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The interaction of tropomodulin with tropomyosin stabilizes thin filaments in cardiac myocytes

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Actin (thin) filament length regulation and stability are essential for striated muscle function. To determine the role of the actin filament pointed end capping protein, tropomodulin1 (Tmod1), with tropomyosin, we generated monoclonal antibodies (mAb17 and mAb8) against Tmod1 that specifically disrupted its interaction with tropomyosin in vitro. Microinjection of mAb17 or mAb8 into chick cardiac myocytes caused a dramatic loss of the thin filaments, as revealed by immunofluorescence deconvolution microscopy. Real-time imaging of live myocytes expressing green fluorescent protein–α-tropomyosin and microinjected with mAb17 revealed that the thin filaments depolymerized from their pointed ends. In a thin filament reconstitution assay, stabilization of the filaments before the addition of mAb17 prevented the loss of thin filaments. These studies indicate that the interaction of Tmod1 with tropomyosin is critical for thin filament stability. These data, together with previous studies, indicate that Tmod1 is a multifunctional protein: its actin filament capping activity prevents thin filament elongation, whereas its interaction with tropomyosin prevents thin filament depolymerization.

Introduction

The regulation of actin dynamics in eukaryotic cells is essential for diverse processes including migration, membrane protrusion, cytokinesis, and contraction. Striated muscle has proven to be a powerful model system for investigating actin (thin) filament dynamics because within myofibrils, actin monomers are precisely assembled and maintained in filaments with strikingly uniform lengths. These properties are critical for proper contractile function and appear to involve several mechanisms, including interactions with other sarcomeric proteins.

At the Z-lines, the borders of individual sarcomeres, the barbed (plus or fast-growing) ends of the thin filaments are capped by CapZ; thereby, the thin filaments are prevented from elongating or shortening from their barbed ends. Along the length of the thin filaments, tropomyosin, an α-helical rodlike molecule, forms hetero- and homodimers in a head-to-tail fashion. One well-established role of tropomyosin is to cooperate with the troponin complex in regulating the Ca^{2+}-dependent actomyosin interaction (Huxley, 1969; for review see Cooke, 1997). However, mounting evidence from a number of in vitro studies indicate that tropomyosin also functions to stabilize the thin filaments. Tropomyosin increases filament stiffness, prevents fragmentation and bending, and prevents depolymerization of actin monomers from their pointed (minus or slow-growing) ends (Wegner, 1982; Broschat et al., 1989; Weigt et al., 1990; Adami et al., 2002). Tropomyosin also enhances actin filament assembly and physically protects thin filaments from the depolymerizing effects of ADF/cofilin or gelsolin (Ono and Ono, 2002; Nyakern-Meazza et al., 2002; for review see Cooper, 2002). In vivo studies have revealed a critical role for tropomyosin in proper muscle function. For example, homozygous α-tropomyosin null mice are embryonic lethal, whereas heterozygous knockout mice show no obvious phenotype (Blanchard et al., 1997; Rethinasamy et al., 1998). In *Drosophila*, mutations of one muscle tropomyosin isoform (*Tm1*) result in disruption of peripheral, but not central, myofibrillar organization, as well as alterations in force-generating properties (Kreuz et al., 1996). Studies in *Caenorhabditis elegans* demonstrate that suppression of tropomyosin expression leads to disorganized sarcomeric actin filaments and muscle paralysis (Ono and Ono, 2002). However, the precise mechanisms by which tropomyosin

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contributes to thin filament stability remain unclear, particularly in vertebrate muscle.

Another sarcomeric protein known to be critical for thin filament length regulation is tropomodulin 1 (Tmod1/E-Tmod), which caps the pointed ends of the thin filaments in cardiac muscle cells as well as in other cell types (for review see Weber, 1999). Unlike other actin capping proteins, Tmod1 also binds tropomyosin. Tmod1 completely blocks elongation and depolymerization from the pointed ends of actin filaments in the presence of tropomyosin in vitro ($K_d < 50$ pM). However, in the absence of tropomyosin, Tmod1 has a lower affinity for the pointed ends ($K_d = 0.1$–0.2 μM) and its capping activity is down-regulated >1,000-fold (Weber et al., 1994, 1999). Based on additional biochemical analyses, it was proposed that Tmod1 may also contribute to thin filament length regulation by preventing additional tropomyosin molecules from binding to the pointed ends of the actin filaments, thus acting as a tropomyosin capping molecule (Wegner, 1979; Fowler, 1990; Fowler et al., 1993).

Tmod1's dual interactions with tropomyosin and actin filaments are reflected by its distinct structure. Its COOH-terminal half is compact and tightly folded (Kostyukova et al., 2000). It is this half of the molecule that possesses the primary actin filament capping activity (Fowler et al., 2003). In contrast, Tmod1's NH_2-terminal half is highly flexible and elongated (Kostyukova et al., 2000), and contains overlapping binding sites for both muscle and nonmuscle tropomyosins (Babcock and Fowler, 1994; Vera et al., 2000). Tmod1 also interacts with the extreme NH_2-terminal modules of the giant striated muscle protein, nebulin. Although the significance of this interaction is unknown, it is intriguing to speculate that its role is consistent with nebulin's proposed function as a thin filament ruler that defines their uniform lengths (McElhinny et al., 2001). The existence of several Tmod1-interacting molecules in striated muscle suggests that Tmod1 may play multiple roles in thin filament length regulation.

The physiological roles of Tmod1 in cardiac muscle cells have been investigated in several studies. For example, decreasing levels of endogenous Tmod1 in rat cardiac myocytes resulted in abnormally long thin filaments (Sussman et al., 1998a). In contrast, overexpression of Tmod1 in rat and chick cardiac myocytes, or Sanpodo (a Tmod homologue) in *Drosophila,* resulted in shorter thin filaments (Sussman et al., 1998a; Littlefield et al., 2001; Mardahl-Dumesnil and Fowler, 2001). Transgenic mice overexpressing Tmod1 (TOT) in their myocardium exhibited myofibril disarray and dilated cardiomyopathy (Sussman et al., 1998b), and Tmod1$^{-/-}$ mouse embryos die at approximately day E10, suggesting an essential role for Tmod1 in myofibril assembly (Chu et al., 2003; unpublished data). Therefore, the levels of Tmod1 expression are important in maintaining the lengths of thin filaments and myofibril architecture in vivo.

The functional role of Tmod1's actin filament pointed end capping activity has been investigated directly in studies using primary cultures of chick cardiac myocytes. Microinjection of a monoclonal antibody that specifically blocked Tmod1's actin capping activity (but not its interaction with tropomyosin), resulted in an abnormal elongation of the actin filaments from their pointed ends and abolished contractile activity (Gregorio et al., 1995). Thus, Tmod1's actin filament capping activity is required to maintain the lengths of the mature thin filaments in cardiac muscle. Interestingly, in these studies, Tmod1 and tropomyosin remained associated with the thin filaments. Therefore, it has remained unclear what exactly is the function of Tmod1–tropomyosin interactions in thin filament length regulation.

Here, we sought to investigate the functional properties of the interaction of Tmod1 with tropomyosin on thin filament length and stability in cardiac muscle. We determined that blocking the interaction of Tmod1 with tropomyosin in live cardiac myocytes resulted in a dramatic loss of thin filaments and subsequent contractile activity. The disappearance of thin filaments was visualized in real time and occurred from their pointed ends toward the Z-line. Our data indicate that the actin- and tropomyosin-binding activities of Tmod1 have unique and complementary functional roles. The actin capping activity of Tmod1 inhibits actin elongation and maintains the lengths of the thin filaments (Gregorio et al., 1995), whereas the tropomyosin-binding domain of Tmod1 appears to stabilize the thin filaments by preventing depolymerization from their pointed ends. These studies indicate that the regulated activity of Tmod1 is essential for proper muscle function.

Results

Two anti-Tmod1 monoclonal antibodies specifically recognize the NH_2-terminal region of Tmod1 and perturb its interaction with tropomyosin

To study the functional significance of the interaction of Tmod1 with tropomyosin, we first sought to identify monoclonal anti-Tmod1 antibodies that disrupted the interaction of the molecules in vitro. Previous work showed that the extreme NH_2-terminal end of Tmod1 (amino acids 6–34) is essential for binding to skeletal muscle tropomyosin (Babcock and Fowler, 1994). We generated two monoclonal antibodies to chicken Tmod1 (mAb8 and mAb17) that specifically recognized the NH_2-terminal end of Tmod1 by Western blot analysis (Fig. 1 b). Both antibodies recognized full-length Tmod1 (Fig. 1 b, lane 1), and recombinant Tmod1 fragments including residues 6–184, 6–94 and 6–57, but failed to recognize fragments including residues 35–359 and 95–359 (data shown for mAb17). These results demonstrated that mAb17 and mAb8 recognize epitopes within the NH_2-terminal residues 6–34 of Tmod1, which contain the skeletal muscle tropomyosin binding site. To demonstrate the specificity of these antibodies, Western blot analyses of embryonic chick cardiac myocyte extracts were performed, revealing that mAb17 and mAb8 specifically recognized a single 40-kD polypeptide, corresponding to the molecular mass of Tmod1 (Fig. 1 c).

To determine whether mAb17 and mAb8 inhibited tropomyosin binding to Tmod1, full-length recombinant chick Tmod1 (25 nM) was absorbed onto nitrocellulose dots. The dots were incubated with increasing concentra-

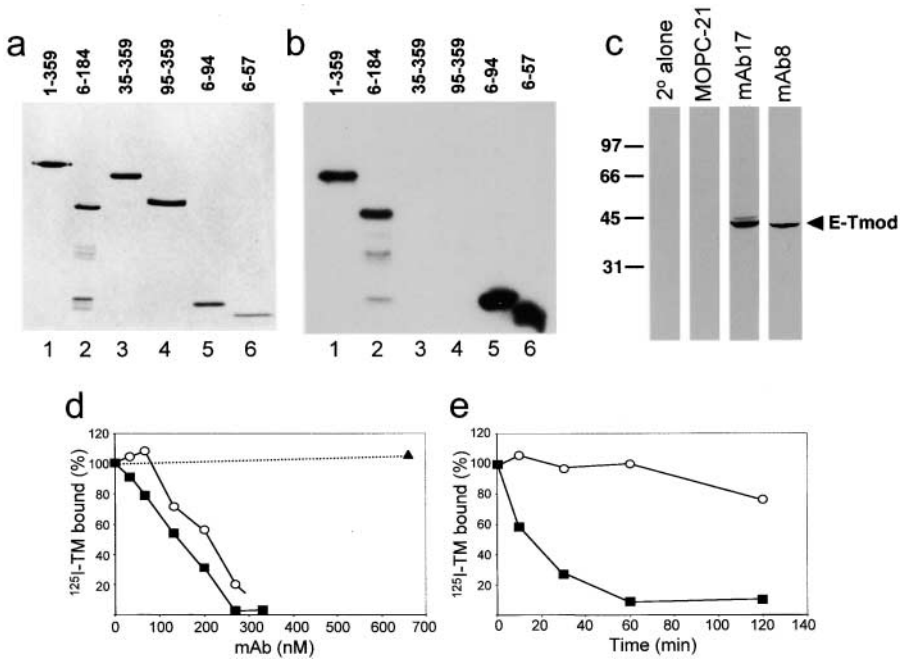

Figure 1. **mAb17 and mAb8 specifically bind to the NH_2-terminal Tmod1 residues 6–57, recognize Tmod1 in embryonic chick heart lysates, and disrupt its interaction with tropomyosin.** (a) Coomassie blue–stained gel of full-length Tmod1 (lane 1,1–359) and Tmod1 fragments (lanes 2–6, residues 6–184, 35–359, 95–359, 6–94, and 6–5, respectively). (b) Western blots of samples in panel a were probed with mAb17 that recognized full-length (lane 1) and NH_2-terminal Tmod1 (lanes 2, 5, and 6), but not COOH-terminal Tmod1 (lanes 3 and 4). (c) Western blots of embryonic heart extracts were probed with secondary antibodies alone (2° alone), MOPC-21, mAb17, or mAb8. mAb8 and mAb17 detected a band at ~40 kD, corresponding to Tmod1. (d) Tmod1 nitrocellulose dots were preincubated with mAb8, mAb17, or mAb9, followed by incubation with ^{125}I-tropomyosin (^{125}I-TM). Preincubating the Tmod1 dots with increasing concentrations of mAb17 (closed squares) or mAb8 (open circles) resulted in a dramatic decrease in the percentage of tropomyosin that bound to Tmod1, as compared with mAb9 which did not disrupt the interaction (closed triangle). (e) Tmod1 dots were incubated with ^{125}I-tropomyosin (^{125}I-TM), followed by incubation with mAb8 (closed squares), or MOPC21 (open circles) for varying periods of time. Addition of mAb8 resulted in a significant dissociation of tropomyosin from Tmod1.

tions (0–700 nM) of mAb8 or mAb17 before the addition of ^{125}I-labeled skeletal muscle tropomyosin. This experiment demonstrated that a 10-fold molar excess over Tmod1 of either mAb17 (open circles) or mAb8 (closed squares) completely abolished ^{125}I-tropomyosin binding to Tmod1 (Fig. 1 d). In contrast, a 28-fold molar excess of mAb9 (closed triangle), an antibody that recognizes Tmod1's COOH-terminal end and blocks its actin filament capping activity (Gregorio et al., 1995), had no effect on ^{125}I-tropomyosin binding to Tmod1 (Fig. 1 d). To test whether mAb17 and mAb8 would also promote the dissociation of ^{125}I-tropomyosin from Tmod1, nitrocellulose dots of Tmod1 were preincubated with ^{125}I-tropomyosin followed by incubation with a 13-fold molar excess of those antibodies with respect to Tmod1. Both mAb17 and mAb8 promoted dissociation of tropomyosin from Tmod1 within 60 min (Fig. 1 e, data shown for mAb8, closed squares). In contrast, addition of equivalent molar ratios of MOPC-21 did not result in a significant dissociation of tropomyosin from Tmod1 (open circles). Fab fragments of mAb8 also inhibited the interaction of Tmod1 with tropomyosin indicating that this effect was not caused by cross-linking Tmod1 by the bivalent antibody (unpublished data). These results demonstrated that both mAb17 and mAb8 specifically disrupted the interaction of Tmod1 with tropomyosin in vitro.

Disrupting the interaction of Tmod1 with tropomyosin in live cardiac myocytes results in a reversible disassembly of thin filaments and cessation of beating

We next used mAb17 and mAb8 in microinjection studies designed to investigate the interaction of Tmod1 with tropomyosin in the context of living cardiac myocytes. Day 3–5 cardiac myocytes were microinjected with mAb17 or mAb8 and incubated for 1 h after injection. The myocytes were fixed and stained for thin filament components. A few cells microinjected with mAb17 or mAb8 demonstrated a thin filament pointed end striated staining pattern for Tmod1 and unperturbed actin filaments (unpublished data). However, the majority (>80%) of myocytes injected with mAb17 (Fig. 2 c) or mAb8 (Fig. 2 e) exhibited a dramatic loss of actin filaments, as determined by staining with fluorescently conjugated phalloidin (Fig. 2, d and f), compared with the normal striated appearance of sarcomeric actin staining in cells injected with the control antibody MOPC-21 (Fig. 2 b) or in surrounding uninjected cells (Fig. 2 d, bottom). Additionally, staining with anti–cardiac actin antibodies demonstrated that the absence of actin staining was not due to an artifact from inhibition of phalloidin staining (Fig. 2 j). Notably, no Tmod1 striations were detected (i.e., Tmod appeared diffused in the cytoplasm) in the cells microinjected with mAb17 or mAb8, suggesting that disrupting the interaction of Tmod1 with tropomyosin promoted Tmod1's dissociation from thin filament pointed ends (Fig. 2, c and e). In the majority of cells (70–75%) perturbed by the microinjection of mAb17 or mAb8, no actin filaments were detected (not depicted), whereas in other cells remnants remained (Fig. 2, d, f, and j). Cells injected with Fab fragments of mAb17 exhibited an identical phenotype, indicating that the loss of sarcomeric actin filament staining was not due to Tmod1 cross-linking or sequestration in the cells (Fig. 2, i and j). The effect of mAb8 and mAb17 was also not due to nonspecific effects of introducing any anti-Tmod1 monoclonal antibody into cardiac myo-

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cytes. Microinjection of mAb95, another anti-Tmod1 antibody that recognizes an epitope close to the middle of the molecule (unpublished data), or microinjection of mAb9, that recognizes the COOH-terminal region of Tmod1 and disrupts its capping activity, did not result in the loss of actin filament striations (Fig. 2, g and h; Gregorio et al., 1995). In additional control experiments to examine for any nonspecific effects on actin filaments, we found that microinjection of mAb17 into fibroblasts that contain actin stress fibers but no detectable Tmod1 (Gregorio and Fowler, 1995) resulted in no observable effects on actin staining (Fig. 2, k and l).

To determine whether injection of the function-blocking antibodies had toxic effects on the myocytes, we tested whether the phenotype was reversible by culturing the microinjected cells for longer time intervals to allow for potential degradation of the mAb17 Fabs and/or sequestration of antibodies by newly synthesized Tmod1 before fixation. In this experiment, cardiac myocytes plated on coverslips with numbered grids (to identify injected cells) were injected with mAb17 Fabs, incubated 24–48 h, fixed, and viewed. Notably, at these later time points, a partial reappearance of actin filament striations was clearly seen at 24 h. By 48 h, a full recovery was observed in all cells that had been injected with mAb17 Fabs, but had no detectable staining for the mAbs (Fig. 2 m). These data indicate that the loss of sarcomeric actin filaments was reversible.

Next, to determine whether mAb17-induced Tmod1 and actin disassembly was accompanied by disassembly of tropomyosin, cells microinjected with mAb17 Fabs were stained for tropomyosin. A loss of tropomyosin staining was clearly observed, which is consistent with the loss of actin filaments (see Fig. 4 b), indicating that the entire thin filament had disassembled. This result suggested that the interaction of Tmod1 with tropomyosin is necessary to stabilize the association of tropomyosin with the actin filaments in cardiac myocyte sarcomeres.

As a complementary approach to using anti-Tmod1 antibodies to disrupt Tmod1's interaction with tropomyosin, we microinjected a recombinant NH_2-terminal fragment of Tmod1 (1–130) into cardiac myocytes, containing the tropomyosin binding site (Babcock and Fowler, 1994; Greenfield and Fowler, 2002). Again, a loss of actin filaments was observed (Fig. 2 o), likely due to a dominant-negative mechanism; that is, the injected Tmod1 fragment containing amino acids 1–130, likely prevented endogenous Tmod1 from binding to endogenous tropomyosin. These experiments support the hypothesis that the interaction of Tmod1 with tropomyosin is critical for actin filament stability.

The dramatic disappearance of thin filaments in microinjected cells suggested that contractile activity of the cells would be inhibited. Indeed, beating activity was dramatically diminished in cells microinjected with mAb17. Only ~5% of those myocytes injected with mAb17 were observed to beat 1 h after injection, compared with ~88% of those cells injected with MOPC-21 (Table I). 100% of the uninjected cells that were beating at the onset of the experiment

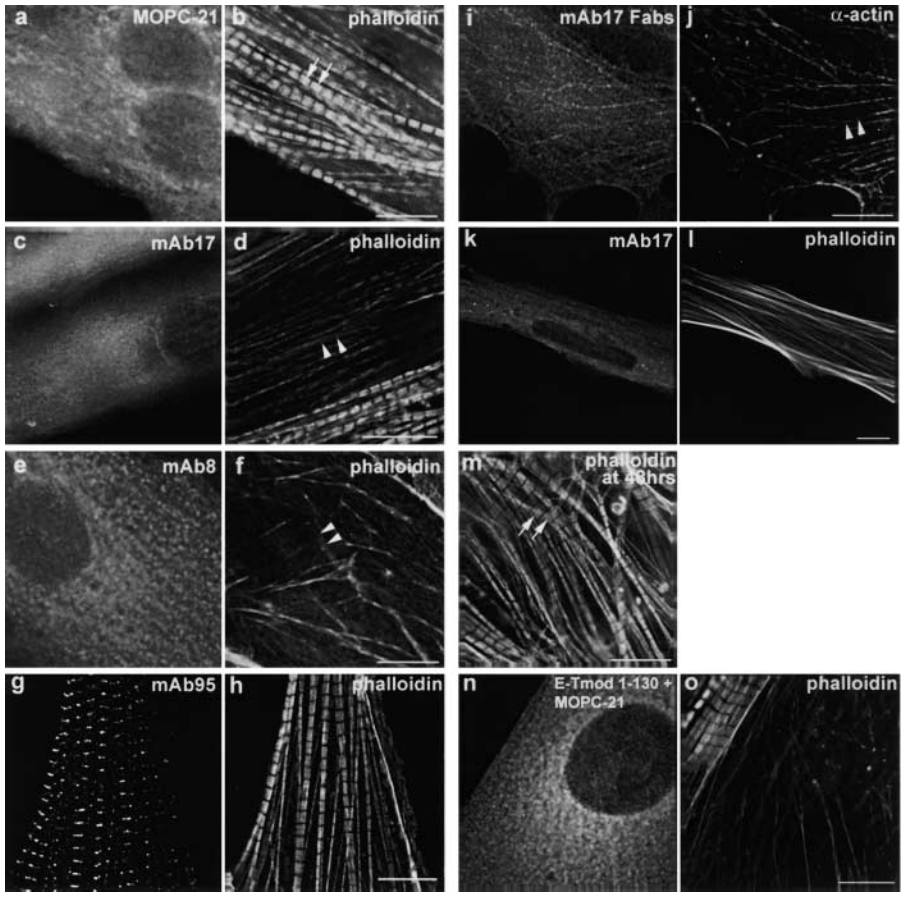

Figure 2. **Disruption of the interaction between Tmod1 and tropomyosin in live cardiac myocytes results in a loss of actin (thin) filaments.** Cardiac myocytes were microinjected with MOPC-21 (a and b), mAb17 (c and d), mAb17 Fab fragments (i and j), mAb8 (e and f), or mAb95 (g and h). Injected cells were observed using an AlexaFluor 594–conjugated anti–mouse IgG to detect the injected antibody (a, c, and e). Microinjection of mAb17 or mAb8 resulted in a loss of actin filaments as detected by AlexaFluor 488 phalloidin (d and f, arrowheads) or using anti–α-actin antibodies (j, arrowheads). Normal actin filaments were seen in myocytes injected with MOPC-21 (b, arrows) or with mAb95 (h). Fibroblasts were also microinjected with mAb17 (k and l); no perturbation of actin filaments was seen (l). To test for recovery, injected cells were incubated for 48 h before staining. Actin filaments were easily visualized at this time point (m, arrows). A recombinant Tmod1 fragment containing residues 1–130, mixed with MOPC-21 (n, to identify injected cells) was microinjected into cardiac myocytes and incubated for 1 h. Microinjection of the Tmod1 fragment containing the tropomyosin binding site resulted in a loss of actin filaments (o). Bars, 10 μm.

Table I. **mAb17 inhibits the contractile activity of chick cardiac myocyte**

mAb	No. of injected myocytes	Percentage of cells beating
MOPC-21	251	87.8 ± 3.0
17	255	5.6 ± 5.1[a]

[a]$P < 0.001$ by *t* test.

remained beating after 1 h (unpublished data). In summary, these data suggest that the Tmod1–tropomyosin interaction is critical for thin filament stability and for contractile activity in cardiac myocytes.

Thin filaments depolymerize from their pointed ends in live cardiac myocytes

The data presented thus far indicated that thin filament stability was compromised in cells in which the interaction of Tmod1 with tropomyosin was disrupted. To address the mechanism of how this disassembly occurred, we collected time-lapse images of cells expressing GFP–α-tropomyosin that were microinjected with mAb17. GFP–α-tropomyosin assembled along the entire length of the thin filaments in all myocytes where it was expressed (Helfman et al., 1999). After injection with mAb17, striated GFP–α-tropomyosin was observed to dissociate directly from the pointed ends of the thin filaments in live cells (i.e., the GFP–α-tropomyosin detectable at the Z-lines remained at similar intensities, whereas the intensity from the pointed ends greatly diminished). This dissociation from the pointed ends was first noticeable at 30 min, with progressive dissociation only from the pointed ends up to 60 min (Fig. 3); i.e., no detectable GFP–α-tropomyosin intensity was lost from the sides of the filaments or from the Z-lines. In contrast, injection of MOPC-21 had no effect on the fluorescence intensity of GFP–α-tropomyosin. Interestingly, the depolymerization effect on the thin filaments appeared to occur with slower kinetics than that observed in myocytes injected with mAb17, fixed, and then stained. We speculate that this slower depolymerization effect was due to overexpression of α-tropomyosin in myocytes that typically do not have a considerable pool of soluble tropomyosin (Gregorio and Fowler, 1995) and/or alterations in the stoichiometry of tropomyosin isoform expression that may have further stabilized the thin filaments. The results from this experiment directly show that the loss of thin filaments is due to the depolymerization of the filaments from their pointed ends in cells. Furthermore, this observation also suggests that Tmod1 acts as a "cap" to stabilize tropomyosin's association with the actin filament pointed ends (Fowler et al., 1993), functioning with tropomyosin to prevent actin filament depolymerization.

Thin filament disassembly is accompanied by perturbation of Z-lines, thick filaments, and titin filaments

To determine the effect that mAb17-induced thin filament disassembly had on overall sarcomere organization, cells microinjected with MOPC-21 Fabs or mAb17 Fabs were costained using AlexaFluor 488 phalloidin, together with antibodies against α-actinin, the major Z-line component; myosin, the major thick filament component; as well as I-band and M-line epitopes of titin. In cells injected with mAb17 Fabs, α-actinin (Fig. 4 d, red), myosin (Fig. 4 f, red), and titin N2A and A168–170 epitopes (Fig. 4, h and j, red), appeared disrupted along with the loss of actin filaments (Fig. 4, d, f, h, and j, green). However, in some regions, closely spaced remnants of a periodic striated staining pattern for α-actinin, titin, and myosin were observed clearly in the absence of actin (Fig. 4, arrowheads). To confirm this observation, cells injected with mAb17 Fabs (Fig. 4 l) were triple labeled for actin (green), α-actinin (red), and M-line titin (blue). In some cells injected with mAb17 Fabs, an alternating staining pattern of α-actinin and M-line titin (corresponding to alternating Z- and M-lines), was observed in the absence of actin filaments in the identical myofibrils. These data support previous studies that revealed that the organization of thick filaments, as well as other sarcomeric components, remained relatively intact in the absence of thin filaments (von Arx et al., 1995; Linke et al., 1999). Therefore, the perturbation of other sarcomeric filament sys-

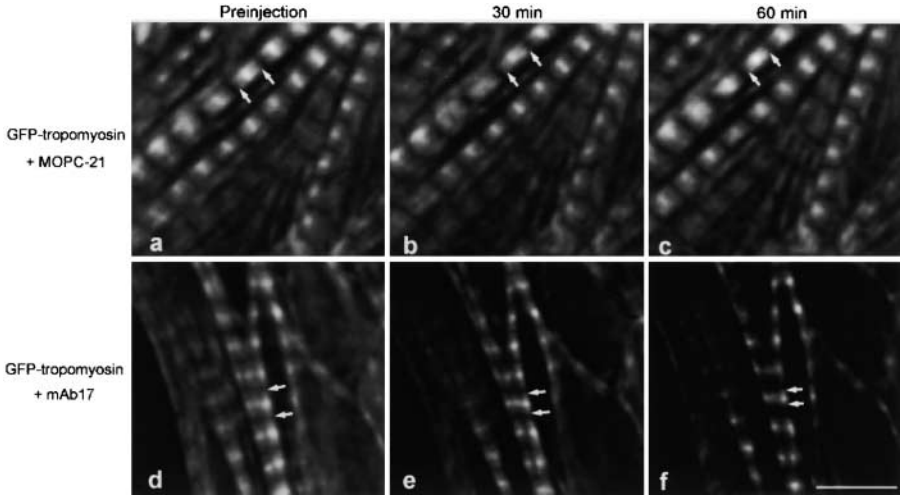

Figure 3. **Depolymerization of thin filaments from their pointed ends was visualized in live cells expressing GFP–α-tropomyosin and microinjected with mAb17.** Cardiac myocytes expressing GFP–α-tropomyosin were microinjected with MOPC-21 (a–c) or mAb17 (d–f). Images were recorded every 15 min for 1 h (data shown for preinjection, 30 and 60 min). After 30 and 60 min, cells injected with mAb17 displayed thin filament shortening from their pointed ends (a–f, arrows). No depolymerization was observed in cells microinjected with MOPC-21. Bar, 5 μm.

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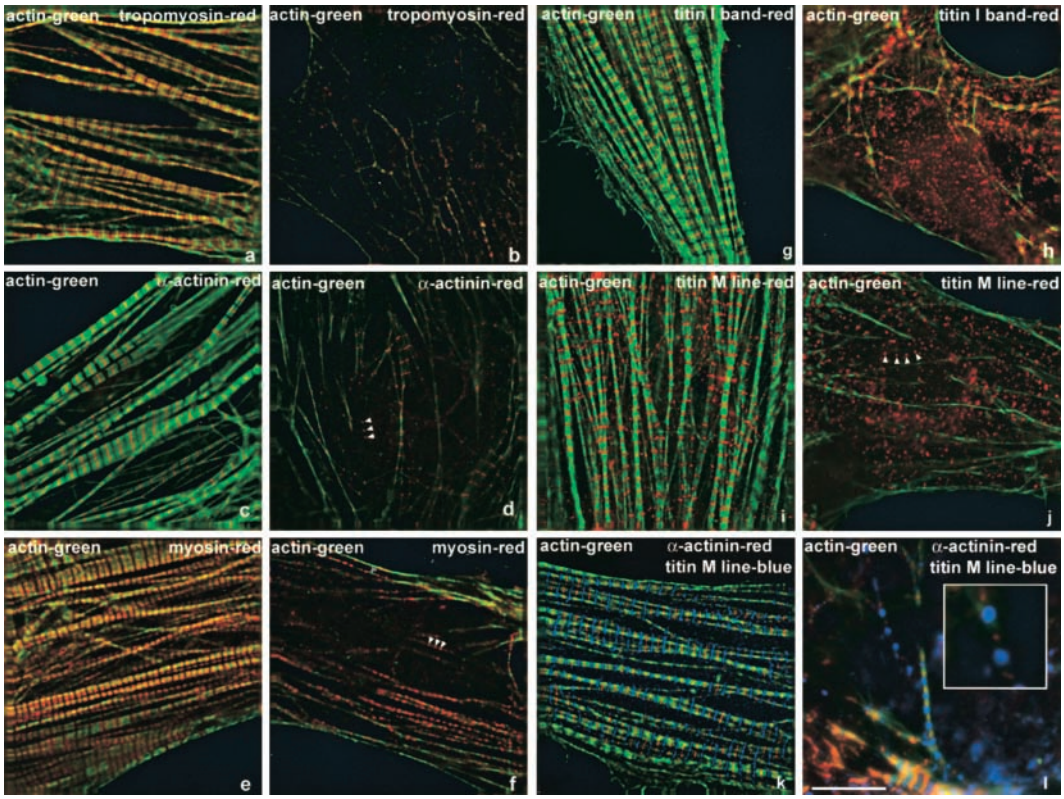

Figure 4. **Disrupting the interaction between Tmod and tropomyosin in live cardiac myocytes results in a perturbation of other sarcomeric components.** Cardiac myocytes were microinjected with MOPC-21 Fabs (a, c, e, g, i, and k) or mAb17 Fabs (b, d, f, h, j, and l), incubated for 1 h, fixed, and stained for tropomyosin (a and b, red), α-actinin (c, d, k, and l, red), myosin (e and f, red), I-band titin (g and h, red) or M-line titin (i and j, red; k and l, blue). Myocytes injected with mAb17 Fabs showed a loss of tropomyosin consistent with the loss of actin filaments (b). Myocytes injected with mAb17 or mAb17 Fabs displayed a perturbed staining pattern for α-actinin (d), myosin (f), and titin (h and j). Strikingly, myocytes injected with mAb17 Fabs displayed alternating M-line titin (l, blue) and α-actinin (l, red) staining in the absence of detectable actin (l, green). (inset) Enlarged view of l. (arrowheads) Closely spaced remnants of striated staining patterns. Bar, 10 μm.

tems, as well as Z-lines, was likely secondary to the loss of the thin filaments observed.

Stabilization of actin filaments in a thin filament reconstitution assay, before the disruption of the interaction of Tmod1 with tropomyosin, prevents thin filament depolymerization

To gain additional insights into the mechanism of the thin filament disruption phenotype, we used a thin filament reconstitution assay. This assay allows us to selectively study the assembly properties of individual thin filament components (in the absence of thick filament components), as well as interfere with specific thin filament protein interactions. Using this assay, we previously demonstrated that binding of Tmod1 to the pointed ends of actin filaments required the prior reconstitution of tropomyosin along the actin filaments in "ghost myofibrils" (Gregorio and Fowler, 1995). In brief, ghost myofibrils were prepared by permeabilizing cardiac myocytes with saponin and extracting them in 0.5 M KCl. Under these conditions, many sarcomeric components including the thick filaments (Fig. 5 a) and the thin filament components, tropomyosin (Fig. 5 b) and Tmod1 (Fig. 5 c), were removed (Gregorio and Fowler, 1995), as shown by a lack of immunofluorescent detection. Actin filaments (Fig. 5, d and g), as well as the major Z-line component α-actinin, and titin filaments remained intact in the extracted cells (unpublished data). Purified tropomyosin (Fig. 5 e) and recombinant Tmod1 (Fig. 5 f) were reconstituted on the existing actin filaments (Fig. 5 g) and assembled in their typical sarcomeric distributions. In fact, actin filaments alone, or those with tropomyosin plus Tmod alone, were stable for >60 min at room temperature (unpublished data). Remarkably, the addition of exogenous tropomyosin followed by a mixture of recombinant Tmod1 with mAb17 (Fig. 5, l–o), or Tmod1 with mAb8 (Fig. 5, p–s), resulted in a dramatic loss of actin filaments (Fig. 5, m and q, respectively): a phenotype that appeared identical to that observed in intact cells. In contrast, reconstitution of tropomyosin followed by the addition of a mixture of Tmod1 with MOPC-21 (Fig. 5, h–k), the addition of mAb8 in the absence of Tmod1 (Fig. 5, t–v), or the addition of mAb8 with Tmod1 in the absence of reconstituted tropomyosin (unpublished data) had no effect on the actin filaments. These data indicate that once the actin–tropomyosin filaments are capped by Tmod1, the interaction between Tmod1 and tropomyosin must be intact to prevent depolymerization of the thin filaments (see Discussion).

To determine if stabilizing the actin filaments before addition of mAb17 or mAb8 with Tmod1 would affect

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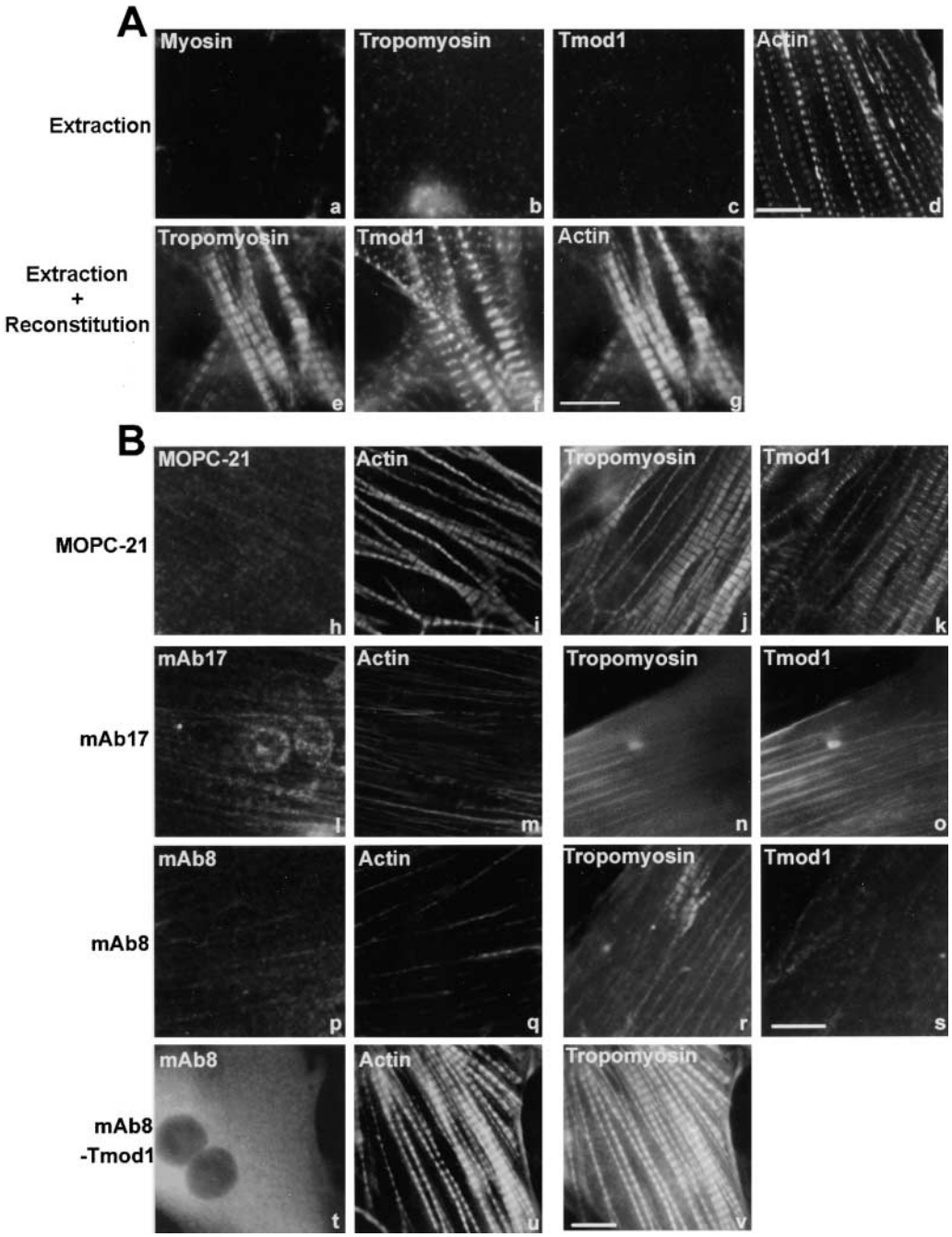

Figure 5. **The disruption of the Tmod1–tropomyosin interaction in permeabilized and extracted cardiac myocytes results in the loss of actin filaments.** (A) Cardiac myocytes permeabilized with saponin and extracted in 0.5 M KCl were stained for myosin (a), tropomyosin (b), or Tmod1 (c): all three sarcomeric components were extracted, whereas the actin filaments were unaffected (c and d, costained). Biotinylated tropomyosin (e) and recombinant Tmod1 (f) were reconstituted onto the ghost myofibrils (g). (B) Cardiac myocytes extracted and reconstituted with biotinylated tropomyosin followed by a mixture of Tmod1 with MOPC-21 (h–k) showed no disruption of the striated actin filaments (i), tropomyosin (j), and Tmod1 (k) upon addition of MOPC-21 (h and i; j and k, both costained). In contrast, the addition of mAb17 (l–o) or mAb8 (p–s) resulted in the loss of actin filaments (m and q), tropomyosin (n and r), and Tmod1 (o and s) (n and o; r and s, both costained). The addition of mAb8 (t) in the absence of recombinant Tmod1 had no effect on the actin filaments (u) or on tropomyosin (v). Bars, 10 μm.

the loss of thin filaments, actin filaments were first stabilized with jasplakinolide or phalloidin, before the reconstitution of tropomyosin and Tmod1 (Fig. 6, data shown for phalloidin treatment). Strikingly, both tropomyosin (Fig. 6, c, g, and k) and Tmod1 (Fig. 6, d, h, and l) remained striated along the stabilized myofibrils after the addition of the Tmod1–mAb17 mixture (Fig. 6 e) or Tmod1–mAb8 mixture (Fig. 6 i). Thus, stabilization of the actin filaments via phalloidin or jasplakinolide, before disruption of the interaction of Tmod1 and tropomyosin, was sufficient to prevent thin filament depolymerization. These data from the reconstitution assay also suggest that the thin filament disassembly phenotype observed is a direct effect of disrupting the interaction of Tmod1 with

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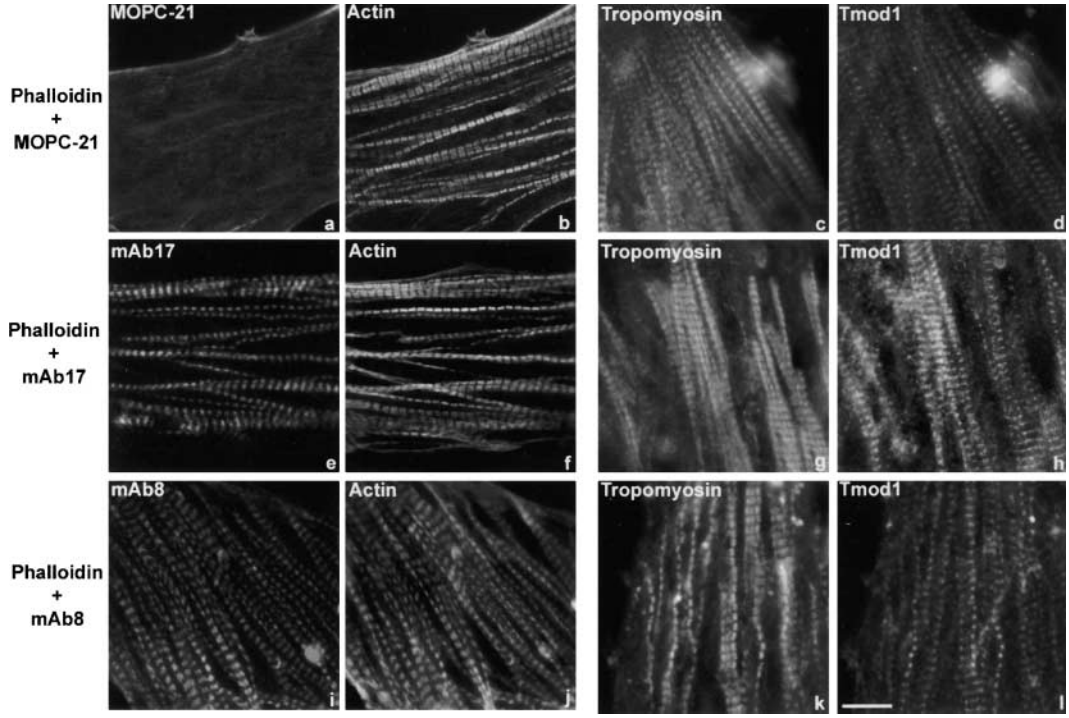

Figure 6. **Stabilization of thin filaments by phalloidin before addition of mAb17 or mAb8 prevents thin filament disruption.** Cardiac myocytes were extracted and actin filaments were stabilized with AlexaFluor 488 phalloidin before the addition of tropomyosin and Tmod1. MOPC-21 was added to the myocytes (a–d) and had no apparent effect on actin filaments (b), tropomyosin (c), and Tmod1 (d) (a and b; c and d, both costained). Notably, the addition of mAb17 (e–h) or mAb8 (i–l) along with the purified Tmod1 had no effect on actin filaments (f and j), tropomyosin (g and k), and Tmod1 (h and l), which were stabilized by phalloidin (e,f; i,j; g,h; k,l, all costained). Bar, 10 μm.

tropomyosin and is likely not influenced by other soluble, regulatory, or sarcomeric components.

Discussion

In this work, we specifically blocked the interaction of Tmod1 with one of its three known binding partners, tropomyosin, in live chick cardiac myocytes. We disrupted this interaction by microinjecting function-blocking antibodies against the NH_2-terminal region of Tmod1 and by microinjecting a recombinant NH_2-terminal Tmod1 fragment containing the tropomyosin binding site. These approaches resulted in a striking phenotype: a loss of thin filaments and cessation of contractile activity. To further investigate the mechanism of this observed phenotype, we transfected myocytes with GFP–α-tropomyosin, which assembled along the lengths of the thin filaments, and then microinjected these cells with mAb17. This live cell imaging strategy allowed us to visualize the loss of thin filaments in real time, revealing that the thin filaments depolymerized progressively from their pointed ends. Results from a cell permeabilization assay further revealed that the loss of thin filaments, upon perturbation of the interaction of Tmod1 with tropomyosin, is prevented by the prior stabilization of the actin filaments with phalloidin or jasplakinolide. These observations reveal exciting, novel functions for both tropomyosin and Tmod1. For tropomyosin, our study provides direct support for its proposed role as a thin filament stabilizing component in vertebrate muscle. For Tmod1, we found that its two distinct structural and ligand-binding regions, the NH_2-terminal tropomyosin-binding domain and COOH-terminal actin capping domain, have unique roles at the pointed ends of the thin filaments that can be distinguished in the context of live myocytes. The interaction of Tmod1 with tropomyosin at the pointed ends is critical for maintaining the integrity of thin filaments, by preventing their depolymerization in live cardiac myocytes (Fig. 7, I–III). The phenotype we observed is strikingly different from the phenotype obtained from inhibiting the interaction of Tmod1 with actin, which revealed that Tmod1's capping activity is required to prevent abnormal elongation of the thin filaments from their pointed ends (Gregorio et al., 1995) (Fig. 7, IV–VI). Together, we conclude that Tmod1 is a multifunctional protein in cardiac muscle, involved in maintaining the lengths and stability of the thin filaments.

The exact mechanisms by which Tmod1–tropomyosin interactions are critical for thin filament stability are puzzling based on many observations, mainly from in vitro studies, that show that tropomyosin–coated actin filaments are stable in the absence of Tmod1. For example, actin can polymerize into long, stable filaments in the absence of tropomyosin and Tmod1; and actin filaments saturated with tropomyosin (but lacking Tmod1) depolymerize more slowly from the pointed ends compared with naked actin filaments. However, the ability of tropomyosin alone to prevent actin depolymerization from the pointed ends in vitro depends on the presence of a considerable excess of free tropomyosin over the amount necessary to coat the actin filaments (Broschat, 1990; Weber et al., 1994, 1999). This is because binding of the terminal tropomyosin molecule at

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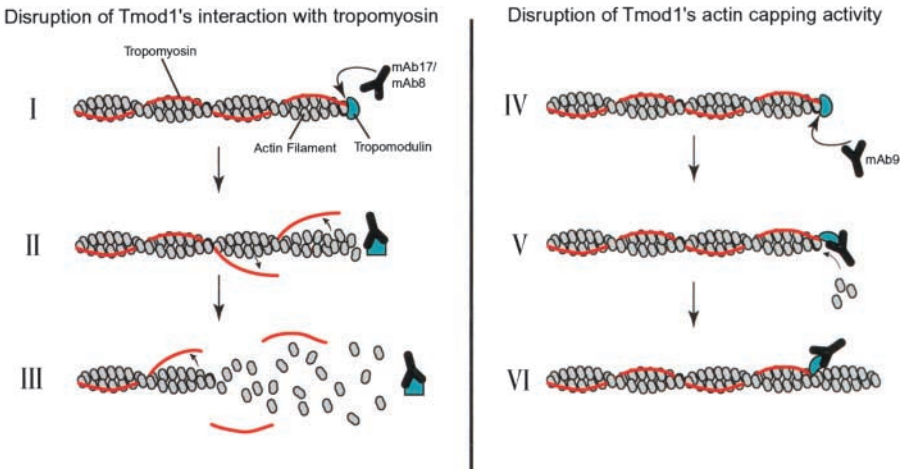

Figure 7. **Model of two identified functional domains of Tmod1.** (I and IV) Stable thin filaments are comprised of actin (gray), tropomyosin (red), and Tmod1 (blue cap). (II) Disrupting the interaction of Tmod1 and tropomyosin (red) with mAb17 or mAb8 (black) appears to alter the functional properties of Tmod1 (blue square), which imparts instability at the pointed ends of the thin filaments. Note, the shape change is meant to indicate that Tmod1 acquires a new functionality, but the exact alteration is unknown. (III) The conferred instability results in a loss of thin filaments via depolymerization from the pointed ends. (V) In contrast, disrupting Tmod1's actin capping activity with mAb9 results in an elongation of actin filaments at the pointed ends (VI). These results suggest at least two functional roles for Tmod1: stabilizing/preventing depolymerization of thin filaments through its interaction with tropomyosin, and inhibiting elongation/maintenance of actin filaments lengths through its actin capping activity.

the pointed filament end is significantly weaker than that of the other tropomyosin molecules, which self-associate with neighboring tropomyosins along the thin filaments (Wegner, 1979; Weber et al., 1999). In contrast, actin filaments coated with tropomyosin and capped by Tmod1 at their pointed ends are significantly more stable because Tmod1 enhances the ability of tropomyosin to reduce pointed end depolymerization (Weber et al., 1994, 1999). Thus, Tmod1 may specifically promote stronger binding of the terminal tropomyosin molecule to the thin filaments by virtue of its ability to bind both actin and tropomyosin at the pointed ends (Weber et al., 1999). Because there is essentially no excess free tropomyosin in chick cardiac myocytes (Gregorio and Fowler, 1995) (i.e., all the available tropomyosin is assembled onto the thin filaments and as a result, tropomyosin concentrations are limiting), we speculate that Tmod1 interactions with tropomyosin may be essential to anchor the terminal tropomyosin molecules to the thin filament pointed ends in vivo. Thus, specific perturbation of Tmod1–tropomyosin interactions in cardiac myocytes may lead to weakened binding of the terminal tropomyosin molecule at thin filament pointed ends followed by cooperative dissociation or "unzipping" of the entire tropomyosin polymer from the thin filaments. This would be followed by actin filament depolymerization, as we observed when Tmod1–tropomyosin interactions were inhibited by mAb8 or mAb17. In this model, Tmod1 functions as a cap for both the terminal tropomyosin and actin molecules, thereby affecting pointed end stability, as well as tropomyosin and actin dynamics.

Although this mechanism is an attractive model, it does not fully account for a surprising result from our cardiac myocyte thin filament reconstitution assays. Actin filaments appear to be relatively stable in extracted cells (myocyte ghosts) in the absence (or presence) of tropomyosin and Tmod1; however, the addition of the anti–NH_2-terminal Tmod1 antibodies mAb8 or mAb17 appear to transform Tmod1 (Fig. 7 III) such that it catalytically imparts instability on what would otherwise be a relatively stable structure. In other words, in these experiments, Tmod1 plus mAb8 or mAb17 appears to behave like a "depolymerase" for tropomyosin–actin filaments. Perhaps the binding of these antibodies leads to a structural alteration in Tmod1 so that it now actively disrupts actin–actin or actin–tropomyosin interactions. Alternatively, such an altered Tmod1 may interact differently with the giant protein nebulin (see Discussion), causing a propagated destabilization down the length of the filament, resulting in its disassembly. It is also plausible that a structural alteration occurs in the thin filament once Tmod1 has associated with the pointed ends, causing the filaments to become unstable if Tmod1–tropomyosin interactions are perturbed. Our current experiments are addressing these intriguing issues.

An interesting, yet complex, question that arises is, how does Tmod1 function with tropomyosin in stabilizing the thin filaments given the fact that the pointed end components are dynamic? GFP-Tmod1 and fluorescently labeled actin monomers rapidly associate and dissociate from the pointed ends in chick embryonic cardiac myocytes (Littlefield et al., 2001). Additionally, epitope-tagged tropomyosin molecules, but not epitope-tagged troponin I molecules, preferentially assemble onto the pointed ends of the thin filaments in adult rat cardiac myocytes (Michele et al., 1999). These studies and our results indicate that the slow-growing, pointed ends of the thin filaments are not only dynamic with respect to the rapid exchange of actin, tropomyosin, and Tmod1 but are the critical site for both length regulation and overall stability of the thin filaments. Exactly how this occurs remains to be determined.

Although Tmod1 is a critical component for maintaining and stabilizing thin filament lengths, there is no evidence that Tmod1 is involved in specifying their lengths. However, several groups have identified nebulin, the Tmod1 and tropomyosin-binding protein, as the prime candidate molecule for functioning as a "ruler" to specify the precise lengths of the thin filaments in skeletal muscle and, more recently, in cardiac muscle (Fock and Hinssen, 2002; Kazmierski et al., 2003; for review see McElhinny et al., 2003). In support of this hypothesis, nebulin participates in the initial stages of assembly of I-Z-I bodies, precursor structures that mature

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into definitive Z-lines and I-bands (Ojima et al., 1999). Additionally, in many studies nebulin is observed in a striated pattern before the thin filaments attain their mature lengths, which is consistent with the idea that nebulin dictates thin filament architecture and restricts filament lengths (Shimada et al., 1996; Moncman and Wang, 1996). Nebulin may also act with tropomyosin as thin filament stabilizers during myofibril assembly. Importantly, recent reports have suggested a critical role for tropomyosin in myofibril assembly. Specifically, genetic analysis of the Unc-60B (homologue of cofilin/ADF) mutant in *C. elegans* (Ono and Ono, 2002) indicated that actin filament assembly depends on a balance between actin stabilization by tropomyosin and actin disassembly mediated by cofilin/ADF. Tropomyosin has also been implicated in playing an important role in zebrafish myofibrillogenesis based on analyses of cardiac troponin T (TNNT2) mutants; the loss of Tnnt2 expression in silent heart (sih) mutants resulted in a significant reduction in tropomyosin levels, causing sarcomere loss and myocyte disarray (Sehnert et al., 2002). Furthermore, tropomyosin is important for myofibrillogenesis in the amphibian, Mexican axolotl; that is, when mouse α-tropomyosin was introduced into mutant hearts (that showed a reduction of overall tropomyosin expression), myofibrillogenesis and contractile activity were restored (Zajdel et al., 1998). Given these data, it is tempting to speculate the following model of thin filament assembly: when the actin–tropomyosin filaments attain their mature lengths, nebulin's NH_2-terminal modules target Tmod1 to the pointed ends of the thin filaments. Once Tmod1 assembles, it functions to cap the actin and tropomyosin polymers, thus stabilizing and maintaining the lengths of the thin filaments at their pointed ends. We speculate that nebulin, tropomyosin, and Tmod1 play complementary, critical roles in controlling thin filament lengths and stability. Furthermore, the regulated interactions among these three thin filament components appear to be essential for proper myofibril assembly, structure, and function.

Materials and methods

Purified proteins and anti-Tmod1 antibodies

Recombinant full-length chicken Tmod1 and Tmod1 fragments were generated in *Escherichia coli* as described in Babcock and Fowler (1994). Monoclonal antibodies against recombinant chicken Tmod1 were generated by C. Grant (Custom Monoclonals, Sacramento, CA) and purified from hybridoma supernatants using a protein G–Sepharose 4 Fast Flow column (Amersham Pharmacia Biotech). The purified antibodies were dialyzed to 1.0 mg/ml in injection buffer (1 mM Tris and 25 mM KCl, pH 7.4). An irrelevant monoclonal antibody of the same isotype, MOPC-21 (Sigma-Aldrich), was used as a control (Gregorio et al., 1995). Fabs of mAb17 and MOPC-21 were generated by incubating the purified antibodies with papain-conjugated beads (Sigma-Aldrich), followed by isolation on a Mono Q anion exchange column. Rabbit skeletal tropomyosin was purified as described previously (Fowler, 1990).

Western blot analysis

0.5 μg of full-length or fragments of Tmod1, and 20 ng of full-length Tmod1 and equivalent molar amounts of fragments, were loaded for Coomassie blue staining and Western blot analysis, respectively. Blots were probed as described previously (Gregorio et al., 1995).

The specificity of the anti-Tmod1 antibodies was determined from day 6 embryonic chick hearts. In brief, hearts were dissected, snap-frozen, and ground in liquid nitrogen. The powder was solubilized in 2× SDS sample buffer, run on a 10% gel, and transferred to nitrocellulose. Nitrocellulose strips were incubated with either 0.5 μg/ml of MOPC-21, mAb8, mAb17 or 1.0 μg/ml mAb17 Fabs followed by HRP-conjugated anti–mouse IgG (1:20,000). Blots were incubated in Super Signal chemiluminescent substrate (Pierce Chemical Co.) and exposed to film (Biomax MR; Kodak).

Antibody competition and dissociation assays

5 pmol of full-length recombinant chicken Tmod1 were absorbed onto nitrocellulose as spots using a dot blot apparatus and preincubated with 0–700 nM of mAb8, 9, or 17 for 3 h at RT in a final volume of 200 μl (25 nM Tmod1). The molar ratio of mAb to Tmod1 varied from 1.3:1 at the lowest antibody concentration used (33 nM) to 28:1 at the highest concentration used (700 nM). After several washes, dots were incubated overnight at 4°C with 33 nM ^{125}I-Bolton Hunter–labeled rabbit skeletal muscle tropomyosin in binding buffer (20 mM Hepes, 80 mM KCl, 2 mM $MgCl_2$,1 mM DTT, 0.2% Triton X-100, and 20 mg/ml BSA), and washed to remove unbound tropomyosin. For the dissociation assay, 5 pmol Tmod1 were adsorbed onto nitrocellulose dots and incubated with 33 nM ^{125}I-tropomyosin overnight at 4°C. After several washes, the Tmod1 dots were incubated with 330 nM mAb8 or MOPC-21 (molar ratio of mAb/Tmod = 13:1). The amount of bound ^{125}I-tropomyosin was quantified in a γ counter.

Cell culture and microinjection procedures

Cardiac myocytes were isolated from day 6 embryonic chick hearts (Gregorio and Fowler, 1995). Isolated cells were plated at 10^6 cells/dish in 35-mm culture dishes containing CELLocate gridded coverslips (Eppendorf). Cells cultured for 3–5 d were injected with a 0.3–1.0-mg/ml solution of mAbs, Fabs, or purified Tmod1 fragments in injection buffer using a Transjector (model 5246; Eppendorf) and micromanipulator (model 5171; Eppendorf). Injected cells were incubated for 1–48 h before fixation.

Beating assays were performed by injecting beating cardiac myocytes cultured for 4 d with mAb17 or MOPC-21. Cells were incubated for 1 h at 37°C and the percentage of injected cells that were beating was determined. Greater than 60 cells/coverslip were analyzed and the experiment was performed in triplicate. Numbered grids on coverslips allowed for the identification of microinjected cells.

Indirect immunofluorescence and deconvolution microscopy

Cardiac myocytes were fixed 1 h after injection in 5% formaldehyde/PBS for 10 min, washed in PBS, and permeabilized in 0.2% Triton X-100/PBS for 15 min. Coverslips were blocked in 2% BSA/1% donkey serum/PBS for 30 min. Microinjected cells were stained with an AlexaFluor 594–conjugated goat anti–mouse IgG (1:1,000) or a donkey Texas red–conjugated anti–mouse IgG (Fab specific; 1:100) to detect the injected antibody. Actin was visualized using an AlexaFluor 488–, 594– or 647 phalloidin, or a monoclonal anti–cardiac actin antibody (Ac1-20.4.2; 1:10; American Research Products) followed by a donkey Texas red–conjugated anti–mouse IgG (Fc specific) antibody (1:100). For triple labeling, cells within numbered grids on the coverslip were injected with mAb17 Fabs or MOPC-21 Fabs and incubated for 1 h before fixation. Cells were incubated with monoclonal sarcomeric anti–α-actinin antibodies (1:1,500; EA-53; Sigma-Aldrich), anti–myomesin B4 antibodies (1:50; provided by J-.C. Perriard and E. Ehler, Institute for Cell Biology, Zurich, Switzerland; Grove et al., 1984), or anti–myosin F59 antibodies (1:10; provided by F. Stockdale, Stanford University, Stanford, CA) followed by FITC-conjugated anti–mouse Fc-specific IgG (1:100). Anti–titin A168-A170 (1:100) or titin N2A antibodies (10 μg/ml) (Centner et al., 2000) were added, followed by Cy5-conjugated donkey anti–rabbit IgG antibodies (1:600). Cells were incubated in AlexaFluor 594 phalloidin. AlexaFluor-conjugated antibodies and phalloidin were purchased from Molecular Probes. All other fluorescent antibodies were purchased from Jackson ImmunoResearch Laboratories. Cells were analyzed on a microscope (model IX70; Olympus). Micrographs were recorded as digital images (with Z-series containing 0.15-μm sections) using a CCD camera (model Series 300; Photometrics) and deconvolved using DeltaVision software (Applied Precision).

Transfection and live cell imaging

For live cell imaging, transfection was performed by incubating 1 μg pEGFP–rat–α-tropomyosin (gift from J.-C. Perriard; Helfman et al., 1999) with 7 μl Cytofectene (Bio-Rad Laboratories) and 100 μl Opti-MEM (Life Technologies) for 15 min at RT and adding the mixture to cardiac myocytes cultured for 24 h 3–4 d after transfection, cells were injected with mAb17 or MOPC-21. Coverslips were placed into a Focht Live-cell Chamber apparatus (Bioptechs) and Z-series of injected cells were imaged every 15 min. Staining with anti–α-actinin antibodies allowed us to determine the direction of depolymerization.

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Cell permeabilization assay

The assay was performed as described previously (Gregorio and Fowler, 1995). Day 6 cardiac myocytes were permeabilized in relaxing buffer (0.12 M KCl, 4 mM $MgCl_2$, 20 mM Tris-HCl, pH 6.8, 4 mM EGTA, 4 mM ATP, and 0.2 mg/ml saponin) for 10 min at 0°C, and then were extracted in a high salt buffer (0.5 M KCl, 10 mM sodium pyrophosphate, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 6.5, 1 mM EGTA, and 0.2 mg/ml saponin) for 10 min at RT. Extracted cells were washed in incubation buffer (20 mM KCl, 5 mM Tris-HCl, pH 6.8, 0.1 mM $CaCl_2$, 0.1 mM ATP, and 0.2 mg/ml saponin) for 15 s. To confirm the extraction, cells were fixed and stained with anti–myosin F59, anti–tropomyosin CH1 (Lin et al., 1985), or anti-Tmod1 monoclonal 95 antibodies (1:50; Almenar-Queralt et al., 1999) followed by AlexaFluor 594–conjugated goat anti–mouse IgG (1:800) and AlexaFluor 488 phalloidin. To reconstitute Tmod1 onto the myofibrils, cells were first incubated with 50 μg/ml biotinylated tropomyosin in 0.1 mM KCl, and 0.1 M Hepes, pH 7.5 for 10 min, rinsed, and then were incubated with recombinant Tmod1 at 70 μg/ml in 80 mM KCl, 2 mM $MgCl_2$, 0.1 mM DTT, and 20 mM Hepes, pH 7.3, in the presence of either 0.2 mg/ml of mAb17, mAb8, MOPC-21 or 0.05 mg/ml mAb17 Fabs for 25 min. After rinsing the cells in rigor buffer (60 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM Tris-HCl, pH 6.8, and 0.2 mg/ml saponin) the cells were fixed and stained for biotinylated tropomyosin using FITC-conjugated avidin (1:200; Zymed Laboratories) and Tmod1 using rabbit anti-Tmod1 1844 antibodies (1:100) followed by Cy5-conjugated donkey anti–rabbit IgG antibodies (1:500). Actin was visualized using AlexaFluor 594 phalloidin. Stabilization of actin filaments was performed by adding either AlexaFluor 594 phalloidin for 20 min or 1 μM jasplakinolide (Molecular Probes) in DMSO for 7 min after the cells were extracted. Myocytes treated with actin-stabilizing agents were reconstituted with tropomyosin and Tmod1, fixed, and stained as described in the section Indirect immunofluorescence and deconvolution microscopy.

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