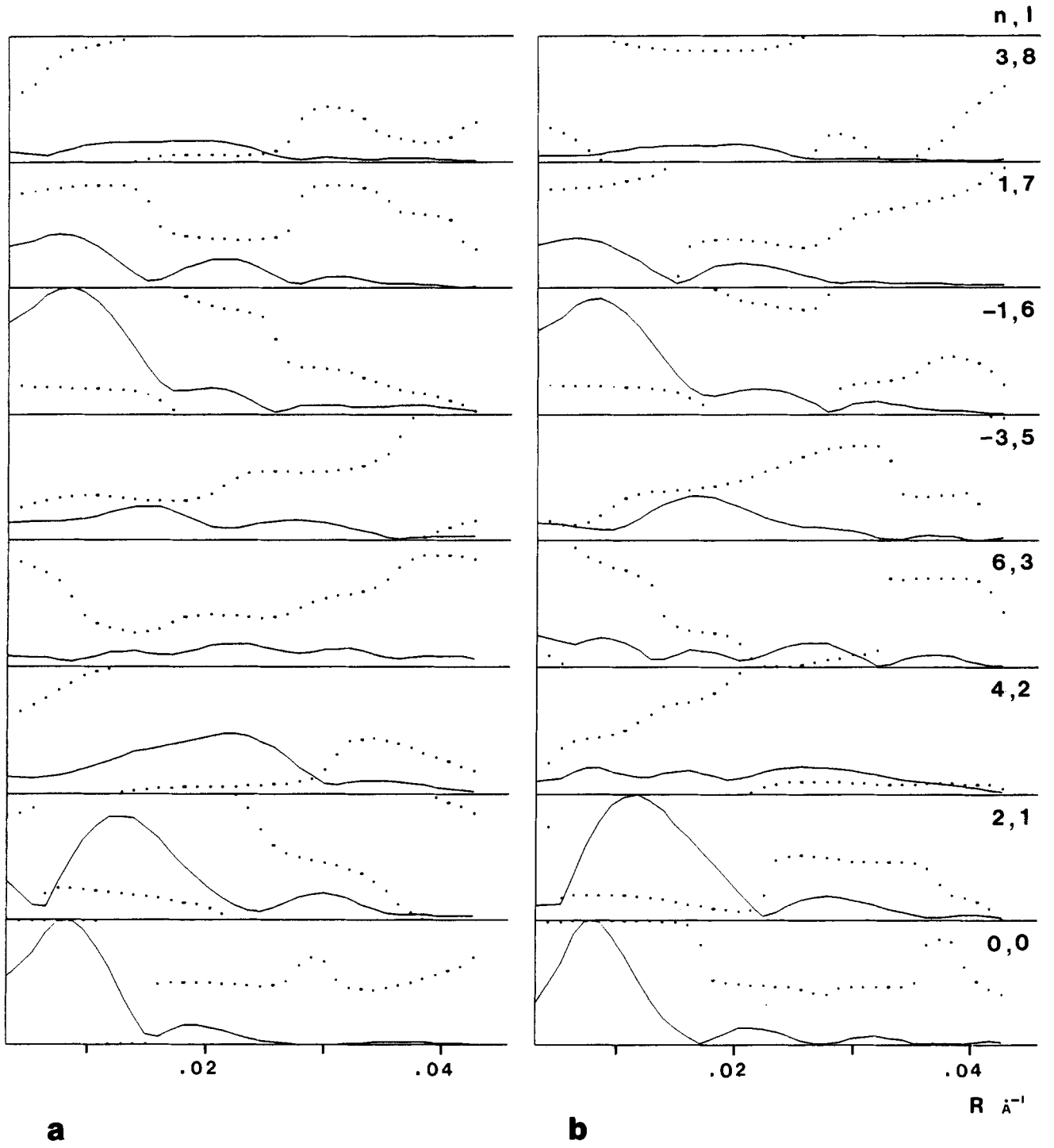

Figure 3. The average amplitudes and phases along layer lines in the thin filament transforms, derived from eight data sets each of (*a*) thin filaments in EGTA; (*b*) thin filaments treated with CaCM. Amplitudes are represented by the solid lines, phases by the dotted lines; both data sets have been brought to the same phase origin. Rotational orders (*n*) and layer line numbers (*l*) allowed by the 13/6 symmetry of the actin helix are shown.

Discussion

We have succeeded in visualizing axially continuous strands of protein on the surface of smooth muscle thin filaments by helical reconstruction. Although these strands are not visible in all reconstructions, our thin filament preparations have component stoichiometries of caldesmon, tropomyosin, and actin that are close to those of native filaments (Lehman et al., 1989; Marston, 1990); it therefore is likely that there is primarily one population of filaments and not two separate ones (with and without strands) in our samples. In addition, anti-caldesmon immuno-precipitates more than 80% of thin filaments in such preparations (Marston and Lehman, 1985), supporting the view that there is essentially one popu-

Table I. Phase Agreement between Sets of Layer Lines

	Q	ω	ψ	$\Delta\psi$	RSC
EGTA series (8 sides):					
Average	21	2.3	50	36	1.00
(range)	(18–25)	(1.3–3.9)	(44–55)	(31–46)	(0.92–1.06)
>10% F_{max}			36 (28–42)	44 (30–53)	
CaCM series (8 sides):					
Average	18	4.1	51	27	1.00
(range)	(11–24)	(1.5–8.2)	(46–54)	(15–38)	(0.84–1.09)
>10% F_{max}			32 (20–42)	43 (26–48)	

Columns 1–4, values in degrees;
Q, intra-filament near/far side phase residual;
ω, out-of-plane tilt;
ψ, inter-filament phase residual relative to preliminary average;
$\Delta\psi$, up/down phase residual;
RSC, radial scaling factor;
>10% F_{max}, phase residuals using only those points with amplitudes greater than 10% of the maximum non-equatorial amplitude.

lation of thin filaments present as defined by protein content. Although local deficiencies in caldesmon or tropomyosin content cannot be ruled out, the two proteins regulate actin cooperatively (e.g., Marston and Redwood, 1993), suggesting that occasional gaps in caldesmon or tropomyosin continuity along the thin filaments may not be functionally significant. Given the biochemical evidence for the existence of one filament population, the fraction of negatively stained filaments in which strands are visible by eye or by helical reconstruction is very unlikely to represent a structurally distinct sub-population, and it is much more likely that variations in negative staining make the strands visible only in some filaments and not in others. We have accordingly selected for inclusion in the averages only those filaments whose reconstructions showed strands, reasoning that these filaments merely reveal clearly a structure that is present but not necessarily visible in the entire population of filaments. We should emphasize that we have never seen strands in any other positions (in a given chemical state) than the ones we describe here. Despite the inferred local variations in strand visibility, the overall quality of the negative staining is good, judged from the fact that the actin region of our reconstructions is very similar to the same region in reconstructions of unstained, frozen-hydrated skeletal muscle thin filaments (Milligan et al., 1990).

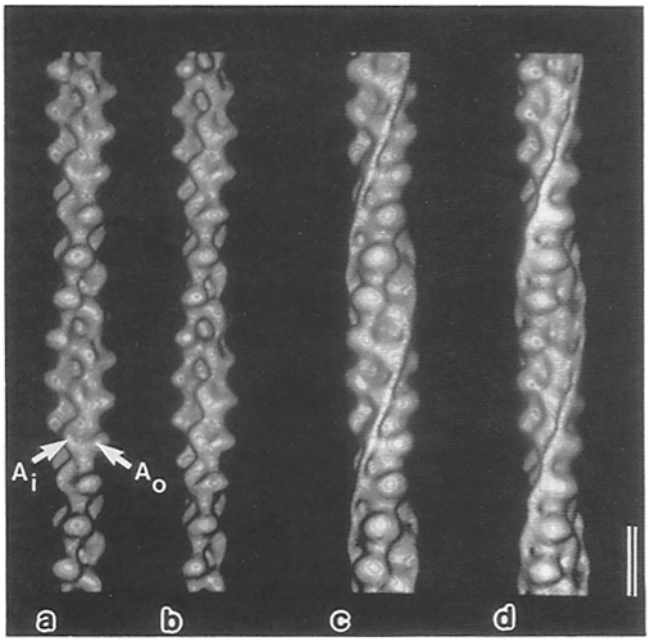

Figure 4. Surface views of the reconstructed densities. (*a* and *c*) thin filaments in EGTA; (*b* and *d*) thin filaments treated with CaCM. Two density levels in each map are shown: (*a* and *b*) a high level, to show the shape and connectivity of the actin monomers, which are very similar in the two maps; (*c* and *d*) a lower level, to show the helically wound strands of density on the surface of actin. The density level chosen gives the strands a diameter of ~20 Å. The inner A_i and outer A_o domains of one actin monomer are marked. Note the different positions of the strands, running along the A_i domains in *c* and lying between A_i and A_o in *d*. Bar, 100 Å.

Both smooth muscle tropomyosin (Matsumura and Lin, 1982) and caldesmon (Graceffa et al., 1988) are elongated molecules, and either or both could contribute to the strands seen in smooth muscle thin filaments. Although strands are detected both in EGTA and after treatment with CaCM, caldesmon (but not tropomyosin) dissociates from the filaments in CaCM. Thus tropomyosin presumably makes the major contribution to the strand. The fact that tropomyosin does not dissociate from thin filaments either during preparation or after CaCM treatment argues strongly that thin filaments contain a full tropomyosin complement; hence the failure to visualize tropomyosin strands in some filaments is not due to its absence and is more likely to be a result of inadequate local staining—perhaps because stain penetrates into the protein. Caldesmon itself can be cross-linked through its COOH-terminal region to sites on the outer actin domain (Adams et al., 1990; Bartegi et al., 1990; Graceffa and Jancsó, 1991; Graceffa et al., 1993). However we see no strand-like density in this region of our maps of filaments in EGTA, suggesting that the average position of caldesmon over its 800 Å length is not well defined, or that most of the molecule apart from the COOH-terminal domain contributes to the strand on the inner actin domain in EGTA where it is unresolved from tropomyosin. The extended 600 Å-long single α-helical segment of caldesmon (cf. Marston and Redwood, 1991) might be expected to require close association with tropomyosin for stabilization of its structure. It may be significant that strands (of tropomyosin/troponin) have not been seen in reconstructions of frozen-hydrated skeletal muscle thin filaments in EGTA, suggesting that their position is disordered (Milligan, R. A., personal communication). Thus it is possible that we see strands clearly because negative staining tends to trap tropomyosin in a particular position in EGTA. There is no a priori reason, however, for expecting skeletal and smooth muscle thin filaments to have the same structure, and we think it more likely that strand visibility is influenced by the presence of additional thin filament proteins such as caldesmon. Whether stain also has an effect will have to be resolved by cryo-electron microscopy of smooth muscle filaments.

Our reconstructions do, however, indicate that the presence of caldesmon in thin filaments can affect the position

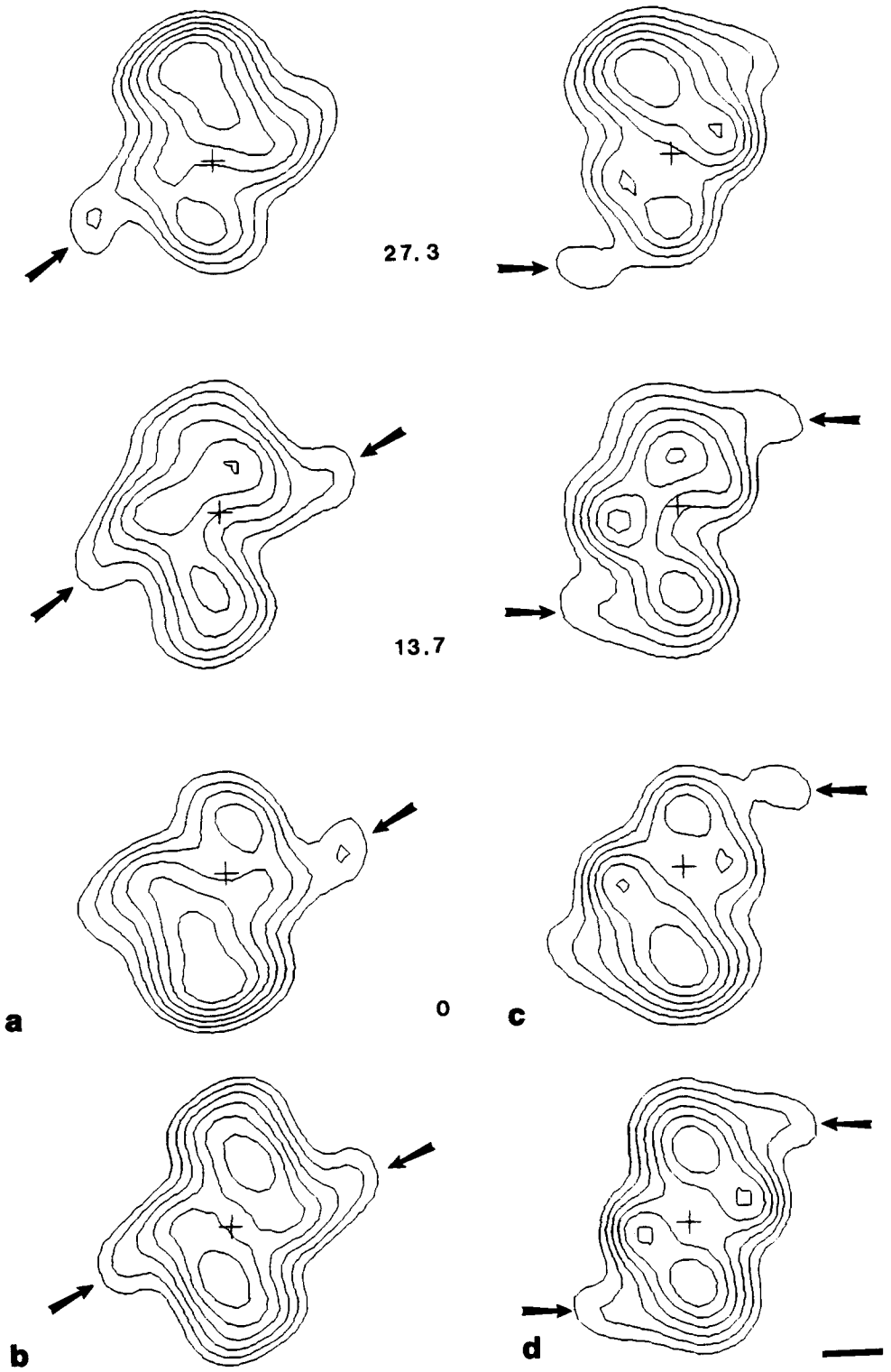

Figure 5. (*a* and *c*) Sections through the density maps, perpendicular to the filament axis, at three levels (z = 0, 13.7 Å, 27.3 Å) through one monomer-repeat of the actin helix. (*b* and *d*) Projections of the densities along the $n = 2$ helical tracks onto the plane $z = 13.7$ Å ("helical projections"). (*a* and *b*) EGTA-treated and (*c* and *d*) CaCM-treated thin filaments; note the difference in the surface density profiles. The tropomyosin strand is indicated by the arrows. The filament axis is marked with a cross in each section. Bar, 20 Å.

of tropomyosin relative to actin. X-ray studies of oriented gels of reconstituted thin filaments reveal no differences between the structures of actin/tropomyosin/caldesmon and of actin/tropomyosin at low caldesmon stoichiometries; but at higher stoichiometries, changes in X-ray intensities suggested that caldesmon was bound in a different position from tropomyosin so that the two molecules were diffracting out of phase (Popp and Holmes, 1992). This effect could be due to the COOH-terminal region of caldesmon binding to the outer domain of actin, whereas tropomyosin is close to the inner domain. In our reconstructions, the strands are centered at a radius of ~40–45 Å in both EGTA and CaCM, somewhat greater than the 35–38 Å value determined for tropomyosin on actin in the absence of additional proteins by Popp and Holmes (1992). In the absence of caldesmon, our reconstructions show tropomyosin located about mid-way

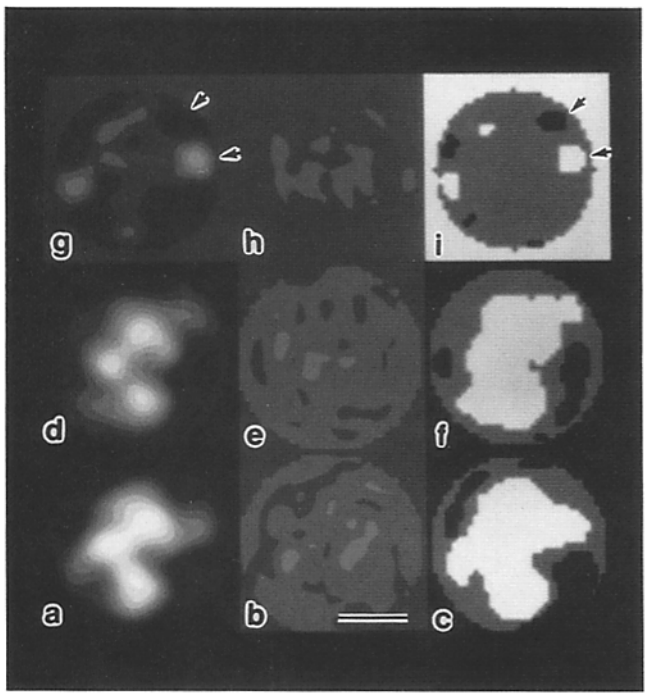

Figure 6. Density maps (at the level z = 13.7 Å in Fig. 5) for (*a*) EGTA-treated filaments and (*d*) CaCM-treated filaments; (*b* and *e*) the standard deviations of the densities at each point in *a* and *d*; (*c* and *f*) the regions of maps *a* and *d* where the density is significantly different from zero at the 99% confidence level; (*g*) the "difference density" when map *d* is subtracted from map *a*; (*h*) the standard errors in the difference map; and (*i*) the regions of the difference map that are statistically significant at the 99.5% confidence level (*white*, significant positive difference; *black*, significant negative difference). Note the pair of positive and negative density peaks (*arrows*) on one side of the filament in *g* and *i*, which reflect the ~20° azimuthal rotation of the "strand" density; another weaker pair is present on the other side. Other minor peaks in *i* may reflect residual errors in the alignment and scaling of the two maps. Bar, 50 Å.

azimuthally between the outer and inner domains of actin (although making closer contact with the outer domain), a position that would be consistent with X-ray diffraction data from oriented actin-tropomyosin gels (Popp and Holmes, 1992).

In the presence of EGTA, caldesmon appears to keep tropomyosin away from the main myosin-binding site known to be on the outer domain of actin (Milligan et al., 1990). The effect of caldesmon on tropomyosin therefore appears to differ from that of troponin, which is thought to constrain tropomyosin (in the absence of Ca^{2+}) to adopt a position on the outer actin domain, where it may interfere sterically with myosin binding. Smooth muscle tropomyosin is thus unlikely to participate in steric hindrance of myosin binding in EGTA. Caldesmon itself has been suggested to inhibit myosin binding by a steric mechanism (Chalovich et al., 1987; Velaz et al., 1990); our reconstructions would be consistent with the idea that caldesmon's COOH-terminal region alone may be responsible for competing with myosin binding to discrete actin molecules occurring on the thin filaments at ~800 Å intervals, but there is no evidence from our work of steric interference by caldesmon over the intervening 13–14 actin molecules (cf. Marston and Redwood, 1993).

Although CaCM lowers the affinity of caldesmon for actin/tropomyosin, and this alone may fully counteract the effect of caldesmon (Sobue et al., 1981), the mechanism of the relief of inhibition by caldesmon in vertebrate smooth muscle in vivo remains uncertain. We have used CaCM to lower caldesmon affinity, and at the low concentrations of caldesmon and thin filaments that we used caldesmon completely dissociated. In intact smooth muscle, protein concentrations are much higher and calmodulin-induced dissociation is not thought to occur in vivo (Clark et al., 1986; Lehman, 1986; Smith et al., 1987); nevertheless the effects associated with the diminished caldesmon affinity for actin and tropomyosin may be responsible for caldesmon disinhibition in vivo, even though dissociation is not complete. Indeed complete dissociation is possible and may well be the mechanism for relieving caldesmon inhibition in non-muscle systems (Yamashiro et al., 1990, 1991; Hosoya et al., 1993). Since we do not know the position of tropomyosin in the presence of CaCM and bound caldesmon, i.e., in the "on" state of the smooth muscle thin filament in vivo, it could differ from the position we have detected in the absence of bound caldesmon. The latter position, however, may be the disinhibited position of tropomyosin that is essential for thin filament action in cytokinesis (Hosoya et al., 1993).

The possible differences in on and off positions of tropomyosin in smooth and skeletal muscle thin filaments may relate to quantitative differences in the enzymatic behavior of the two systems. For example, at low myosin/actin ratios, tropomyosin activates smooth muscle actomyosin ATPase 4–5 times; such an effect is not apparent in skeletal muscle preparations (Chacko et al., 1977; Sobieszek and Small, 1977; Chacko, 1981; Lehrer and Morris, 1984; Williams et al., 1984). This activation is the result of both an increase in V_{max} of ATP hydrolysis and the binding of actin and myosin (Chacko and Eisenberg, 1990), and caldesmon blocks tropomyosin activation. This activation may require a particular steric relationship between tropomyosin and actin, and caldesmon-induced movement of tropomyosin away

Table II. Quantification of Caldesmon Dissociation by Ca^{2+}/Calmodulin

	Relative stain intensity of bands	
	actin/tropomyosin ratio	actin/caldesmon ratio
Isolated thin filaments	2.43 ± 0.09	7.88 ± 0.97
CaCM-treated thin filaments	2.49 ± 0.11	102.9 ± 2.05

Note that calponin is present at a level ~25% of that in whole muscle relative to actin, and that it is not dissociated from the thin filaments by CaCM; in fact, definitive evidence for CaCM binding to calponin that is associated with thin filaments is lacking. The calponin levels present in our filament preparations are tenfold lower than that needed to inhibit actomyosin ATPase.

from this position may prevent activation. Moreover, caldesmon inhibits cooperative potentiation of the interaction of myosin with actin during ATPase activity (Horiuchi and Chacko, 1989). This process is mediated by tropomyosin and is exhibited by both smooth and skeletal actomyosin. Again a preferred association of tropomyosin and actin may be required for potentiation, which could be disrupted by caldesmon-induced movement of tropomyosin. While constraints imposed on tropomyosin by caldesmon may disrupt potentiation, myosin-docking on actin may be unaffected, a process which in turn could give rise to the latch-state observed at low CaCM concentration.

We conclude that the apparent influence of caldesmon on tropomyosin position could in principle explain its ability to modulate actomyosin ATPase and smooth muscle contractility. Fine-tuning of tropomyosin position on actin by third proteins could provide a versatile means for modulating thin filament reactivity. Such mechanisms could underlie the graded responses of contractile elements to a variety of stimuli during smooth muscle contraction, as well as in such cellular processes as cytokinesis, exocytosis, and signal transduction.

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